Autoimmunity to Neuronal Antigens in Multiple Sclerosis

Autoimmuniteit tegen neuronale antigenen in multiple sclerose

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GENERAL INTRODUCTION

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MULTIPLE SCLEROSIS

Clinical aspects

Multiple sclerosis (MS), first identified as a separate neurological disease by the French neurologist Jean-Martin Charcot (Charcot, 1868), is the most common disease of the central nervous system (CNS) in young adults. Affecting more than 2 million people worldwide, MS classically starts with visual problems and tingling sensations and in many patients develops into permanent disability frequently involving paralysis and spasticity (Matthews, 1985). Cognitive functions such as memory, attention and visuospacial abilities are frequently impaired, while language skills are preserved (Bobholz and Rao, 2003).

Neuropathological or radiological examination of MS patients reveals the presence of sclerotic lesions or plaques, typically located in the periventricular white matter. Other preferential sites for lesions include the optic nerve, brainstem, cerebellum, and the (cervical) spinal cord. The grey matter may also be affected especially in secondary progressive disease (Vercellino *et al.*, 2005). The lesions are sites of extensive demyelination, inflammation and axonal degeneration. The immune-mediated removal of myelin sheaths results in an impaired or complete loss of axonal conduction, subsequently leading to neurological dysfunction. Adaptive mechanisms, like a redistribution of sodium channels (Waxman *et al.*, 2004) or remyelination, may result in restoration of nerve conduction and resolution of clinical symptoms.

Due to the plasticity of the brain, the disease course in most patients starts as a relapsing-remitting (RR-MS) disease i.e. acute periods of worsening followed by full or partial recovery (Lublin and Reingold, 1996). Within 20 years after initial symptoms approximately 80% of these patients convert to secondary progressive disease (SP-MS) (Weinshenker *et al.*, 1989). It is generally assumed that this transition is due to the accumulation of axonal loss and exhaustion of restorative mechanisms (Trapp *et al.*, 1999) (Figure 1). While myelin can be replaced to a large extent, regeneration of axons is far more limited in the CNS. About 5-10% of MS patients develop primary progressive (PP-MS) disease typified by progressive spastic paraplegia due to involvement of the spinal cord (Miller and Leary, 2007).

Aetiology

Although the actual cause of MS remains to be elucidated, it is thought that genetic and environmental factors contribute to the development of disease. The genetic contribution is mainly restricted to the human leukocyte antigen (HLA) region. For example, heterozygosity or homozygosity for the HLA-DRB1*1501 allele increases the susceptibility of MS by 3.4-fold and 7.5-fold respectively (Barcellos *et al.*, 2006). Other susceptibility alleles include the DRB5*0101, DQA1*0102 and the

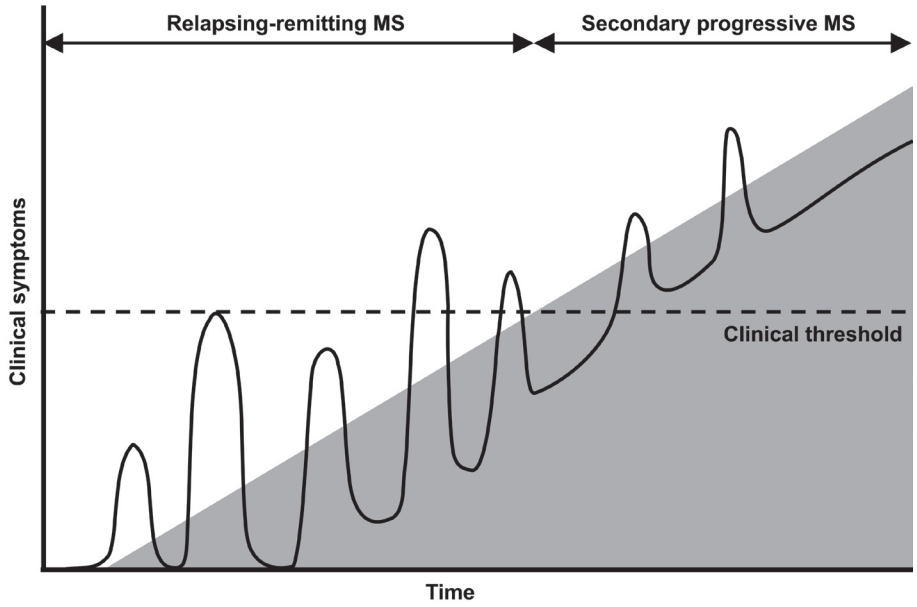

Figure 1. Relation of axonal damage with clinical disease. Axonal damage (grey) starts early in disease and gradually accumulates over time. Initially, clinical exacerbations are followed by complete remissions due to compensatory mechanisms in the central nervous system. However, when axonal damage passes beyond a clinical threshold (dotted line), restorative mechanisms are exhausted and disease converts to a secondary progressive stage, characterized by continuous deterioration in between superimposed relapses. Based on Trapp *et al.* (1999).

DQB1*0602 alleles. Co-inheritance of the DRB1*1501 allele with HLA-DRB1*14 completely abrogates the risk, indicating the existence of both susceptibility and resistance alleles (Ramagopalan *et al.*, 2007). That certain HLA-alleles increase the risk of developing MS might be explained by a preferential presentation of myelin peptides resulting in activation of encephalitic T cells. On the other hand, loading of myelin peptides onto HLA-molecules in the thymus may delete autoreactive T cells and hence results in a protective effect (Ramagopalan *et al.*, 2007). Non-HLA genetic risk factors are polymorphisms in the interleukin (IL)-7 receptor gene (Lundmark *et al.*, 2007), the IL-2 receptor gene (Hafler *et al.*, 2007) and in the 3' untranslated region of the IL-23 gene (Illes *et al.*, 2007). These genetic associations underscore the importance of T and B-cell proliferation in MS.

Environmental factors involved in the susceptibility for MS include sunlight exposure, infections and smoking (Giovannoni and Ebers, 2007). The increased incidence of MS in countries more distant from the equator supports the idea that sunlight influences

the development of MS. In addition, studies with identical twins showed that higher sunlight exposure during childhood decreases the risk of getting MS (Islam *et al.*, 2007). This effect might be attributed to vitamin D, of which a higher serum level has been shown to be protective (Munger *et al.*, 2006). Of note, vitamin D has been shown to reduce disease in the animal model experimental autoimmune encephalomyelitis (EAE) possibly via immunomodulatory effects on T cells (Spach *et al.*, 2006). Another environmental factor that has been implicated in the aetiology of MS is the exposure to microbial or viral infections, such as Epstein-Barr virus (EBV). Almost all (> 99%) MS patients are seropositive for EBV compared to 90% in controls (Ascherio and Munch, 2000). The risk to develop MS is higher in persons with a symptomatic EBV-infection (2.3-fold increase) or in people with high titres of EBV-antibody (Levin *et al.*, 2005, Thacker *et al.*, 2006). EBV-infected B cells and plasma cells have been shown to accumulate in ectopic B-cell follicles, creating a site of viral persistence within the brain (Serafini *et al.*, 2007). This is also reflected by the finding of EBV-reactive oligoclonal bands in the CSF of MS patients (Rand *et al.*, 2000). How EBV can predispose for MS is not known, but it is suggested that EBV-infection causes immortalization of autoreactive B cells leading to the (intrathecal) production of autoantibodies (Pender, 2003).

Therapy

For a long period MS therapy was restricted to the treatment of severe relapses using immunosuppressive drugs such as (methyl)prednisolone. Currently, therapy is more focussed on modulating inflammatory responses. For example interferon-β acts disease modifying through multiple mechanisms e.g. by skewing towards a Th2 response (Rudick *et al.*, 1998) or by directly affecting the blood-brain barrier (BBB) resulting in reduced monocyte migration (Floris *et al.*, 2002). Glatiramer acetate, also called copaxone, is also effective in reducing (30%) the relapse rate in RR-MS patients (Johnson *et al.*, 1995). Its therapeutic effect may rely on promoting Th2 responses or by restoring regulatory T-cell function (Weber *et al.*, 2007). Based on animal studies in which blocking adhesion molecules prevented CNS leukocyte infiltration and subsequent disease (Yednock *et al.*, 1992), humanized monoclonal antibodies have been developed against very late antigen-4 (VLA-4; Natalizumab®). However, despite beneficial effects on the disease course (Polman *et al.*, 2006), the drug also resulted in progressive multifocal leukencephalopathy (PML) in two patients, due to activation of latent viruses in the CNS (Kleinschmidt-DeMasters and Tyler, 2005). Possibly, the combination of Natalizumab® with interferon-β has rendered the brain severely immuno-suppressed. Currently, Natalizumab® is again used for treating MS, but only as monotherapy and under strict conditions.

A number of other potential drugs and strategies are currently under investigation, including statins, antibodies directed to CD20 on B cells (Rituximab®), fingolimod

(FTY720), rapamycin, cannabinoid-based drugs, T-cell depletion (Campath-1) and DNA-vaccination (Kleinschnitz *et al.*, 2007, Petereit *et al.*, 2007).

As a final point, it is noteworthy that while the immune-modulating therapies benefit RR-MS patients they are not effective for PP-MS patients (Leary *et al.*, 2003), suggesting that in progressive disease autoimmunity plays a minor role. Other treatment strategies, e.g. neuroprotection using riluzole (Killestein *et al.*, 2005), may be more effective for this group of patients.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Animal models play an important role in the investigation of fundamental disease mechanisms as well as in testing the efficacy of novel compounds or therapeutic regimens for use in MS. Demyelination in the CNS can be induced using viruses, chemicals or by sensitization with CNS proteins resulting in EAE. Recently also spontaneous EAE models have become available, by generating transgenic mice with myelin-specific T-cell receptors and / or immunoglobulins directed to myelin antigens. Table 1 shows an overview of models used for MS research.

Of all models, EAE is the most widely used and is induced with CNS homogenates, purified myelin proteins or myelin peptides emulsified in complete Freund's adjuvant (CFA). Whether strains are susceptible for EAE is dependent on the genetic background but also of the preparation of the adjuvant. Adjuvants made by sonication contain smaller droplets and the neuroantigens are more concentrated in the intermicellar space compared to adjuvants made by syringe extrusion (Fillmore *et al.*, 2003, Maatta *et al.*, 1996). In addition, many EAE models use *Bordetella pertussis* or pertussis toxin to increase disease incidence and/or severity. Historically described to be crucial for opening of the BBB (Linthicum *et al.*, 1982), recently it was shown that it could also mediate its effect by increasing the number of highly encephalitic Th17 cells (Hofstetter *et al.*, 2007).

The clinical disease course is highly variable between but also within animal strains, for example Biozzi antibody high (ABH) mice develop a chronic relapsing-remitting disease following immunization with myelin oligodendrocyte glycoprotein (MOG)8-21 peptide whereas the MOG35-55 peptide results in more progressive chronic disease. The occurrence of high levels of anti-myelin antibodies in the relapse phase has been implicated in the development of demyelination, which is abundant in this stage but low in the acute phase of disease (Morris-Downes *et al.*, 2002). Due to these features and the development of axonal damage and secondary progressive disease, the Biozzi ABH mouse model is a good model to mimic MS and study pathological mechanisms involved in disease (Amor *et al.*, 2005). Other mouse strains, e.g. C57BL/6 mice, do

Table 1. Commonly used animal models for multiple sclerosis.

Type	Examples	Features	Reference
Viral	• TMEV infection	Viral persistence causes chronic demyelination	Oleszak *et al.* (2004)
	• SFV infection	Demyelination and clinical disease dependent on virus strain	Fazakerley and Walker (2003)
Toxin	• Cuprizone oral feeding or ethidium bromide injection	Oligodendrocyte death; axon loss dependent on age	Blakemore (1982) Irvine and Blakemore (2006)
Autoimmune (EAE)	• Lewis rats: active immunization or T-cell transfer	Monophasic disease, minimal demyelination	Swanborg (2001)
	• Dark agouti rats: immunization with MOG or SCH in IFA	Chronic relapsing disease	Lorentzen *et al.* (1995) Storch *et al.* (1998)
	• Biozzi ABH mice immunized with MOG, PLP or SCH	Chronic relapsing disease	Amor *et al.* (2005) Baker *et al.* (1990)
	• C57BL/6 mice immunized with MOG35-55	Chronic progressive disease	Slavin *et al.* (1998)
	• Rhesus macaques immunized with MOG or MBP	Hyperacute disease resembling acute disseminated encephalomyelitis	Kerlero de Rosbo *et al.* (2000) Van Lambalgen and Jonker (1987)
	• Common marmosets immunized with MOG or human white matter	Progressive or relapsing-remitting disease	Brok *et al.* (2001) Massacesi *et al.* (1995)
Spontaneous	• Humanized transgenic mice with TCR for MBP85-99 and HLA-DR15	High incidence of paralysis after 6 months	Ellmerich *et al.* (2005)
	• Transgenic mice with TCR for MOG	Spontaneous optic neuritis	Bettelli *et al.* (2003)

Abbreviations: ABH, antibody high; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; SCH, spinal cord homogenate; SFV, Semliki forest virus; TCR, T-cell receptor; TMEV, Theiler's murine encephalomyelitis virus.

not have the capacity of mounting a pathogenic antibody response to MOG and are therefore less suitable for studying factors involved in antibody-mediated demyelination (Bourquin *et al.*, 2003, Gold *et al.*, 2006).

Although the EAE model has led to the development of therapies such as copaxone and Natalizumab® for use in MS, it has failed to predict the adverse effects of Natalizumab® treatment (Steinman and Zamvil, 2005). In addition, while treatment with antibodies against the cytokine tumour necrosis factor (TNF)-α ameliorates EAE, the effect in MS is totally the opposite (Selmaj and Raine, 1995). One of the reasons for this discrepancy could be that EAE animals are mostly treated for a short period (weeks) compared to patients with MS (years). In addition, animals are kept in a relatively clean environment, which does not mimic the situation in humans. Finally, there are many differences between the rodent and the human immune system, which could result in unexpected drug effects (Mestas and Hughes, 2004). Therefore experimental models in non-human primates may be relevant as an intermediate step to the clinic as these species have been considered to be genetically closer to humans (Brok *et al.*, 2001). However, therapies in non-human primates have been shown to be vastly different as shown by the drastic response to anti-CD28 therapy in humans whereas little effect was observed in rhesus monkeys (Suntharalingam *et al.*, 2006).

In summary, provided that the correct model is chosen, EAE can be valuable to study fundamental mechanisms of demyelinating disease. Also it can be used as a proof-of-principle to test new therapeutic approaches; however, one should always be aware that it does not fully replicate the pathomechanistic heterogeneity of the human disease.

NEUROPATHOLOGY

Post-mortem CNS tissue is widely used to study which molecular and cellular mechanisms are involved in the development of MS lesions. As already mentioned before, the pathological hallmarks of the lesion are inflammation, demyelination and axonal degeneration. Box 1 describes the different subtypes of MS lesions distinguished on basis of the presence and location of inflammatory cells and demyelination.

Inflammation

Among the inflammatory cells in the MS lesion, HLA class II positive macrophages dominate. These cells invade the myelin sheath, generally at the node of Ranvier, strip the membranes off the axon, and subsequently phagocytose and degrade the myelin (Lampert, 1983). In EAE, it has been shown that blood-derived macrophages play a crucial role, since depletion using clodronate-containing liposomes completely

Box 1. Lesion stages in multiple sclerosis brain.

Using general histology methods such as haematoxilin and eosin (H&E) or Kluver-Barrera / luxol fast blue (LFB) stain in combination with immunohistochemistry for HLA-DR and myelin antigens, lesions can be classified into several stages. The lesion classification used in this thesis is that of Bö and Trapp (Bo *et al.*, 1994) modified by De Groot and Van der Valk (Van der Valk and De Groot, 2000). MRI-guided lesion sampling is used since it results in an increased yield of demyelinating and preactive lesions (De Groot *et al.*, 2001). According to the distribution of inflammatory cells and the presence of demyelination, five lesion stages are discriminated:

- Preactive: the white matter contains activated microglial cells (strongly positive for HLA-DR) and a few perivascular inflammatory cells, however there is no demyelination.
- Active demyelinating: the white matter area is hypercellular due to macrophages and microglia which are phagocytosing myelin sheaths surrounding axons, giving them a 'foamy' appearance. The presence of granules within macrophages that are immunopositive for particular myelin antigens (MOG) or chemical stains (LFB) indicate recent phagocytosis. Axonal damage is prominent (Ferguson *et al.*, 1997).
- Chronic active: the lesion contains a hypocellular centre and a hypercellular rim with myelin-containing macrophages indicating ongoing demyelination and axonal damage.
- Chronic inactive: the lesion is hypocellular although some residual inflammatory activity may be present at the borders of the lesion. Hypertrophic astrocytes cause gliosis, resulting in the typical scar-like appearance of MS lesions.

Normal-appearing white matter (NAWM) of MS patients is often used as control reference tissue, however some pathology may be present such as axonal (Wallerian) degeneration.

abrogated the development of clinical disease (Huitinga *et al.*, 1990). The ability of Th1-skewed T cells to activate macrophages and to induce clinical disease in animals has led to the assumption that MS is also Th1-mediated disease. This scenario however is under serious debate (Lassmann and Ransohoff, 2004) as the pathology of EAE greatly differs from MS. For example, in MS lesions, $CD8^+$ cytotoxic T cells dominate over $CD4^+$ T cells (Babbe *et al.*, 2000) in contrast to EAE (Baker *et al.*, 1990). Moreover the finding of ectopic B-cell follicles in meninges of MS patients (Serafini *et al.*, 2004) argues for

a more differentiated view on the role of Th1 cells and suggests the involvement of a humoral immune response in the pathogenesis of MS.

Demyelination

Only little is known about demyelinating lesions in early MS. Lucchinetti and colleagues addressed this by studying both autopsy and biopsy tissues from early RR-MS patients. They observed that lesions could be classified into four different patterns of demyelination. Pattern I and II were consistent with a T-cell dependent, macrophage mediated myelin destruction. Only in pattern II lesions antibody and complement deposition was present on degenerating myelin sheaths. In pattern III, oligodendrocytes were showing signs of apoptosis and a preferential loss of myelin-associated glycoprotein (MAG) over other myelin proteins was observed. In pattern IV lesions oligodendrocytes were also primarily affected but without signs of apoptosis and preferential loss of MAG. Strikingly, lesions within the same patient were similar. The authors postulate that lesion heterogeneity is a reflection of separate mechanisms of demyelination that exist in different subsets of patients (Lassmann *et al.*, 2001, Lucchinetti *et al.*, 2000). This has important implications for therapy, for example patients with antibody-mediated pattern II lesions may be more responsive to plasmapheresis or intravenous immunoglobulin treatment compared to patients with other lesions patterns. Preliminary studies indicate that patients with pattern II lesions indeed benefit from plasmapheresis (Keegan *et al.*, 2005).

The concept of heterogeneity among lesions remains somewhat controversial and it has been suggested that it could rather be explained by lesional evolution. This view is based on studies in acute fatal cases of MS showing the presence of apoptotic oligodendrocytes in areas where myelin is still relatively intact and only few leukocytes are present. This stage has been suggested to precede macrophage recruitment and phagocytic clearance of myelin (Barnett *et al.*, 2006, Barnett and Prineas, 2004).

While different patterns of demyelination may exist early in disease, this is probably not the case in patients with longer disease duration. In a recent study using an unselected pool of autopsy material from patients with a disease duration ranging from 7-51 years, lesional heterogeneity was not detected. Lesions uniformly showed complement and antibody deposition on myelin and within myelin-containing macrophages, suggestive of pattern II lesions. Oligodendrocyte apoptosis was observed only occasionally (Breij *et al.*, 2008). This study suggests that, in contrast to early disease, there is a common mechanism of demyelination in established MS.

Axonal degeneration

Despite Charcot's detailed description in 1868 of axonal damage in MS it was only in the last decade that researchers returned to examine this aspect of the disease

in any detail (Trapp *et al.*, 1998). In addition to inflammation and loss of myelin, pathological studies reveal axonal injury and loss, grey matter involvement and atrophy of the brain and spinal cord of MS patients (Box 2). The average loss of axons in chronically demyelinated spinal cord lesions of MS patients was even 68% (Bjartmar *et al.*, 2000). Acute axonal damage is associated with axonal swelling and bulb formation as a consequence of axonal transection which can be assessed by silver impregnation and immunostaining of β-amyloid precursor protein (β-APP) that accumulates due to disruption of axonal transport (Ferguson *et al.*, 1997, Koo *et al.*, 1990). Axonal damage in MS is also monitored by increased immunoreactivity for SMI-32 which detects non-phosphorylated neurofilaments (NP-NF) (Trapp *et al.*, 1998). Dephosphorylation of neurofilaments can be a consequence of demyelination and it is not known whether it is reversible. Thus, the mere presence of NP-NF may not necessarily indicate axonal damage and morphologic appearance of axons must be taken into account when evaluating axonal damage. Of note, NP-NF have been shown to be more vulnerable to proteolysis via calpains (Pant and Veeranna, 1995), therefore dephosphorylation of neurofilaments may be an early event in axonal degeneration. Ultrastructurally, degenerating axons are characterized by an axoplasma full of floccular dense bodies, swelling of mitochondria and disintegration of neurofilaments (Lampert, 1967). Damage is not restricted to the axon as demonstrated by the presence of apoptotic neuronal cell bodies in cortical lesions (Peterson *et al.*, 2001, Vercellino *et al.*, 2005). Despite such detailed pathology the mechanisms underlying the axonal and neuronal damage and whether the damage is a primary or secondary event in MS is not known and deserve further study. Nevertheless these observations have not only highlighted the importance of axonal pathology in MS, but have also lead to the concept that its pathogenesis involves a neurodegenerative component.

Advances in imaging and spectroscopy have provided crucial insights into the dynamics of axonal changes in MS and their relationship to the inflammatory component of the disease. However, these approaches rely on surrogate markers to assess pathological changes in the CNS and in all probability underestimate the neurodegenerative component of the disease since conventional imaging is unable to quantify the extent of damage in grey and NAWM (Kidd *et al.*, 1999). In principal gadolinium enhanced magnetic resonance imaging (MRI) is used to identify sites of increased BBB permeability and inflammation, while local concentration of N-acetylaspartate (NAA) provides a marker of neuronal and axonal integrity that can be assessed by magnetic resonance spectroscopy (MRS) (Bjartmar *et al.*, 2000). These techniques provide a unique view of the anatomical distribution of lesions and the extent of brain and spinal cord atrophy as the disease progresses. Not only are brain atrophy and decreased levels of NAA seen very early in MS, but also there is a trend for neurodegeneration (as defined by these parameters) to become dissociated

Box 2. Axonal and neuronal changes in MS and EAE.

A number of morphological and molecular changes has been described to occur in axons and/or neurons during MS and EAE. Some may be reversible while other processes inevitably lead to axonal degeneration and loss.

- Transection of axons resulting in the formation of terminal ovoids or end bulbs (Trapp *et al.*, 1998).
- Impaired axonal transport as indicated by accumulation of β-APP (Ferguson *et al.*, 1997, Kornek *et al.*, 2000).
- Axonal cytoskeletal changes: dephosphorylation of neurofilaments, reversible destabilisation of microtubules in neurites (EAE) (Shriver and Dittel, 2006).
- Mitochondrial dysfunction: respiratory complexes I and III gene expression as well as enzymatic activity is decreased in MS cortex (Dutta *et al.*, 2006).
- Altered expression of glutamate receptors: the metabotropic mGluR1α is upregulated on axons in MS lesions and colocalizes with β-APP and SMI-32 immunoreactivity (Geurts *et al.*, 2003).
- Altered calcium homeostasis: expression of the plasma-membrane Ca^{2+}-ATPase 2 (PMCA2), an calcium pump expressed primarily in neurons, is severely reduced at the onset of EAE (Nicot *et al.*, 2003, Nicot *et al.*, 2005). In addition, the pore-forming subunit of the voltage-gated calcium channel colocalises with β-APP on axons in EAE (Kornek *et al.*, 2001).
- Upregulation of MHC class I expression on neurons and axons in MS (Hoftberger *et al.*, 2004). Probably this functions to reduce the number of excitatory synapses on neuronal cell bodies by microglia, a process known as synaptic stripping (Oliveira *et al.*, 2004).
- Dendritic beading (EAE) (Zhu *et al.*, 2003) and neurite transection (MS) (Peterson *et al.*, 2001).
- Synaptic pathology (EAE): reversible reduction of immunoreactivity for the synaptic proteins synaptophysin and synapsin I during disease (Zhu *et al.*, 2003).
- Apoptosis of neurons (Peterson *et al.*, 2001, Vercellino *et al.*, 2005), caspase-3 expression in neurons and axons during EAE (Ahmed *et al.*, 2002).

from the MRI measures of inflammatory activity (Bergers *et al.*, 2002, Pascual *et al.*, 2007). This suggests that chronic neurodegeneration in MS can be mediated by effector mechanisms distinct from those responsible for acute axonal injury in the inflammatory demyelinating lesions. The question that arises is: what are the effector mechanisms responsible for the continuing destruction of nerve cells and axons?

MECHANISMS OF AXONAL DEGENERATION

Among the mechanisms that might contribute to axonal pathology are: 1) disruption of trophic support provided by glial cells due to chronic demyelination, 2) generation of neurotoxic factors by chronically activated microglia or macrophages, 3) energy imbalance or oxidative stress and 4) primary autoimmune attack on axons (Figure 2). Evidence supporting each of these mechanisms will be discussed.

Chronic demyelination

Already in 1989, chronic demyelination was suggested to cause axonal degeneration in EAE (Raine and Cross, 1989). Guinea pigs with chronic relapsing EAE (CREAE) (18-36 months after immunization with spinal cord homogenate) had numerous axonal spheroids filled with vacuoles or with axoplasmic organelles. The authors proposed that long-term demyelination disturbs interactions of axons with glial cells, leading to a block in axonal transport and disruption of the axonal cytoskeleton. Indeed studies with mice deficient for the myelin protein MAG show that interaction of MAG with gangliosides on axons is necessary for axon-myelin stability by influencing neurofilament spacing and axonal caliber (Pan *et al.*, 2005, Yin *et al.*, 1998). Similarly, PLP deficiency in both mice and humans results in axonal degeneration due to impaired fast axonal transport (Edgar *et al.*, 2004, Garbern *et al.*, 2002, Griffiths *et al.*, 1998). Also overexpression of PLP in myelin is deleterious by causing activation of macrophages/microglia and influx of $CD8^+$ T cells (Ip *et al.*, 2006), indicating that axon-glial interactions must be tightly regulated.

Inflammation

Axonal damage, as shown by APP accumulation, is positively correlated with the number of macrophages (Bitsch *et al.*, 2000) in MS lesions. In addition, macrophages are often found in close association with damaged axons (Trapp *et al.*, 1998). This has lead to the 'bystander damage' hypothesis. In the course of myelin phagocytosis macrophages and microglia produce various factors, like reactive oxygen and nitrogen species (ROS and RNI) and cytokines that could in a bystander manner also lead to axon damage. For example macrophages are able to secrete nitric oxide (NO) and exposure of NO at physiological levels to electrically active axons causes acute Wallerian degeneration (Smith *et al.*, 2001). In addition, macrophages may secrete high levels of glutamate through the enzyme glutaminase causing excitotoxic death of axons (Werner *et al.*, 2001). Indeed, MS patients have elevated levels of glutamate in the CSF (Sarchielli *et al.*, 2003). The role of glutamate is further shown in EAE where treatment with NBQX, a glutamate receptor antagonist, significantly reduces clinical disease (Pitt *et al.*, 2000).

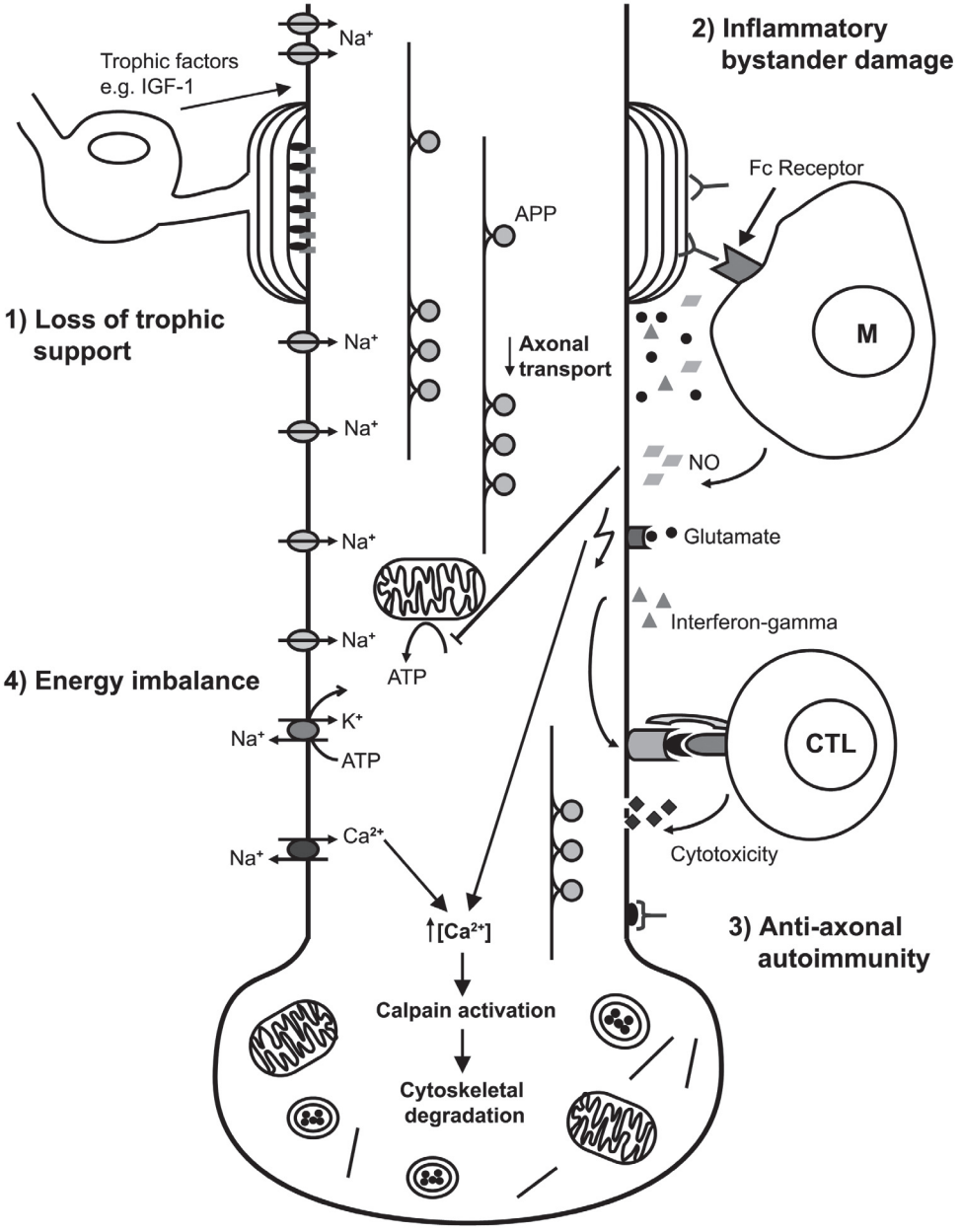
Trophic factors
e.g. IGF-1
Na+
1) Loss of trophic support
Na+
Na+
Na+
Na+
APP
Axonal transport
2) Inflammatory bystander damage
Fc Receptor
M
NO
Glutamate
Interferon-gamma
CTL
Cytotoxicity
3) Anti-axonal autoimmunity
ATP
4) Energy imbalance
Na+
K+
ATP
Na+
Ca2+
↑[Ca2+]
Calpain activation
Cytoskeletal degradation

Figure 2. Overview of mechanisms resulting in axonal damage. Axonal damage can occur as a consequence of demyelination **(1)** either because direct interactions between myelin and axons are lost, or because trophic support is lacking, i.e. soluble factors produced by oligodendrocytes that promote axonal survival (IGF-1, insulin-like growth factor-1). This may result in reduced axonal transport. During the course of myelin phagocytosis, inflammatory cells may induce bystander axonal damage by producing soluble toxic factors including cytokines and free radicals **(2)**. Nitric oxide (NO) directly inhibits the activity of mitochondrial enzymes that produce adenosine tri-phosphate (ATP) while high levels of glutamate cause continuous stimulation of glutamate receptors leading to excitotoxicity. Interferon-γ upregulates MHC class I expression on axons thereby axons become vulnerable to cytotoxic T lymphocyte (CTL)-mediated killing. Another example of anti-axonal autoimmunity **(3)** is binding of antibodies that recognize axonal antigens leading to complement deposition and subsequent damage. Finally, energy imbalance **(4)** may develop due to a redistribution of Na^+ channels, requiring a higher level of ATP to repolarize the axonal membrane. The depletion of ATP causes reversal of the ATP-independent Na^+/Ca^{2+} exchanger resulting in calcium influx. Increase of calcium levels subsequently activates calpains that degrade cytoskeletal proteins and induce axonal degeneration.

Energy imbalance

Another mechanism of axonal damage may be energy imbalance due to altered ion homeostasis (Dutta and Trapp, 2007, Stys, 2005). In order to restore conduction after demyelination, axons show a redistribution of sodium channels (Waxman *et al.*, 2004). This results in an increase of energy demand, since the Na^+/K^+ ion pump, needed for repolarization requires ATP. However the activity of mitochondrial enzymes (complexes I and III) in MS brain is reduced (Dutta *et al.*, 2006, Lu *et al.*, 2000), which may be caused by nitric oxide and its metabolite peroxynitrate (Brown and Borutaite, 2002). The ATP depletion leads to an increase in Na^+ levels which in turn reverses the ATP-independent Na^+/Ca^{2+} exchanger, resulting in increased intracellular Ca^{2+} levels (Li *et al.*, 2000). Chronic increases in Ca^{2+} subsequently activate phospholipases and proteases such as calpains to degrade the cytoskeleton, including neurofilaments (Stys and Jiang, 2002). Calpains are indeed upregulated in MS lesions and in neurons during EAE (Guyton *et al.*, 2005, Shields *et al.*, 1999). Also the Na^+/Ca^{2+} exchanger has been observed to co-localize with Na^+ channels in damaged axons (Craner *et al.*, 2004).

Pharmacological interference in the cascade causing an increase in axonal Ca^{2+} concentration may be a novel therapy to prevent axonal degeneration in MS. Indeed, administration of the Na^+ channel blockers phenytoin and lamotrigine to EAE animals prevented axonal degeneration and ameliorated EAE (Bechtold *et al.*, 2006, Lo *et al.*, 2003).

Anti-axonal autoimmunity

Finally, we propose in this thesis that autoimmune response directed primarily to axonal/neuronal antigens may be the cause of axonal degeneration, like is the case for myelin damage. Autoimmune axonal damage could be mediated by $CD8^+$ cytotoxic cells or by $CD4^+$ T cells that activate macrophages or alternatively stimulate B cells to

produce anti neuronal antibodies. $CD8^+$ T cells correlate with axonal damage (Bitsch *et al.*, 2000) and dominate in MS lesions (Babbe *et al.*, 2000). In theory, these $CD8^+$ T cells can cause lysis of axon/neurons through interaction with HLA class I, which is expressed on neurons and axons in MS lesions (Hoftberger *et al.*, 2004). *In vitro*, MHC class I can be induced in axons that were electrically inactive after stimulation with IFN-γ (Neumann *et al.*, 1995). These MHC class I positive axons became vulnerable for $CD8^+$ T-cell mediated cytotoxicity (Medana *et al.*, 2001). Although T-cell responses to gangliosides, neuron specific enolase and arrestin have been described in MS patients (Forooghian *et al.*, 2007, Pender *et al.*, 2003), it is so far not known whether these are mediated by $CD8^+$ or $CD4^+$ T cells and whether they are pathogenic.

Antibodies to various neuronal antigens have been found in sera or CSF of MS patients. These target antigens include neurofilament light (NF-L) (Ehling *et al.*, 2004), neurofilament medium (Bartos *et al.*, 2007), gangliosides (Sadatipour *et al.*, 1998), antibodies to axolemma proteins (Rawes *et al.*, 1997) and neurofascin (Mathey *et al.*, 2007). Axon-reactive B cells are present in the CSF and brain of MS patients and antibodies binding to axons are present in lesions (Zhang *et al.*, 2005a, Zhang *et al.*, 2005b). Increased levels of antibodies to NF-L are found in progressive patients (Silber *et al.*, 2002) and antibody levels correlate with cerebral atrophy (Eikelenboom *et al.*, 2003), suggesting that these antibodies could play a pathogenic role. However, firm evidence that antineuronal immune responses are able to induce axonal damage *in vivo* is still lacking.

AIM AND OUTLINE OF THE THESIS

While it is known that antibodies to various neuronal antigens are present in MS patients, a number of other questions regarding anti neuronal autoimmunity in MS remain unanswered. The most important question and the central theme in this thesis is whether autoimmunity to neuronal/axonal antigens can result in axonal damage in MS.

Since it has been reported that autoimmunity to neuronal antigens occurs in several other neurodegenerative diseases we wondered whether there was existing evidence for anti neuronal autoimmunity as an *inducer* of neurodegeneration in those diseases and, if so, via which mechanism it could mediate its pathogenic effect **(Chapter 2)**.

In contrast to anti neuronal antibodies, surprisingly little is known about anti neuronal T-cell responses in MS patients. Therefore we studied the proliferative responses of peripheral blood mononuclear cells (PBMC) to NF-L, which was previously identified as a target for antibodies in MS **(Chapter 3)**. The type of response was characterized by studying the cytokine profile and was compared to the autoimmune response to the myelin protein MOG and to the responses observed in control subjects.

We next hypothesized that these antineuronal T-cell responses were initiated by antigen presenting cells (APC) which had phagocytosed damaged axons, like is known for myelin sheaths. This was investigated by examining the presence of neuronal antigens in APC in the CNS of MS patients **(Chapter 4).** We subsequently studied whether APC containing these neuronal antigens were also present in the cervical lymph nodes (CLN) of MS patients and animals with EAE **(Chapter 5)**.

In order to determine whether autoimmune responses to NF-L could result in axonal degeneration and neurological disease we induced an autoimmune response to NF-L in Biozzi ABH mice **(Chapter 6)**. This strain of mice was selected for its ability to develop high levels of antibodies and for its susceptibility to several other experimental autoimmune diseases (Amor *et al.*, 2005). The novel animal model that we consequently developed was compared with the classical MOG-induced EAE. This enabled us to answer the question whether anti-axonal autoimmune responses can preferentially target axons for degeneration **(Chapter 7)**.

Finally, how these results help to answer the main question and what implications this will have for MS patients is discussed in **Chapter 8**.

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AUTOIMMUNITY TO NEURONAL PROTEINS IN NEUROLOGICAL DISORDERS

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ABSTRACT

Autoreactive T-cell responses directed to myelin proteins in the central nervous system (CNS) are widely believed to be fundamental in the pathology of multiple sclerosis (MS). This view has dominated the research field in MS for many years and led to the reliance on experimental models of neurological disease following immunization with myelin antigens. Such experimental diseases have developed as the central paradigm to investigate underlying mechanisms operating in MS as well as the preclinical development of therapeutic strategies.

This long-standing concept has recently shifted and evidence is rapidly accumulating indicating a significant role of axonal damage and neurodegeneration in disease. Indeed axonal damage is now considered to be the major cause of irreversible neurological disability in MS patients. One correlate of axonal damage in MS is the presence of antibodies against neurofilament light protein, a major component of the axonal cytoskeleton. Contrary to extensive literature on pathogenic myelin autoimmunity the possible pathogenic role of autoimmunity to axonal antigens in MS has so far been ignored.

As validation of the concept that autoimmunity directed to neuronal antigens plays a role in disease we define four criteria. First, that autoreactivity to neuronal antigens is consistently present in neurological disease in humans and experimental neurological disease in animals. Secondly, that transfer of such neuronal reactive T cells and/or antibodies are pathogenic either *in vivo* or *in vitro* model systems. Thirdly, that immunization with neuronal antigens induces pathogenic autoimmune responses and leads to neurological disease in experimental animals and finally that inhibition of such responses ameliorates neurological disease.

Using these criteria we review the evidence for autoimmunity to neuronal antigens in several neurodegenerative disorders such as paraneoplastic disorders, and discuss how similar mechanisms may also operate in MS. We postulate that clinical disease in MS models may not only be due to autoimmune attack on myelin, but that neurons and axons can also be target for pathogenic autoimmunity. Understanding the factors leading to neuronal injury and neurodegenerative disorders is key to the development of effective therapies to prevent progression of disease and irreversible damage.

INTRODUCTION

In this chapter we will review the evidence for autoimmunity to neurons and axons in neurological diseases and suggest how this may also be relevant in the pathogenesis of MS.

The view that autoreactive T cells directed to myelin proteins in the CNS are fundamental in the pathology of demyelinating disorders such as MS has dominated neuroimmunology research for many years. This has led to the reliance on experimental models of paralytic neurological disease, namely experimental autoimmune encephalomyelitis (EAE), induced in susceptible animals by immunization of myelin proteins. EAE has developed as the central paradigm to investigate the underlying mechanisms operating in MS in humans and heavily influenced the preclinical development of therapeutic strategies. In particular such approaches have focused on the role of $CD4^+$ helper T cells since several lines of evidence support the held notion that $CD4^+$ T cells play a crucial role in mediating the development of inflammatory lesions in both MS and EAE. Furthermore, histopathology of actively developing MS lesions has revealed the presence of many activated $CD4^+$ T cells in such lesions, and therapeutic intervention that targets helper T-cell functions in MS patients has at least partly positive clinical effects. More recently the long-standing concept that pathogenic autoreactive T cells are critical for myelin damage and disease in MS has shifted. Evidence is now rapidly accumulating indicating a significant role of axonal damage and neurodegeneration in disease (Trapp *et al.*, 1998). Indeed axonal damage is now considered to be the major cause of irreversible neurological disability in MS patients. One correlate of axonal damage in MS is the presence of antibodies against neurofilament light protein, a major component of the axonal cytoskeleton (Eikelenboom *et al.*, 2003). Contrary to extensive literature on pathogenic myelin autoimmunity the possible pathogenic role of autoimmunity to axonal antigens in MS has so far been ignored. Recent experimental data indicate that immunity to axonal antigens, as described in MS, is pathogenic rather than acting merely as a surrogate marker for axonal degeneration.

That autoimmune responses to neuronal antigens occur in neurodegenerative disorders other than MS is well documented (for an overview refer to Table 1). For example many paraneoplastic neurological disorders (PND) are thought to be caused by an autoimmune reaction directed against 'onconeural' antigens expressed by neurons and tumour cells (summarized in Table 2). In Lambert-Eaton myasthenic syndrome (LEMS) and neuromyotonia there are indications that disease is mediated by autoantibodies to antigens within the nervous system. Likewise, in other neurodegenerative diseases such as ALS, Parkinson's disease and Alzheimer's disease antibodies to neuronal proteins are present in patients and may contribute to disease. It is probable that, like myelin autoimmunity, pathogenic immunity to neuronal antigens is more common than originally thought and may be an important mechanism underlying neuronal damage and axonal damage observed in many neurological diseases including MS.

The question now is whether there is also evidence for involvement of autoimmunity to neuronal antigens in CNS disorders such as MS as well as other neurodegenerative disorders. Clearly this is of fundamental importance to the perspective of therapy in

Table 1. Distribution of neuronal antigens, immune responses and disease association.

Antigens	Expression and role	Disease association with antineuronal antigens	Reference
Neurofilament light	Neuronal cytoskeleton, control of axonal caliber and transport	ALS MS	Couratier *et al.* (1998) Silber *et al*. (2002)
Neurofilament heavy	Neuronal cytoskeleton, control of axonal caliber and transport	Alzheimer's disease GBS	Soussan *et al.* (1994) Terryberry *et al.* (1998)
GluR3	Glutamate receptor subunit on postsynaptic membrane	Rasmussen's encephalitis	Levite and Hermelin (1999)
Aldolase C **γ-enolase** **Pyruvate kinase**	40, 45, 60 and 98 kDa proteins of neuron	Basal ganglia disease Encephalitis lethargica Sydenham's Chorea Tourette's syndrome	Martino and Giovannoni (2004) Dale *et al.* (2004) Church *et al.* (2003) Hallet *et al.* (2000)
Tau	Microtubule-associated protein that stabilizes neuronal microtubules	Alzheimer's disease	Rosenmann *et al.* (2006b)
NR2A and NR2B	Neurons in hippocampus, amygdala and hypothalamus, subunits of NMDA receptor	SLE	Huerta *et al.* (2006) Sakic *et al.* (2005) DeGiorgio *et al.* (2001)
β-III-tubulin	Neuronal cytoskeleton	Brain trauma	Skoda *et al.* (2006)
Gangliosides (GM1, GQ1b, GD1b, GD1a, GalNAc-GD1a)	Cell membranes, enriched in neurons	ALS Miller-Fisher syndrome Cerebellar ataxia Ataxic sensory neuropathy	Hughes and Cornblath (2005) Willison (2005)
GAD	Synapses of GABAergic neurons	Stiff person syndrome in association with diabetes	Schloot *et al.* (1999)

neurological disorders by targeting the pathogenic antineuronal responses. In MS several concepts have suggested how autoimmunity to myelin antigens may arise. For example similar structures on viruses and myelin antigens may lead to crossreactive immune responses - so called molecular mimicry. Yet another idea suggests that the progression of neurological disease is due to the phenomenon of determinant spreading. In this view the repertoire of the anti-myelin T-cell responses that drives the disease becomes increasingly dynamic with the generation of new reactivities recognizing antigens released with each new wave of disease. With these ideas in mind we discuss how autoimmunity to neuronal antigens may arise and, rather than focus on the role of

Table 2. Paraneoplastic neurological disorders.

Antigens	Antibody	Expression, distribution and role	Tumor	Neurological disease association	Reference
Hu antigens	Anti-Hu (ANNA-1)	35-40 kDa neuron specific RNA binding proteins	Small cell lung carcinoma	Hu encephalopathy, Subacute sensory neuronopathy, Paraneoplastic brainstem encephalomyelitis, Limbic encephalitis	Tanaka *et al.* (2004) Rousseau *et al.* (2005)
Yo (PCA-1) **Cdr62** **Cdr34**	Anti-Yo	Purkinje cells 62 kDa intracellular binds to myc	Ovarian and breast carcinomas	Cerebellar ataxia	Tanaka (2004)
Ri	anti-Ri (ANNA-2)	Neuronal nuclei 55 and 80 kDa, Neuron specific RNA binding protein	Small cell carcinoma, Breast carcinoma	Paraneoplastic opsoclonus myoclonus ataxia	Fadare and Hart (2004)
Ma antigens Ma1 (PNMA-1) Ma2 (Ta/PNMA-2) Ma3 (PNMA-3)	Anti-Ma1 Anti-Ma2 Anti-Ma3	Ma-1 37 kDa neuron, testis Ma-2 40 kDa neurons of limbic system Ma-3 37kDa neuron, testis	Testicular small cell lung carcinoma	Limbic encephalitis	Gultekin *et al.* (2000)
Tr	Tr cell location, cytosol and outer surface of ER	Purkinje cells	Hodgkin's lymphoma	Tr syndrome	Bernal *et al.* (2003)
Amphiphysin	Anti- amphiphysin	128kDa pre-synaptic nerve terminals	Lung and thymoma	Stiff person syndrome	Folli *et al.* (1993)
AChR	Anti-AChR	Motor endplates transmembrane	thymoma	Myasthenia Gravis	Vincent *et al.* (1999)
Voltage gated calcium channels	Anti-VGCC		Small cell lung carcininoma	Lambert Eaton syndrome, RA, SLE and ALS	Vincent *et al.* (1999) Vincent (2006)
Voltage gated potassium channels	Anti-VGKC			Limbic encephalitis	Kleopa *et al.* (2006)
mGluR1	Anti-mGluR1	Cerebellar Purkinje cells	Hodgkin's lymphoma	Ataxia	Coesmans *et al.* (2003)

pathogenic T cells, as is common in MS, we will discuss the evidence for autoantibodies in neurodegenerative disorders. Understanding the mechanisms by which autoimmunity to neuronal antigens leads to neuronal injury and neurodegenerative disorders is key to the development of effective therapies to prevent progression of disease and irreversible damage.

Neurons and axons as targets of the immune response

The immune responses must maximize the removal of harmful microorganisms while protecting the healthy tissues and cells. In the CNS this protection is exemplified in part by the presence of the blood-brain barrier (BBB) and low level expression of molecules on neurons required for immune activation as well as expression of molecules crucial for tissue protection and repair.

To exert a pathogenic effect on neurons and axons T cells need to recognize antigenic peptides presented by the major histocompatibility complex (MHC) molecules. Antigens derived from endogenously synthesized proteins are bound to MHC class I molecules and are recognized by $CD8^+$ T cells whereas antigens derived from proteins, internalized by endocytosis and processed in vesicles, are bound to MHC class II molecules and recognized by $CD4^+$ T cells. However, in health the CNS and in particular neurons are protected in part from pathogenic immunity by BBB but also by expression of low levels of MHC molecules. The situation is different in disease where the expression of MHC molecules is dependent on the type of tissue injury. In MS lesions neurons and axons express class I but not MHC class II molecules (Hoftberger *et al.*, 2004, Neumann *et al.*, 1995) and thus may be target for MHC class I restricted killing. Indeed $CD8^+$ cytotoxic T-cell induced neuronal damage in dorsal root ganglia is a prominent feature in viral infections such as lentivirus infections (Zhu *et al.*, 2006) although the exact role of T cells in neurodegenerative disorders is unknown.

EVIDENCE FOR ANTINEURONAL AUTOIMMUNITY IN NEUROLOGICAL DISEASES

The precise role of pathogenic autoimmunity to self antigens including myelin antigens, as has been described in MS or indeed neuronal antigens in human neurological disorders is difficult to establish. The mere presence of antibodies and T-cell responses to CNS antigens in human disease is clearly insufficient to assume that autoimmunity is relevant to disease or damage in the CNS. Firm evidence for the role of autoimmunity to axonal antigens in neurological disorders heavily relies on the pathogenic effect of autoreactive T cells and antibodies in cell culture and *in vivo* or following immunization of experimental animals with neuronal proteins. Nevertheless

many neuronal antigens and immune responses to neuronal antigens have been associated with human disorders (Table 1) in particular with the PND (Table 2).

To rationally examine the evidence for a pathogenic effect of autoimmunity to axonal antigens in neurological disorders we believe that the evidence should fulfil Witebsky's criteria (Rose and Bona, 1993). These include *indirect* (circumstantial) evidence from clinical clues in patients i.e. a) the autoantibody or T cell should be present in the patient with disease; b) the autoantibody and/or T cell recognizes the specific antigen, and *direct* evidence in which c) antibodies or T cells from patients are pathogenic to neuronal cells *in vitro* or induce neurological disease in laboratory animals and d) immunization of the animal with the antigen should mimic the disease in humans.

Most of the direct evidence for a pathogenic role of antineuronal autoimmunity in humans comes from studying the effects of antibodies and T cells from patients with neurological disease *in vitro* on neuronal cell cultures. Adoptive transfer of T cells and antibodies *in vivo* has proven to be more difficult probably due to the difficulty in directing antibodies into the PNS or CNS due to the blood nerve barrier or BBB. Furthermore the pathogenic action of antibodies may rely on uptake by or entry into neurons via species specific pathways e.g. FcR receptors (FcR). In the case of FcR, murine receptors are known to have different functions than those of humans and moreover the Fc portion of human immunoglobulins may not be recognized by rodent FcR. Likewise, activated T cells from humans may not recognize antigen due to sequence differences or due to inadequate presentation by murine antigen presenting cells. Thus transferring immunoglobulin or T cells from patients to mice or other animals must be viewed with caution.

Despite these limitations, animal models can be crucial tools in defining the pathogenesis of human diseases and are essential to develop and examine the safety of therapeutic interventions for patients with complex neuroimmunological syndromes. For example, the autoimmune basis of MS is well-accepted due to rodent and primate models which replicate many but not all of the pathological features (Amor *et al.*, 2005, Brok *et al.*, 2001). It is worth noting that animal models themselves must also be viewed with caution as being an analogue rather than the exact copy of the human counterpart, because they invariably differ to some degree from the human disease.

Due to the wide variety of biological tools available, rodent models are the most widely used system to probe the mechanisms underlying human disorders. Moreover, the extensive numbers of mutant mice deficient in specific immune factors allow detailed investigation of the disease models. These include mice deficient in FcR, B cells or T-cell receptor (TCR), or severe combined immunodeficiency (SCID) mice which can be reconstituted to examine individual components of the immune response or indeed 'humanized' by reconstituting with human cells. For several neurodegenerative diseases such as Parkinson's disease, models in non-human primates (Eslamboli, 2005) may

be more relevant since behavioural studies such as cognitive changes can be better investigated in higher species (Philippens *et al.*, 2000).

Taking these factors into account here we discuss the evidence for pathogenic responses of T cells and antibodies to neuronal antigens from patients with neurological disease *in vitro.* In addition, we will outline the major models of diseases in which autoimmunity to neuronal structures have been demonstrated (summarized in Table 3). These experimental models provide evidence that autoimmunity to neuronal antigens may contribute to the disease process rather than act as markers of neuronal damage, or are secondary to neuronal damage, or being an epiphenomenon.

Disorders of the neuromuscular junction

Autoimmunity to antigens of the neuromuscular junction is observed in several neurological disorders such as the well-known disorder myasthenia gravis (MG) in which antibodies to acetylcholine receptor (AChR), an important neurotransmitter receptor results in weakness and fatigue (Vincent, 2006, Vincent *et al.*, 1999). Approximately 10% of MG cases is associated with thymomas and therefore considered to belong to the group of diseases known as paraneoplastic disorders (see below). That antibodies to AChR may be pathogenic comes from indirect evidence in patients. Anti-AChR antibodies are observed in over 80% of MG patients where increasing levels of autoantibody correlate with deterioration in disease. In addition, MG patients respond to intravenous immunoglobulin (IVIg) which is thought to block the pathogenic antibodies. In mothers with MG anti-AChR antibodies may cross the placenta and bind to fetal AChR resulting in antibody-mediated arthrogryposis multiplex congenita providing more direct evidence of autoimmunity in disease. However firm evidence for a pathogenic role of autoantibodies was first observed following passive transfer of serum from MG patients to mice (Toyka *et al.*, 1975). Following injection of the serum, mice developed neuromuscular defects associated with a reduction in the numbers of AChR. In a follow up study the active fraction in the serum was identified as IgG and the effect was enhanced by complement component C3 (Toyka *et al.*, 1977). To address the role of autoreactive T cells in MG Wang *et al.* (1999) transferred either $CD4^+$ or $CD8^+$ T lymphocytes from MG patients to SCID mice. Here it was clearly shown that $CD4^+$ T cells were crucial for the induction of experimental MG by providing 'help' to B cells in the production of pathogenic anti-AChR antibodies. As well as autoimmunity to AChR MG is also associated with muscle atrophy in which antibodies to the muscle-specific kinase (MuSK) are observed. The role of antibodies and T cells to MuSK using approaches as described for AChR is unknown.

Antibodies to voltage-gated Ca^{2+} or K^+ channels (VGCC or VGKC) have been associated with a variety of clinical disorders in which defects in neuronal signalling and synaptic transmission are observed (Vincent, 2006). For example autoantibodies

Table 3. Neurological disease in animals induced by immunization with neuronal antigens or (components of) bacteria leading to antineuronal autoimmunity.

Human disease	Animal species/ strain	Immunization/procedure	Reference
Myasthenia gravis	Mouse	Torpedo AChR in CFA	Christadoss *et al.* (2000)
	Mouse	MuSK extracellular domain	Jha *et al.* (2006)
	Rabbit	MuSK extracellular domain	Shigemoto *et al.* (2006)
	Rhesus monkey	Torpedo AchR	Toro-Goyco *et al.* (1986)
LEMS	Rat and mouse	Synaptotagmin peptides	Takamori *et al.* (1994)
GBS	Mouse	Lipooligosaccharide of *B. melitensis*	Watanabe *et al.* (2005)
	Rabbit	Ganglioside or galactocerebrosides *C. jejuni* lipooligosaccharide	Caporale *et al.* (2006) Saida *et al.* (1981) Yuki et al. (2004)
	Guinea pig	*C. jejuni* lipooligosaccharide	Shu *et al.* (2006)
Paraneoplastic syndrome HuD PNMA1 (Ma1)	Mouse Rat	Immunization with plasmid coding HuD and implantation with neuroblastoma cell line Immunization and transfer of Ma-specific T cells	Carpentier *et al.* (1998) Pellkofer *et al.* (2004)
Stiff-person syndrome	Rat	Passive transfer SPS patients' Ig to amphiphysin	Sommer *et al.* (2005)
Diabetic neuropathy	Mouse and rat	Neuropathy in diabetic mice and following T-cell transfer	Calcutt (2002) Schmidt *et al.* (2003) Bour-Jordan *et al.* (2005)
Sydenham's chorea Tourette's syndrome	Mouse (SJL/J)	Homogenate of group A beta-haemolytic streptococcal bacteria in CFA	Hoffman *et al.* (2004)
Amyotrophic lateral sclerosis	Guinea pig	Bovine spinal motor neurons Spinal cord ventral horn homogenate Choline acetyltransferase	Engelhardt *et al.* (1990) Engelhardt *et al.* (1997)
Alzheimer's disease	Rat Rat Mouse Rat Rat Guinea pig	Cholinergic neurons Peptides of beta-synuclein Recombinant human tau protein Cholinergic Torpedo neurons NF-H protein of cholinergic neurons Septal cholinergic neurons	Chapman *et al.* (1989) Mor *et al.* (2003) Rosenmann *et al.* (2006a) Michaelson *et al.* (1990) Oron *et al.* (1997) Kalman *et al.* (1997)
Parkinson's disease	Guinea pig	Homogenates of bovine mesencephalon Dopaminergic MES 23.5 cells	Appel *et al.* (1992) Le *et al.* (1995)
Epilepsy Rasmussen's syndrome	Mouse Rabbit	Glu3RB peptide aa 372-395 Glu3R fragment aa residues 245-457	Levite and Hermelin (1999) Twyman et al (1995)
SLE	MRL/lpr mouse	Spontaneous	Hikawa *et al.* (1997) Ballok *et al.* (2004)

to the Shaker type (Kv1) K^+ channels are associated with peripheral nerve excitability, neuromyotonia and limbic encephalitis (Kleopa *et al.*, 2006). In Lambert-Eaton myastenic syndrome (LEMS) the pathogenic role for antibodies to nerve terminal VGCC has been demonstrated in animals following chronic administration of plasma, serum or IgG to mice. Following transfer, electrophysiological and ultrastructural findings similar to those seen in patients are observed (Flink and Atchison, 2003). These studies also showed a reduction in amplitude of Ca^{2+} currents through P/Q-type channels. In addition, transfer of antibodies to the presynaptic VGKC, the autoantigen in neuromyotonia, into mice also results in defects in neuromuscular transmission.

As a model for MG, experimental autoimmune myasthenia gravis (EAMG) can be induced in many rodents by immunization with AChR from *Torpedo california* neurons or mouse AChR in complete Freund's adjuvant (Christadoss *et al.*, 2000). As with many models in mice (Drachman *et al.*, 1998) the MHC class II genes influence autoimmunity to AChR since MHC class II-restricted $CD4^+$ cells are crucial for induction of anti-AChR antibodies. Moreover tolerance to AChR or dominant peptide epitopes prevents EAMG by blocking the action of these pathogenic T cells. EAMG may also be induced following immunization of H-2(a), H-2(b), H-2(bm12) and H-2(d) mice with recombinant rat-MuSK extracellular domain (Jha *et al.*, 2006) although variations in clinical signs are observed. In rabbits immunization with the MuSK ectodomain protein induces MG-like muscle weakness with a reduction of AChR clustering at the NMJs (Shigemoto *et al.*, 2006) which more closely mimics the human disease.

Immunization with peptides of synaptotagmin, one of the functionally VGCC-associated proteins, induced electrophysiological changes following repeated injections of Lewis rats (Takamori *et al.*, 1994). Whether active immunization with VGKC in experimental animals will mimic channelopathies in humans is unknown.

Experimental models in higher species have also been developed, which are crucial for development of novel therapies as well as the study of effects of the disease on higher functions not possible in rodents. EAMG in rhesus monkeys may be induced with purified AChR from *Torpedo california* (Toro-Goyco *et al.*, 1986). Rhesus monkeys immunized with three doses of 80 mg protein at two-week intervals developed antibodies and clinical signs of disease correlating with the levels of antibodies induced. Histological examination revealed muscular atrophy, fibrous degeneration and lymphocyte infiltration in the lesions characteristic of MG.

In summary MG serves as the prototypic disease fulfilling many criteria demonstrating a role for autoimmunity to neuronal antigens in disease. Not only do antibodies to MG correlate with disease but *in utero* transfer of antibodies in MG patients and transfer of human antibodies to AChR to animals mimics MG in humans. Evidence is also observed following immunization of animals with purified AChR thus fulfilling Witebsky's criteria for pathogenic autoimmunity to axonal antigens in MG.

Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) consists of several subtypes of monophasic disorders associated with inflammation and demyelination of the peripheral nervous system. GBS and related syndromes, such as Miller-Fisher syndrome (MFS) are related to bacterial and viral infections and thought to be due to antibody responses to gangliosides such as GM1 and GQ1b. In approximately 25% of patients with GBS, particularly the axonal form, disease occurs after infection with *Campylobacter jejuni* which contains ganglioside-like structures similar to those of peripheral nerves. It has therefore been suggested that disease is induced via the mechanism of molecular mimicry. That antibodies to neuronal structures in GBS may also involve the CNS was reported by Bae and Kim, (2005) who reported that serum antibodies to GD1b and GM1 were elevated in a patient with GBS presenting with cerebellar ataxia. Further evidence is observed in Bickerstaff's encephalitis, a rare condition related to MFS involving the CNS in which antibodies to GQ1b gangliosides are thought to play a pathogenic role.

The pathogenicity of antibodies to gangliosides in GBS is proven by studies whereby transfer of serum and immunoglobulin from GBS patients induces neurodegenerative changes *in vivo* and physiological and pathological effects in culture. For example GBS sera containing anti-ganglioside antibodies as well as monoclonal antibodies raised against gangliosides induce neuronal cell lysis in culture by targeting specific cell surface gangliosides and this effect is complement dependent (Zhang *et al.*, 2004). Furthermore purified IgG from GBS patients' sera induces postsynaptic block of mouse myotubes expressing native embryonic-type nAChR channels or HEK 293 cells transfected with recombinant nAChR channels (Krampfl *et al.*, 2003, Willison, 2005). The GBS variant, acute motor axonal neuropathy (AMAN) has also been associated with anti-GM1, anti-GM1b, anti-GD1a, and anti-GalNAc-GD1a antibodies. This is supported by the finding that antibodies from patients have been shown to damage motor terminal endplates *in vitro* suggesting a pathogenic role in disease (Hughes and Cornblath, 2005).

In vivo monoclonal antibodies against gangliosides, transferred to mice as hybridomas induced axonal neuropathy affecting a small proportion of nerve fibres whereas purified anti-ganglioside antibodies had no effect despite high titer circulating antibodies (Sheikh *et al.*, 2004). It was suggested that this effect was due to properties of the hybridoma on the blood nerve barrier by allowing direct access of immunoglobulin to the nerve.

Purified IgG from Miller-Fisher syndrome patients induced muscle weakness in adult mice due to inhibition of postsynaptic channels (Buchwald *et al.*, 1998). This effect was also seen with anti-GQ1b antibodies in conjunction with activated complement at the NMJ (Plomp *et al.*, 1999) while injury to motor nerve terminals was associated with antibodies specific for disialoside epitopes on gangliosides GT1a (Halstead *et al.*, 2004).

The variant of GBS, AMAN, is modelled in rabbits following immunization with the lipo-oligosaccharide of *Campylobacter jejuni,* which results in disease by inducing antibodies that cross-react with gangliosides (Yuki *et al.*, 2004). Watanabe *et al.* (2005) also demonstrated that the lipo-oligosaccharide of *Brucella melitensis* may also play a role in GBS since this is also expressing a GM1 ganglioside-like structure that induces strong anti-GM1 ganglioside antibody responses in BALB/c mice resulting in flaccid limb weakness.

As discussed anti-GM1, anti-GM1b, anti-GD1a, and anti-GalNAc-GD1a IgG antibodies are associated with AMAN in humans and have been shown to augment experimental autoimmune neuritis (EAN) in rabbits as well as induce disease following immunization of rabbits with galactocerebrosides or GM1 ganglioside (Caporale *et al.*, 2006, Saida *et al.*, 1981). Paralyzed rabbits developed pathological changes in their peripheral nerves identical to changes seen in human GBS. The pathogenicity of ganglioside structures of lipo-oligosaccharide of *C. jejuni* was also demonstrated following immunization of guinea pigs using the *C. jejuni* strain HB9313, which resulted in neuropathy and axonal degeneration (Shu *et al.*, 2006). Thus evidence for autoimmunity to neuronal structures comes from the finding of autoantibodies in patients, passive transfer of antibodies to laboratory animals as well as experimental disease induced following active immunization with neuronal antigens.

Paraneoplastic neurological disorders

The paraneoplastic neurological disorders (PND) are observed in patients who have cancer along with abnormal neurological symptoms despite the absence of tumours in the nervous system. Common tumours of the breast, ovary and lung express some proteins generally restricted to the nervous system and the patients generate immune responses to these 'nervous system specific' proteins. While autoimmune responses to these neuronal antigens help to suppress the growth of the tumour they also cause damage in the nervous system leading to neurological symptoms (Roberts and Darnell, 2004). PND are thus considered to be due to autoimmune attack on neurons expressing the antigens abnormally expressed by the tumour. The clinical syndromes are very heterogeneous due to the many different regions of the nervous system that may be involved. In many of these disorders antibodies against the tumour and the neurons are detected in the patient's serum and cerebrospinal fluid (Posner, 2003) although it should be noted that for many PND the neuronal antigen expressed by the tumour is unknown (as a summary please refer to Table 2).

The majority of the PND antigens are intracellular and are thus not readily available for recognition by antibodies although, as demonstrated in other pathological conditions, antibodies do gain access to intracellular proteins. Indeed PND antibodies enter neurons and may trigger responses in the neuron such as apoptosis. Alternatively it has been

suggested that tumour cells taken up by dendritic cells in the periphery present not only tumour antigens but also the neuronal antigens present on tumours. In this way the immune response may be activated to respond not only to the tumour but could also elicit $CD8^+$ T-cell responses that subsequently target neurons.

Evidence of the pathogenicity of the antineuronal antibodies is limited to a few cases such as Lambert Eaton syndrome where antibodies to VGKC induce LEMS, as described before, and are involved in peripheral nerve hyperexcitability (Hart *et al.*, 2002). In others the pathogenic role of autoimmune responses requires further study. The antigen Ri encoded by the gene *Nova* is expressed in the brain stem, cortex and motor neurons, and also by neuroblastomas (in children) and lung and ovarian tumors (Fadare and Hart, 2004). Autoimmunity to Ri is associated with the disorder paraneoplastic opsoclonus myoclonus (dancing eyes) ataxia where unlike most PND this disease can be relapsing remitting. In adults opsoclonus myoclonus may follow a viral infection but has, similar to basal ganglia disease, also been reported following pharyngitis due to streptococcal infection in which the antigen was identified as neuroleukin (Candler *et al.*, 2006). In another PND - paraneoplastic cerebellar degeneration, autoimmune responses to antigens of cerebellar Purkinje neurons are expressed by breast or ovarian tumour. Subacute sensory neuronopathy and Hu encephalopathy are associated with responses to the Hu antigens expressed by small cell lung cancer or breast cancer which induce disease involving many regions of the CNS and PNS. In this disease symptoms include memory loss, dorsal root ganglionopathy or more destructive multifocal neurological degeneration. Limbic encephalitis occurs in conjunction with small cell carcinomas of the lung and testicular tumours in which the anti-Ta (anti-Ma2) antibodies are present (Gultekin *et al.*, 2000). Paraneoplastic syndromes also affect the lower motor neurons as described in a patient with breast cancer with antibodies directed against axons and nodes of Ranvier. More detailed studies showed that these antibodies were directed to isoforms of beta IV spectrin - a protein enriched in axonal initial segments and nodes of Ranvier (Berghs *et al.*, 2001). The antigen Tr expressed in the cerebellum is also a target for anti-Tr antibodies in Hodgkin's disease that react with the cytoplasm of Purkinje cells (Bernal *et al.*, 2003).

Compared to MG and LEMS, most neoplastic antigens are intracellular antigens and thus considered unlikely to be targeted by the antibody directly. It is probably for this reason that development of models or demonstration for a direct pathogenic effect of antibodies has been difficult.

Although antibodies to recoverin have been shown to induce apoptosis of cells in cancer associated retinopathy (Adamus, 2003), for others this effect has not be demonstrated. For example, application of anti-Yo or anti-Hu antibodies on primary mouse brain-derived neurons did not kill neurons, but instead induced the expression of cell adhesion molecules and accelerated neuronal differentiation. Similar effects of

serum IgG fractions from patients containing the anti-Yo or the anti-Hu antibody on the cultured neurons were observed, suggesting that their effects were not through the binding of the antibody to specific antigens, but to some other factors contained in IgG fractions (Tanaka *et al.*, 2004).

IgG purified from the serum of patients with Hodgkin's disease paraneoplastic cerebellar ataxia in which the number of Purkinje cells is severely reduced was shown to affect the basal activity of Purkinje cells in mouse brain slice cultures (Coesmans *et al.*, 2003). The number of studies demonstrating the pathogenic effects of antibodies to intracellular paraneoplastic neuroantigens is scarce however earlier studies by Greenlee *et al.* (1995) show that microinjection of anti-Yo antibodies from humans in rats reach the CNS. While anti-Yo antibodies were identified on the dendrites and within the cell body of Purkinje cells, animals did not develop ataxia or cerebellar injury. Additional studies to determine the mechanism of action are required to provide clues to their effect in human disease.

Immunization of mice with a plasmid coding HuD induced a strong and specific anti-Hu response. To model the human disease mice were challenged by implantation of a neuroblastoma cell line that constitutively expresses HuD. In the presence of secreted HuD and subsequent immunity to Hu significant inhibition of tumour growth was observed however the animals did not develop neurological deficits or neuropathological evidence of nervous system pathology (Carpentier *et al.*, 1998, Sillevis Smitt *et al.*, 1996). Likewise it has not been possible to induce experimental disease following passive transfer of anti-Yo antibody and immunization with recombinant Yo protein does not induce damage to Purkinje cells (Tanaka *et al.*, 1995). In humans paraneoplastic cerebellar degeneration is also associated with immune responses to PCD17/cdr2 on Purkinje cells. Sakai *et al.* (2001) demonstrated that while mice immunized with naked PCD17 cDNA developed autoantibodies against Purkinje cells and CTLs from these mice lysed cells pulsed with H-2K-restricted PCD17 peptide, clinical or pathological changes in the cerebellum were absent.

Development of animal models for PND has focused on induction of antibodies to onconeuroantigens and as such has been problematic. As a step to study the involvement of T cells in PND, Pellkofer *et al.* (2004) show that following immunization of rats with Pnma1 (Ma1), transfer of T-cell blasts specific from the rats into naïve animals induced encephalitis indicating a role for T cells in PND. However, animals did not develop clinical disease.

Stiff person syndrome

The "Stiff person syndrome" (SPS) is a rare chronic neurological disorder characterized by progressive stiffness, painful persistent or spasmodic muscle contractions, mostly involving the spine and lower extremities. In 60 to 90% of cases,

non-paraneoplastic forms are associated with the presence of autoantibodies against glutamic acid decarboxylase (GAD), an enzyme that converts the excitatory neurotransmitter glutamate to gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the CNS. The syndrome often occurs spontaneously and in association with type I (insulin-dependent) diabetes mellitus (Schloot *et al.*, 1999). In contrast SPS associated with antibodies to amphiphysin, a protein involved in clathrin-mediated endocytosis, is frequently associated with the paraneoplastic types (Folli *et al.*, 1993). The relevant treatment for SPS is to alter the inhibitory processes controlling muscle activity, control of the immune response and removal of associated neoplasia. High-dose intravenous immunoglobulin (IVIg) is also effective suggesting an involvement of humoral immunity in disease.

While antibodies to GAD were first recognized in an SPS patient they are also observed in patients with chronic cerebellar ataxia, drug-resistant epilepsy and myoclonus, Batten disease (Ramirez-Montealegre *et al.*, 2005) as well juvenile ceroid lipofuscinosis, a fatal paediatric neurological disorder. The serum from patients with juvenile ceroid lipofuscinosis reacted with GAD-positive GABAergic neurons but also with other cell populations (Lim *et al.*, 2006) distinguishing the responses from sera from Stiff-persons syndrome and type-1 diabetes. The role of anti-GAD antibodies is still unclear although decreased GABA synthesis in nerve terminals as a result of antibody interference with exocytosis of GABA may be crucial to disease expression. This is suggested from *in vitro* studies whereby GAD autoantibodies or purified IgG from the cerebrospinal fluid (CSF) or serum from people with SPS were observed to reduce GABA production in crude rat cerebellar extracts (Dinkel *et al.*, 1998). Likewise, Raju *et al.* (2006) showed that patients with SPS had a decrease in the level of GABA(A)-receptor-associated protein (GABARAP), responsible for stability and expression of the GABA(A)-receptor. Furthermore sera from SPS patients immunoprecipitated GABARAP and significantly reduced GABA(A)-receptor expression *in vitro* suggesting that these antibodies may play a role in disease.

In addition to GAD antibodies, IgG antibodies to the intracellular synaptic protein amphiphysin purified from patients not only recognized rat CNS tissue but when injected intraperitoneally, induced stiffness with spasms in rats (Sommer *et al.*, 2005). It is important to mention that in this particular study, MBP reactive T cells were co-transferred to open the blood brain barrier and that high and repeated doses of antibodies were required to induce the clinical signs. Nevertheless these studies elegantly show that in SPS patients antibodies to neuronal antigens not only induced physiological changes *in vitro* but also mimic the human disease in experimental animals. It remains to be seen whether immunization of experimental animals with amphiphysin leads to clinical disease as observed in patients.

Diabetes and rheumatoid arthritis complicated by neuropathy

In other autoimmune disorders such as diabetes and rheumatoid arthritis (RA) antibodies to neuronal structures have also been described and are associated with peripheral neuropathies. Nearly 60% of all people with diabetes suffer from peripheral neuropathy in which the underlying mechanisms are unknown although autoimmune responses to neuronal structures may play a key role. Neurons and pancreatic beta-cells share common determinants such as GAD. In early diabetes GAD-specific antibodies are present and patients with high levels of GAD-65 antibodies were more likely to develop neuropathological complications. Although there is not a clear association between the presence GAD-65 antibodies and diabetic neuropathy autoantibodies were associated with worse peripheral nerve function (Hoeldtke *et al.*, 2000). Serum from diabetic type-1 patients reacts with neuroblastoma cells in culture and induces apoptotic effects on neurons in culture (Vinik *et al.*, 2005).

Using whole-cell recording of rat cerebellar slices it was shown that Ig in CSF from ataxic patients with anti-GAD antibodies reduced the release of GABA (Mitoma *et al.*, 2003). More specifically the pathogenic role of anti-GAD Ig in the CSF in a patient with progressive cerebellar ataxia associated with insulin-dependent diabetes was clearly demonstrated by a selective suppression of the inhibitory postsynaptic currents in Purkinje cells (Ishida *et al.*, 2007). As well as anti-GAD antibodies, anti-neurofilament antibodies are present in patients with RA complicated by peripheral neuropathy and may correlate with extent of neuropathological disease activity (Salih *et al.*, 1998). While the mechanism of neuronal damage is unclear, Towns *et al.* (2005) showed that serum from diabetic patients with neuropathy induced autophagy, a lysosome-dependent degradation pathway of cytoplasmic contents, in the neuronal SH-SY5Y cells. The effect was abolished when serum was incubated with Protein L beads, which bind immunoglobulin, suggesting that autophagy was induced by autoantibodies present in the patients' serum.

Mechanisms underlying the neurological complications in diabetic patients have been studied in the experimental models of diabetes namely non-obese diabetic (NOD), streptozotocin (STZ)-induced diabetic mice (type 1 diabetes), db/db mouse (type 2 diabetes) and diabetic BBW rats (Calcutt, 2002, Schmidt *et al.*, 2003). As well as diabetes these animals also develop neuropathy similar to diabetic patients providing a model to study mechanisms of disease. While the role of antibodies in neuropathy is unknown, autoreactive T cells are observed in the peripheral nerves although neuropathy is not dependent on perforin or Fas pathways (Bour-Jordan *et al.,* 2005). To date similar studies on the mechanisms underlying neuropathy in patients with RA have not been performed in experimental arthritis in animals.

Movement disorders, encephalitis lethargica and infections

In 1916 von Economo described a neurological disease characterized by sleep and associated with basal ganglia signs and neuropsychiatric sequele. While there was speculation that Encephalitis lethargica (EL) was associated with the 1918 influenza pandemic investigators found no evidence of influenza RNA. More recently Dale *et al.* (2004) have described patients with similar characteristics as von Ecomos EL patients which similarly developed parkinsonism and neuropsychiatric symptoms following pharyngitis. Rather than viral etiology, antibodies to streptolysin-O were elevated in 65% of the patients. As in Sydenham's chorea (Church *et al.*, 2003), EL patients develop antibodies against cells within the basal ganglia and respond well to steroids suggesting a role for autoimmunity in disease in particular anti-basal ganglia (neuronal) antibodies. Sydenham's chorea occurs after infection with group A β-haemolytic streptococcal bacteria and is characterized by involuntary, purposeless movements of the limbs. Neurological diseases occurring after such infections also include tics, and dystonia, emotional disorders such as Tourette's syndrome, obsessive-compulsive disorder, anxiety and depression. However, experimental models of these disorders are lacking and the pathological relevance of antibodies is unclear.

Antibodies to basal ganglia antigens in patients with movement disorders probably arise due to cross-reactivity with streptococcal infections although their pathogenicity remains unknown (Martino and Giovannoni, 2004). Serum from patients with Sydenham's chorea, but not other streptococcal disorders, bound to the surface of human neuronal cells and recognized the neuronal glycolytic enzymes aldolase C, γ-enolase, and pyruvate kinase (Dale *et al.*, 2006). Subsequent studies showed that such antibodies were also present in other patients with post-streptococcal infection presenting with a spectrum of movement and psychiatric disorders. The association of the antibodies with the disease was examined *in vitro* whereby serum from patients with Sydenham's chorea were found to induce calcium/calmodulin-dependent protein kinase activity that leads to dopamine release (Kirvan *et al.*, 2006). While further studies are necessary to define the mode of action of antineuronal antibodies in Sydenham's chorea itself, Dale *et al.* (2006) demonstrated that commercial antibodies to neuronal glycolytic enzymes induced apoptosis in cultured neurons.

The relevance of anti-brain antibodies in the movement disorder Tourette's syndrome was examined in rats infused with serum or purified IgG from patients. The antibodies induced involuntary movements in the animals similar to that seen in patients and examination of the CNS demonstrated antibodies bound to the striatal neurons (Hallett *et al.*, 2000). It remains to be determined if this phenomenon occurs in other disorders or if there is a pathogenic role for cell-mediated immune response to basal ganglia antigens.

To establish a model of these disorders SJL/J mice were immunized with homogenates of the group A β-haemolytic streptococcal bacteria in CFA (Hoffman *et al.*, 2004). Mice developed behavioural changes and autoantibodies in the serum that reacted histologically against neurons within the deep cerebellar nuclei, globus pallidus, and thalamus. In addition IgG deposits were observed in the neurons suggesting that antibodies cross-reactive with brain components may play a role in disease.

Autoimmunity to the neuronal antigen heterogeneous nuclear ribonuclear protein A1 (hnRNP-A1) has been demonstrated in patients with human T-lymphotropic virus type 1 (HTLV-1)-associated tropical spastic paraparesis (HAM/TSP). IgG isolated from HAM/TSP patients contains antibodies to neurons as determined by western blot studies on neuronal homogenates and more specifically to proteins identified as hnRNP-A1. A functional significance of such IgG in patients was confirmed using patch clamp recording of neurons from rat brain sections where infusion of IgG inhibited neuronal firing (Levin *et al.*, 2002).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease affecting adults. Research on mechanisms of neurodegeneration has focused on oxidative stress, glutaminergic excitotoxicity and aberrant protein aggregation, such as NF, in the neurons although ALS has also been suggested to be an unconventional autoimmune disease (Drachman and Kuncl, 1989). This is supported by findings of autoimmunity to neuronal antigens since immunoglobulin is present in the motor neurons of ALS patients more frequently than serum from controls (Fishman and Drachman, 1995). IgG and IgM to spinal cord proteins and gangliosides (GM1 is located on the outer surface of motor neurons) are present in the serum and CSF of ALS patients (Annunziata *et al.*, 1995, Niebroj-Dobosz *et al.*, 2006, Niebroj-Dobosz *et al.*, 2004) and responses to NF are associated with the slow evolution of disease in a subgroup of patients (Couratier *et al.*, 1998). In a more extensive study high frequencies of antibodies to tubulin and NF-H were observed in ALS patients but also in patients with other neurological disorders (Terryberry *et al.*, 1998). In culture, IgG from ALS patients recognizes L-type VGCC resulting in an inhibition of dopamine mediated by L-type calcium channels in cultured PC12 cells (Offen *et al.*, 1998) and induces calcium dependent motor neuron cell death that is prevented by preincubating IgG with purified intact L-type VGCC (Smith *et al.*, 1994). That ALS Ig may have a pathogenic effect *in vivo* was demonstrated following injection of mice with IgG from ALS patients. IgG form ALS patients (but not control IgG) was observed in motor neurons and at the neuromuscular junction of mice (Appel *et al.*, 1991). These mice had increased miniature end-plate potential frequency, with normal amplitude and time course and normal resting membrane potential, indicating an increased resting quantal release of ACh from the nerve terminal. Similar studies

were reported by Pullen and Humphreys, (2000) in which Ig from ALS patients was present in the cell bodies of lumbar motor neurons and EM studies revealed histological abnormalities including abnormal formation of Nissl bodies and fragmented Golgi and ER.

Several models of ALS have been developed and have contributed to the understanding of ALS. For example the pathogenic role for autoimmune responses to neuronal antigens was demonstrated in guinea pigs immunized with purified bovine spinal motor neurons or with bovine spinal cord ventral horn homogenate (Engelhardt *et al.*, 1989, Engelhardt *et al.*, 1990). Immunization with purified bovine spinal motor neurons caused signs of lower motor neuron deficits. In comparison guinea pigs immunized with bovine spinal cord ventral horn homogenate developed acute muscle weakness and paresis concomitant with inflammation in the CNS and both upper and lower motor neuron degeneration. As with the human disorder, IgG deposits at the motor end plate and within the motor neurons were observed. Passive transfer with immunoglobulin from guinea pigs with disease into naive mice demonstrated the presence of IgG in motor neurons and at the neuromuscular junction. Similar to the finding following injection of Ig from an ALS patient the mice showed an increase in miniature end-plate potential frequency providing evidence for autoimmune mechanisms in the pathogenesis of both the animal models and human ALS (Appel *et al.*, 1991). Immunization of guinea pigs with choline acetyltransferase was shown to induce lower motoneuron destruction and striated muscle atrophy. As with the other models IgG was detected in lower motor neurons and at the motor end-plate and transfer of antibodies into mice induced selective lower motor neuron damage (Engelhardt *et al.*, 1997).

Alzheimer's disease

Alzheimer's disease (AD) is the most common human neurodegenerative disease characterized by progressive cognitive decline. The conventional paradigm is that abnormally folded proteins accumulate in neurons leading to neuronal death via apoptosis. However, inflammatory processes, and possibly autoimmunity, may also play a role in disease since antibodies, complement components and T cells ($CD4^+$ and $CD8^+$) are observed in the brains of AD patients. Moreover, the presence of antibodies in sera of AD patients targeting specific neuronal antigens such as NF-H has lead to the suggestion that AD should be classified as an autoimmune disorder (D'Andrea, 2005). That immune responses may play a role in AD is supported by the finding that anti-inflammatory approaches reduce disease severity. In addition, T cells from AD patients recognize a major component of the protein aggregates, amyloid-β (Aβ). In the search for autoantigens antibodies to spectrin (Fernandez-Shaw *et al.*, 1997), choline acetyltransferase (Engelhardt *et al.*, 1997), tau protein (Rosenmann *et al.*, 2006b) and aldolase A (Mor *et al.*, 2005) have been reported in AD.

Autoimmunity to neuronal antigens is significantly higher in older (aged 70-79) than in younger (aged 40-59) subjects, which may explain why neurodegenerative disorders generally occur at a later age. More specifically AD patients have IgG antibodies that bind more to ventral root cholinergic neurons which is enriched in phosphorylated NF-H, an observation that distinguished normal aged persons and those with AD (Soussan *et al.*, 1994).

Also in patients with AD antibodies to the extracellular domain of β-amyloid precursor protein (β-APP) induced morphological changes in neuronal cultures consistent with apoptosis and may be the mechanism by which neuronal death in AD occurs (Rohn *et al.*, 2000). Of relevance to disease was the report that injection of purified IgG from AD patients into rat basal forebrain decreased the numbers of cholinergic neurons. Injection of IgG from controls had no effect, suggesting that autoimmunity may play a major role in neurodegeneration in AD patients (Engelhardt *et al.*, 2000).

To examine the pathogenic potential of antibodies to cholinergic neurons Chapman *et al.* (1989) immunized rats with cholinergic neurons. Repeated immunizations induced antibody responses and immunoglobulin was detected in neurons. The animals displayed behavioural deficits involving spatial awareness and memory. In another study, similar signs were observed in rats repeatedly immunized with cholinergic Torpedo neurons (Michaelson *et al.*, 1990). Hippocampal neurons contained IgG and tangle-like structures. Experimental AD was also induced following prolonged immunization of rats with NF-H protein of cholinergic neurons and again revealed IgG in neurons and cognitive deficits (Oron *et al.*, 1997) or immunization of guinea pigs with septal cholinergic neurons (Kalman *et al.*, 1997).

Aβ and β-synuclein are neuronal proteins that accumulate in plaques in AD and PD. Immunization with Aβ or β-synuclein was proposed as a therapy for reducing protein accumulations in the brain. Contrary to the expectations, some treated patients as well as mice immunized with Aβ peptide developed encephalitis (Furlan *et al.*, 2003, Nicoll *et al.*, 2003). Similarly, immunization of rats with peptides of β-synuclein induced pathogenic T cells that resulted in acute paralytic encephalitis and uveitis following adoptive transfer (Mor *et al.*, 2003). In another study mice, immunized with recombinant human tau protein developed neurological deficits and histopathological features of AD and tauopathies, such as the presence of neurofibrillary tangle-like structures, axonal damage, gliosis and inflammation in the CNS (Rosenmann *et al.*, 2006a). Although the clinical signs of immunized mice did not mimic the symptoms observed in human AD, these data do provide proof of principle that autoimmunity to CNS neuronal antigens leads to neuronal deficits suggesting that autoimmunity may also play a role in human disease.

Parkinson's disease

Idiopathic Parkinson's disease (PD) is a progressive neurological disorder characterized by loss of motor control as observed by for example rigidity of muscles and gait dysfunction. The pathology of PD is predominantly the degeneration of dopaminergic (DA) neurons within the substantia nigra and intracytoplasmic inclusions known as Lewy bodies which contain accumulated α-synuclein fibrillar aggregates. The cause of PD is unknown although mutations in e.g. α-synuclein have been implicated in susceptibility to disease. While α-synuclein can form several different aggregate morphologies including oligomers, protofibrils and fibrils, the role of these morphologies in the progression of PD is not known.

It is clear that immune responses play a crucial role in disease since substantia nigra degeneration is associated with microglia activation and anti-inflammatory approaches appear to control disease. $\gamma\delta^+$ T cells are increased in patients with PD (Fiszer *et al.*, 1994) and more recently Orr *et al.* (2005) describe the presence of immunoglobulin on DA neurons and Lewy bodies in PD patients while FcR were expressed by adjacent microglia. Whether the elevated $\gamma\delta^+$ T-cell population in Parkinson's disease reflects previously unrecognized inflammation or may occur also in non-inflammatory disorders remains to be elucidated. The strongest evidence that autoimmunity to DA neurons may play a role in disease comes from studies showing that the CSF of patients is toxic to DA cells in culture (Dahlstrom *et al.*, 1990) via complement-dependent mechanisms (Defazio *et al.*, 1994). Furthermore microinjection of IgG from the sera of PD patients in the substantia nigra of rats induced severe neuronal loss concomitant with microglia activation. That microglia, activated by lipopolysaccharide, induced injury to DA neurons in culture (Le *et al.*, 2001) suggests that activated microglia could also induce neurological disease *in vivo*. The role for Fcγ receptors on microglia in the autoimmune attack on nigral neurons is reflected by the absence of neuronal damage in mice lacking Fcγ receptors following injection of IgG from PD patients (He *et al.*, 2002). More recently Huber *et al.* (2006) showed that antibodies in the sera of PD patients bind to DA neurons and reduce dopamine production suggesting the potential for autoimmunity in disease.

To investigate a pathogenic role for autoimmune mechanisms in PD guinea pigs were immunized with homogenates of bovine mesencephalon (containing neurons of the substantia nigra). While no overt clinical signs of basal ganglia dysfunction were observed animals showed evidence of neuronal damage in the substantia nigra concomitant with a decrease in tyrosine hydroxylase activity and DA content. IgG was observed in neurons in the substantia nigra (Appel *et al.*, 1992). In a follow-up study Le *et al.* (1995) immunized guinea pigs with dopaminergic MES 23.5 cells which resulted in hypokinesia in over 50% of the animals. Severe loss of substantia nigra neurons, decreased tyrosine hydroxylase activity and dopamine content as well as IgG deposition within neurons in the substantial nigra was observed. These data suggest

that a similar mechanism in which autoimmunity to substantia nigra neurons induces neurodegeneration in animals may operate in Parkinson's disease in humans.

Brain trauma

During injury of the nervous system for example as a result of mechanical stress, antigens may become exposed to the immune system and may provoke an autoimmune response. For example, in 40% of patients with traumatic brain injury T cells reactive against myelin antigens are observed (Cox *et al.*, 2006). Yet, the massive release of brain antigens and early expansion of myelin-specific T cells in local lymph nodes does not lead to autoimmune neurological disease, at least in animals (Kwidzinski *et al.*, 2003). Only few studies have examined the presence of autoantibodies to neuronal antigens following brain trauma in humans. Increased levels of NF-L protein in the CSF is observed following cerebrovascular accidents, subacute haemorrhage and severe brain traumas (Van Geel *et al.*, 2005). While these may reflect the degree of axonal damage these antigens may also trigger a pathogenic immune response.

IgG and IgM antibodies to beta-tubulin class III (betaTcIII), which is almost exclusively found in neuronal cytoskeleton is present in the serum of people shortly after brain trauma (Skoda *et al.*, 2006). Also serum from mice with experimental spinal cord injury recognizes CNS proteins and binds to DNA and RNA (Ankeny *et al.*, 2006). In adult rats in which brain trauma was induced, circulating IgG autoantibodies were also directed against neurons (Rudehill *et al.*, 2006). The pathogenicity of these anti-brain antibodies that develop following trauma is currently unclear. Antibodies may be epiphenomenal or even play a beneficial role as antibodies to self antigens help remove dead cells by enhancing phagocytosis (Stein *et al.*, 2002). However, as discussed above antibodies do not generally reach the CNS due to an intact BBB. During mechanical injury to the brain the BBB is compromised and serum antibodies may reach the CNS, where they may promote repair.

In summary, trauma to the CNS does evoke autoimmunity to CNS components and could induce disease although the evidence is weighted to such responses having a protective effect.

Epilepsy

Rasmussen's syndrome (RS) is a rare slowly progressive neurological childhood illness characterized by frequent and severe seizures, loss of motor skills and speech and paralysis on one side of the body. The encephalitis observed is suggested to result from autoimmunity following CMV and EBV infection although this is still controversial. The idea that neuronal damage is due to autoimmunity was suggested by the fact that plasma exchange and IVIg as well as immunosuppressive therapies are beneficial in some patients. Patients have antibodies and T-cell responses directed

to glutamate receptors (GluR3) (Takahashi *et al.*, 2005). The pathogenic effect of the anti-GluR antibodies is reflected by their association with the frequency of seizures. In addition, in cultured neuronal cells, antibodies to GluR3 act as ligands for AMPA subtype of glutamate receptors and evoke glutamate-like ion currents (Twyman *et al.*, 1995) which may explain the clinical phenotype in humans. The pathogenic potential of the AMPA receptor subunit GluR3 in RE was reported by Levite and Hermelin, (1999) who immunized four strains of mice with the Glu3RB peptide (aa 372-395). The mice developed antibodies and T cells to the peptide and behavioural abnormalities but not epilepsy. The murine antibodies bound to neurons *in vitro* and evoked GluR channel activity, mimicking the pathophysiological effects of excess of glutamate inducing neuronal death (Levite *et al.*, 1999). In another study rabbits immunized with peptides of the receptor developed anti-GluR3 antibodies that bound to neuronal cultures and induced apoptosis (Twyman *et al.*, 1995). More relevant was the finding that the rabbits exhibited recurrent episodes of seizures as well as inflammatory lesions in the CNS resembling human RE. Taken together, these studies demonstrate that antibodies to a specific peptide of the GluR can kill neurons by an excitotoxic mechanism, and induce clinical features resembling the human disease RE.

Other neurological disorders

Antibodies to neurofilament proteins have been associated with a variety of disorders including neuropathy with monoclonal gammaopathy of undetermined significance (MGUS). In these patients antibodies to NF-H in the serum bind to neurons (Stubbs *et al.*, 2003). The pathogenic potential of serum containing anti-NF antibodies was demonstrated following injection of rat sciatic nerve. Serum containing anti-NF antibodies but not control serum altered the axonal caliber and induced vesiculation and ovoid formation leading to peripheral nerve conduction block (Stubbs *et al.*, 2003). Furthermore patients with slowly progressive sensory motor axonal neuropathology have a monoclonal IgG-kappa that bound to NF protein (Nemni *et al.*, 1990).

Growing evidence also demonstrates the presence of autoantibodies to neurons in psychiatric disorders such as schizophrenia (Margutti *et al.*, 2006) and in autistic disorders in which patients display impaired socialisation and abnormal patterns of behaviour. While the exact cause of the disorder is unknown, genetic, biochemical and environmental causes have been suggested to play a role. One factor may be abnormal immune responses since patients with autism come from families in which autoimmune disorders cluster. In the autistic person, autoantibodies to myelin proteins and neuronal antigens such as NF are observed (Cohly and Panja, 2005) and altered T-cell functions and cytokine levels (Zimmerman *et al.*, 2005) have been reported. In an extensive study examining responses to human brain proteins serum antibodies in autistic subjects more frequently and intensely responded to antigens in caudate,

putamen and prefrontal cortex as well as cerebella and cingulated gyrus antigens than aged matched controls (Singer *et al.*, 2006).

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disorder in which the CNS may be involved and in which a significant loss of central neurons may occur. In some cases SLE patients present with Parkinsonian-like deficits, changes to the basal ganglia and axonal dysfunction although the underlying mechanisms of such damage are unknown. Antibodies to double stranded DNA, present in the serum of SLE patients, are known to deposit in the kidney and skin contributing to disease. DeGiorgio *et al.* (2001) demonstrated that a subset of the dsDNA antibodies, present in the CSF and serum of SLE patients also recognized NR2A and NR2B subunits of the NMDA receptor and reduced the viability of brain cells *in vitro* (Sakic *et al.*, 2005). Antibody responses to microtubule-associated protein 2 (MAP-2), a cellular protein restricted to neurons, is also a common finding in neuropsychiatric SLE (Williams *et al.*, 2004). That intrathecally synthesized autoantibodies from SLE patients with neuropyschiatric symptoms may be pathogenic is demonstrated by the finding that CSF IgG of SLE patients reduces the viability of a neural stem cell line *in vitro* (Sakic *et al.*, 2005). In a further study anti-NMDAR antibodies in serum and CSF from SLE patients induced cognitive impairment in mice injected with LPS to compromise the BBB integrity. Mice also exhibited neuron damage and memory impairment. Moreover immunoglobulin eluted from the brains of SLE patients, bound to DNA and NMDAR and caused neuronal apoptosis in mouse brains. Despite these findings further studies are necessary to reveal the mechanism of autoimmunity to NMDAR in neuropsychiatric lupus and the approaches by which such patients may be treated (Kowal *et al.*, 2006).

Spontaneous development of lupus-like disease in the MRL/lpr mouse model has been valuable to study mechanism underlying neuropsychiatric SLE. SLE in the MRL/lpr mouse is accompanied by impaired dopamine catabolism and degenerating axon terminals in the mesencephalon (Hikawa *et al.*, 1997). Mice show behavioural dysfunction similar with progression of SLE in humans. The neurodegeneration in MRL/lpr mice (Ballok, 2007) is associated with the cytotoxicity of the IgG fraction CSF on neurons (Sidor *et al.*, 2005) which was attenuated by the immunosuppressive drug cyclophosphamide (Ballok *et al.*, 2004). Although the specificity of the mediators in the CSF is unknown, it is probable that autoimmunity to neurons contributes to pathology in the MRL/lpr mouse.

The pathogenic significance of antibodies to NR2A and NR2B subunits of the NMDA receptor which are present a subset of SLE patients was studied in BALB/C mice in which epinephrine was used to compromise the BBB. These mice shown a decrease in neurons in the lateral amygdala and develop cognitive and emotional behaviour changes (Huerta *et al.*, 2006).

Neuronal and axonal reactive T cells

Many neurological disorders discussed so far have been associated with antibodies to neuronal antigens however in some cases cellular responses are also observed. Evidence that T cells may be involved in the pathogenicity of neurodegenerative disorders comes from the observation of T cells in the nervous system as well as the CSF and blood both in the PNS and CNS disorders. T cells are present in nervous tissue from patients with ALS, MS, GBS as well as patients with paraneoplastic cerebellar degeneration. In many cases it is unclear what these T cells recognize and indeed whether they are pathogenic. The paucity of evidence comes from studies of paraneoplastic disorders. Autoreactive cytotoxic T lymphocytes (CTLs) specific for a cytoplasmic protein of Purkinje cells PCD17/cdr2 are present in the blood of patients (Sakai *et al.*, 2001) suggesting that they may play a role in the ensuing neuronal damage. Likewise T-cell responses to Hu-D peptides are present in patients with neuronal and axonal injury particularly those with the common paraneoplastic neurologic syndrome anti-Hu syndrome (Rousseau *et al.*, 2005). The pathological significance of such responses were demonstrated by Tanaka *et al.* (1999), whereby activated $CD8^+$ T cells from a patient lysed autologous fibroblasts following induced expression of HLA class I and HuD protein. These authors also demonstrated cytotoxic T-cell activity to Yo protein expressed by autologous dendritic cells suggesting that CTLs could be involved in Purkinje cell loss in paraneoplastic cerebellar degeneration (Tanaka *et al.*, 1998).

Conversely culture of human neurons with activated T cells leads to neuronal death through contact-dependent non-MHC class I mechanisms (Giuliani *et al.*, 2003). Neuronal structures may be key targets in disease as demonstrated by elegant studies using two-photon microscopy in living brain tissues (Nitsch *et al.*, 2004). However while these studies provide compelling evidence for a role of T cells in neurodegeneration whether this is the result of an autoimmune phenomenon is unclear. In some disorders such as following facial nerve axotomy while T cells are observed close to injured nerves they appear to confer neuroprotection to injured motor neurons (Ha *et al.*, 2006). This protective role for CNS specific T cells may be why patients with brain injury that developed reactivity to myelin had a much more favourable outcome. However, such a 'protective' role for antibodies is unknown. Likewise CNS trauma following spinal cord injury induces activation of T cells and enhances macrophage activity but this may be concomitant with increased functional impairment (Jones *et al.*, 2005).

EVIDENCE FOR ANTINEURONAL AUTOIMMUNITY IN MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is considered an autoimmune disease of the CNS and is characterized by demyelination and axonal loss. While research in MS has focused on

pathogenic T cells targeting myelin antigens relatively few studies have described a role for pathogenic antibodies to neuronal proteins. Moreover despite Charcot's detailed description of the pathology of MS in a lecture in 1868 on the extent of inflammation and axon loss it has only been recently that researchers returned to examine the mechanism of axonal and neuronal loss in MS. Undoubtedly the development of magnetic resonance spectroscopy has allowed a unique view of the progression of the disease in patients with time. Such approaches have helped clarify the relationship of axonal damage and atrophy of the brain to the irreversible neurological disability. Nevertheless the cause of axonal damage remains unclear. As explained above the role for autoimmunity, in particular autoantibodies to neuronal antigens has been clearly demonstrated in other neurodegenerative disorders. The realisation that MS may also be classified as a neurodegenerative disorder in which autoantibodies to neuronal antigens may also play a role may help to not only uncover the pathogenic role for such autoimmune responses but may also lead to novel therapeutic strategies.

Before completely dismissing the role of neuronal specific T cells in MS it is pertinent to mention a study in which T cells recognizing synapsin, a highly conserved neuronal protein, were detected in MS patients (Polak *et al.*, 2001). However, in the animal model at least, T cells recognizing the myelin protein MBP also recognized synapsin (De Santis *et al.*, 1992) suggesting that myelin specific T cells could also induce neuronal damage via cross-reactivity with neuronal antigens. So far T-cell reactions to other neuronal or axonal proteins have not been described.

Recognition of neurons and thus T-cell activation leading to neurological damage requires the expression of key molecules for antigen presentation, such as MHC and co-stimulatory molecules. For direct attack on neurons this requires neurons and axons to express MHC class I for recognition and killing by $CD8^+$ specific T cells. Although it has become clear that $CD8^+$ T cells greatly outnumber $CD4^+$ T cells in MS lesions (Babbe *et al.*, 2000), very little information is available to show whether or how T cells may kill neurons. In the normal brain, neurons express negligible levels of MHC-class I probably to avoid such lethal consequences. However during inflammation neurons and axons may upregulate MHC class I (Neumann *et al.*, 1995, Redwine *et al.*, 2001). Correspondingly, in MS lesions axons have been found positive for MHC class I (Hoftberger *et al.*, 2004). *In vitro*, it has been shown that axons upregulate MHC class I after IFN-γ and tetrodotoxin treatment, resulting in transection of neurites by cytotoxic T cells (Medana *et al.*, 2001). More evidence for the impact of T cells on neurons comes from studying interactions of PLP-specific T cells in brain slice cultures. While not specific for neurons, activated PLP-specific T cells induced calcium changes in neurons leading to neuronal damage (Nitsch *et al.*, 2004). Likewise MBP-specific T cells are more effective in activating microglia that have axon-damaging ability as observed in organotypic CNS cultures (Gimsa *et al.*, 2000) suggesting that in some way CNS specific T cells augment the CNS damage.

To study the possible involvement of autoimmunity to neuronal antigens in MS several studies have used the experimental model EAE. In rat EAE, depletion of synaptic terminals during acute disease has been described suggesting that neuronal structures may also be target for immune responses. Similar to several of the neurodegenerative diseases in humans and the experimental models rats immunized with bovine myelin, immunoglobulin deposits are observed in the spinal cord neurons (Slavin *et al.*, 1996). This staining may however be due to the cross-reactivity of myelin proteins with neuronal proteins since T cells to MBP also recognize synapsin (De Santis *et al.*, 1992).

The evidence for the presence of antineuronal antibodies in MS is clear. Axon-reactive B cells can be detected in MS lesions and isolated from CSF from patients and immunoglobulin was found attached to axons in the lesions (Zhang *et al.*, 2005a, Zhang *et al.*, 2005b). These B cells were recently shown to be directed against the glycolytic enzymes triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kolln *et al.*, 2006). However, these enzymes are ubiquitously expressed and this does not seem to correspond with the specific CNS involvement in MS. In a review by (DeVries, 2004) evidence is described whereby antibodies to axolemma-enriched fractions may play a role in axonal destruction. Indeed such antiserum has been shown to induce neuronal changes *in vitro* like destruction of neurites and inhibition neurite outgrowth.

Another neuronal protein proposed as an autoimmune target in MS is neurofilament light (NF-L), a cytoskeletal protein expressed in neuronal cells. The protein has been used as a biomarker in the CSF to measure the degree of neuronal damage in MS patients and in animals with EAE (Norgren *et al.*, 2005, Semra *et al.*, 2002). However antibodies to NF-L have been described in both serum and CSF of MS patients (Ehling *et al.*, 2004, Eikelenboom *et al.*, 2003, Silber *et al.*, 2002). Among all subgroups in MS, patients with progressive disease seem to have elevated levels of antibodies to NF-L. In one study, patients with primary progressive MS had higher levels of antibodies to NF-L in the serum, but not in the CSF, than patients with secondary progressive MS (Ehling *et al.*, 2004). In another study, patients with primary progressive MS or secondary progressive MS had higher levels of Abs to NF-L than patients with relapsing-remitting MS (Silber *et al.*, 2002). Significant differences in levels of NF-L Abs between different MS subtypes, however, have not been found in other studies (Eikelenboom *et al.*, 2003), possibly due to differences in the selection of patients.

An important question is whether the antibody levels to NF-L in MS patients are reflecting disease progression. To address this, anti-NF-L indices have been correlated with MRI measures of tissue damage. It appears that the anti-NF-L index in MS patients, especially in the relapsing-remitting subgroup, correlates with MRI measures for axonal loss (Eikelenboom *et al.*, 2003). However, in that study there were no adjustments made for possible confounding factors, such as age which is known to influence both NF-L antibody levels and cerebral atrophy (Ehling *et al.*, 2004, Resnick *et al.*, 2003).

NF-L was also identified as an antigenic protein in MS, using serological proteome analysis. i.e. 2D SDS-PAGE, followed by immunoblotting and mass spectrometry (Almeras *et al.*, 2004). In another paper from that same group, NF-L was identified as a discriminate antigen between MS and healthy controls or Sjögren syndrome patients (Lefranc *et al.*, 2004). In fact, the frequency of sera positive for NF-L was lower in MS patients compared to healthy controls or Sjögren syndrome patients. This finding is in contrast with other studies that describe that antibodies to NF-L are more frequently found in MS patients than in healthy controls (Newcombe *et al.*, 1985).

Thus, while it is clear that antibodies against NF-L protein are present in MS patients the pathogenicity of these anti-NF-L immune responses is not known and requires further studies in experimental animals.

MECHANISMS OF IMMUNE-MEDIATED DAMAGE AND PERSPECTIVES FOR THERAPY

How autoimmunity to neuronal structures develops is debatable. Evidence for cross-reactive immune responses between infectious agents and self-antigens i.e. molecular mimicry is well-accepted for GBS but is nevertheless controversial for other diseases like MS. Exposure to environmental agents such as neurotoxic agents has also been suggested since autoantibodies to neuronal specific antigens such as neurofilaments were prevalent in people exposed to lead or mercury and correlated with degree of exposure. In rats such exposure related to the pathology in the nervous system and antibodies purified from humans and rats exposed to environmental chemicals interfered with neuromuscular function following injection in experimental animals (El-Fawal *et al.*, 1999).

One question is how, following such exposure to infectious agents or environmental neurotoxic agents and neurological damage, does release of neuronal antigens induce autoimmune responses? The CNS has always been regarded as an 'immune-privileged' organ lacking classical lymphatic drainage, however it is now realised that dendritic cells are able to migrate from the CNS to cervical lymph nodes (CLN) (Hatterer *et al.*, 2006). In addition, lymphatic structures in the meninges, present in MS patients, could be potential sites where antigen-presenting cells can come in contact with T cells (Serafini *et al.*, 2006). In MS and EAE, myelin antigens are present in the cervical lymph node (De Vos *et al.*, 2002), but whether this results in pathogenic autoimmunity or a mechanism by which the CNS limits 'pathogenic responses' is still unclear. Alternatively, axonal antigens, released in soluble form upon CNS damage (Galea *et al.*, 2007), may reach the regional lymph nodes although the exact pathways require further study. Harling-Berg *et al.* (1999) showed indeed that injection of proteins in the ventricles or

the brain parenchyma drained to the CLN and this route was very effective for mounting a humoral immune response.

As discussed in this review pathogenic autoimmunity to neuronal structures are present in patients and the question is 'what are the mechanisms by which such responses cause injury to neurons?' One such way is by interfering with neuronal function. Neurofilaments are synthesized and assembled in neuronal cell bodies, transported along axons and degraded in the synapse. In several pathological diseases such as ALS, diabetic neuropathy and Parkinson's disease, where NF aggregates form Lewy bodies, and in neurofibrillary tangles in Alzheimer's disease, neuronal proteins aggregate in the cell bodies and axons. In peripheral disease such as Charcot-Marie-Tooth syndrome (Brownlees *et al.*, 2002) abnormalities in neurofilament assembly and axonal transport is due to mutations in the protein. Such abnormalities in alpha-internexin as well as NF have also been identified in neuronal intermediate filament inclusion disease (Momeni *et al.*, 2006). Aggregation of NF proteins may be due to mutations in the proteins themselves, or alternatively immunological mechanisms may induce abnormal function leading to aggregation for example by cross-linking of antibodies recognizing neuronal structures. Microinjection of antibodies to kinesin and dynein, which are involved in axonal transport of NF, impaired anterograde and retrograde NF transport (Theiss *et al.*, 2005).

FcR-mediated phagocytosis aids the uptake and clearance of, for example, bacteria coated or 'opsonized' with antibodies. Immunoglobulin bound to FcR triggers numerous biological activities in effector cells depending on whether they activate immunoresponsive tyrosine activating motifs (ITAMS) or inhibitory motifs (ITIMs), which are phosphorylated by tyrosine kinases during signal transduction. In the peripheral nervous system FcR are expressed by Schwann cells (Vedeler *et al.*, 1991) and in the CNS FcR are widely expressed by microglia. Few studies have described such expression on neurons *in vivo* (Andoh and Kuraishi, 2004) although expression of FcR is observed on a neuronal cell line *in vitro* (Gorini *et al.*, 1992). The high affinity receptor IgG receptor FcγRI is expressed on primary sensory neurons and is activated by Ig-antigen complex which leads to increased intracellular calcium, and release of neurotransmitters. Excessive release is associated with neuronal damage and disease. As described above Ig from ALS patients is taken up by neurons and may enhance neuronal damage by exacerbating neurotransmitter release. Mohamed *et al.* (2002) elegantly showed that F(ab')2 fragments of Ig from ALS patients are not taken up by neurons and thus do not induce neurotransmitter release. Likewise neurons in mice lacking the gamma subunit of the FcR, fail to take up ALS IgG and the acetylcholine release were markedly reduced. Thus targeting this pathway may help in the treatment of ALS and possibly also other neurodegenerative disorders such as MS.

For several disorders such as MG and paraneoplastic disorders, removing the source

of antibody (tumour) or blocking activity with for example IVIg results in improvement of the clinical disease. IVIg has slight but detectable beneficial effects on diabetic patients presenting with axonal damage and is a recognized therapy in MS. It is unclear whether IVIg is beneficial in MS patients with axonal damage. IVIg is able to enter the CNS only when BBB function is compromised such as during EAE. In MS autoimmunity to myelin proteins has prompted therapeutic regimens such as tolerance strategies to re-establish immunological tolerance to self-antigens and regulate autoimmunity. Tolerance strategies to CNS antigens are highly effective in regulating EAE in particular chronic relapsing EAE (Heijmans *et al.*, 2005, Pryce *et al.*, 2005, Smith *et al.*, 2005). Since antibodies to neuronal and axonal antigens may have a pathogenic role in neurological diseases, tolerance strategies should also target axonal antigens.

CONCLUSIONS

Although MS has long been considered an autoimmune disorder in which aberrant immune responses attack and damage myelin it is also probable that autoimmunity to neuronal antigens plays a role in the neurodegeneration observed in disease. Similar to other neurodegenerative disorders, such as ALS, AD and PD, antibodies to axonal and neuronal proteins are present in sera and CSF of MS patients and on axons in the lesions. It remains to be determined whether neuronal protein specific antibodies augment or exacerbate axonal damage in MS as has been shown with antibodies to myelin. In any case, the draining cervical lymph nodes may play a crucial role, either by mounting a humoral response to neuronal antigens or by suppression of pathogenic autoimmunity. If indeed autoimmunity to neuronal antigens plays a role in the axonal damage seen in MS, or other neurodegenerative disorders, therapeutic strategies designed to inhibit such pathogenic antibodies may be key to the treatment of the destructive progressive disease and axonal damage associated with cognitive changes in chronic MS and neurodegenerative disorders.

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3

T-CELL RESPONSES TO NEUROFILAMENT LIGHT PROTEIN ARE PART OF THE NORMAL IMMUNE REPERTOIRE

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system in which axonal damage and degeneration contribute significantly to the progressive irreversible neurological disability. Similar to pathogenic myelin autoimmunity, autoimmune responses to neuronal antigens may contribute to axonal damage and irreversible disability in MS. Autoantibodies to the axonal cytoskeletal protein neurofilament light (NF-L) are associated with cerebral atrophy in MS, however the T-cell response to NF-L in MS patients has not been examined. Here we identify and characterize T-cell proliferative responses to NF-L as compared to myelin oligodendrocyte glycoprotein (MOG) in MS patients and healthy controls. Using a carboxyfluorescein succinimidyl ester (CFSE) dilution assay we show that while responses to MOG are dominated by $CD3^+CD4^+$ T cells, responses to NF-L were observed in both $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell populations. Both MOG and NF-L-reactive cells expressed $CD45RO^+$, indicative of a memory phenotype. Moreover, in contrast to MOG-stimulation which predominantly induced interferon-γ, both Th1 and Th2-type T-cell responses to NF-L were observed as indicated by the induction of interferon-γ, tumor necrosis factor-α as well as interleukin-4.

The finding of T-cell responses to NF-L in MS patients may reflect transient activation of pathogenic potential but their presence also in healthy controls indicates that these cells are part of the normal immune repertoire.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) in which immune-mediated destruction of myelin results in neurological dysfunction. In addition to extensive myelin loss, damage to axons and neurons is observed already early in disease (Kuhlmann *et al.*, 2002) and is considered to be a good correlate of irreversible neurological symptoms, characteristic of the progressive phase of MS (Trapp *et al.*, 1999). The mechanisms leading to axonal damage are unclear, but may involve direct autoimmune-mediated damage (DeVries, 2004).

Support for this hypothesis comes from a recent study showing that MS serum contains antibodies to neurofascin-186, a neuronal protein that is expressed at the axonal internode and becomes exposed during demyelination (Mathey *et al.*, 2007). Transfer of a monoclonal antibody to neurofascin into rats with mild experimental autoimmune encephalomyelitis (EAE) induces acute axonal damage and disrupted nerve transmission *in vitro* suggesting that circulating antibodies to neurofascin are pathogenic and may contribute to neurological disease in humans. Antibodies to several other neuronal proteins, including neurofilament light (NF-L) (Newcombe *et al.*,

1985, Terryberry *et al.*, 1998), neurofilament medium (Bartos *et al.*, 2007) and other yet unspecified neuronal proteins (Lily *et al.*, 2004, Rawes *et al.*, 1997) are also present in the serum MS patients. NF-L may be a key neuronal target since serum antibodies to NF-L are elevated in progressive MS patients (Ehling *et al.*, 2004, Silber *et al.*, 2002) and correlate with cerebral atrophy in MS (Eikelenboom *et al.*, 2003). Moreover, that immunization of mice with NF-L protein induces axonal degeneration and clinical signs including paralysis and spasticity demonstrates that anti-NF-L autoimmunity is potentially pathogenic and may contribute to progression of neurological disease (Huizinga *et al.*, 2007, Huizinga *et al.*, 2008).

In contrast to the humoral antineuronal response, reports of T-cell responses to neuronal or axonal antigens in MS are limited. Such autoreactive T cells could be involved in promoting the humoral response, but also in inducing a cytotoxic attack against neurons and axons, since neurons and axons express major histocompatibility complex (MHC) class I in MS lesions (Hoftberger *et al.*, 2004). To date, only two studies have shown that T cells from MS patients respond to neuronal antigens i.e. neuron specific enolase, arrestin (Forooghian *et al.*, 2007) and gangliosides (Pender *et al.*, 2003). Indirectly, the presence of antineuronal T cells is suggested by the presence of IgG antibodies to neuronal antigens, indicating class switching which requires the action of specific $CD4^+$ T cells.

Here we characterize the T-cell responses to NF-L in MS patients as well as in healthy controls compared to responses to the myelin protein MOG. That T-cell responses to the cytoskeletal protein NF-L are present to a similar extent in both MS patients and healthy controls indicates that such responses are part of the normal immune repertoire.

METHODS

Patients and control subjects

All experiments were approved by the local ethical committee and informed consent was obtained from all donors that participated in this study.

Patients of the MS outpatient clinic were selected on basis of the diagnosis clinically definite MS according to the Poser criteria (Poser *et al.*, 1983). Clinical details are shown in Table 1. Peripheral blood of patients ($n = 16$) and age-matched healthy controls ($n = 16$) was collected in Vacutainer CPT tubes with sodium heparin (BD Biosciences, Alphen aan den Rijn, The Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation, washed twice with RPMI 1640 medium (BioWhittaker [Lonza], Verviers, Belgium) and subsequently frozen in RPMI 1640 supplemented with 10% dimethyl sulfoxide and 20% heat-inactivated fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO). Cells were stored in liquid nitrogen until use.

Table 1. Clinical details of patients with multiple sclerosis.

Patient	Gender	Age	Diagnosis	Age at onset	CIS-CDMS time	Disease duration	OCB	Treatment
1	F	53	RR-MS	31	12 yr	10 yr	+	none
2	M	44	RR-MS	44	2 mo	2 mo	+	none
3	F	43	SP-MS	32	6 yr	5 yr	-	MP > 4 mo b.s.
4	F	48	SP-MS	34	16 mo	13 yr	-	IFN-β 7 yr
5	F	44	RR-MS	34	7 yr	3 yr	+	none
6	M	38	RR-MS	27	2 yr	9 yr	-	none
7	F	36	RR-MS	26	4 yr	6 yr	-	MP > 4 mo b.s.
8	F	35	RR-MS	18	5 yr	12 yr	+	see *
9	M	48	PP-MS	41	2 yr	5 yr	-	MP > 4 mo b.s.
10	F	61	RR-MS	48	5 yr	8 yr	-	see #
11	M	32	PP-MS	25	1 yr	6 yr	+	none
12	M	63	PP-MS	53	5 yr	5 yr	n.d.	none
13	F	53	PP-MS	42	3 yr	8 yr	-	MP > 4 mo b.s.
14	F	60	PP-MS	37	5 yr	18 yr	+	none
15	M	39	SP-MS	22	2 yr	15 yr	+	see #, §

* underwent interferon (IFN)-β treatment for 5 years and subsequently received copaxone for 14 months until 17 months before blood sampling, received MP > 4 months before sampling. # participated in Linomide trial > 7 years before sampling (active or placebo treatment unknown); § underwent IFN-β treatment for 7 years until 2 years before sampling, received MP > 4 months before sampling. b.s. before sampling; CDMS clinically definite MS; CIS clinically isolated syndrome; MP methyl prednisolone; n.d. not determined; RR-MS relapsing-remitting MS; OCB oligoclonal bands; PP-MS primary progressive MS; SP-MS secondary progressive MS.

Proliferation assays

Thawed PBMC were washed and resuspended in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum (Sanquin, Leiden, The Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (all BioWhittaker [Lonza], Verviers, Belgium). In a round-bottom plate, 2 x 10^5 cells/well were cultured for 7 days in the presence or absence of 10 μg/ml ovalbumin (Worthington Biochemical Corp., Lakewood, NJ), recombinant human MOG (Smith *et al.*, 2005) or recombinant mouse NF-L (Heins *et al.*, 1993) which has 96% sequence homology with human NF-L. The purity of the proteins was assessed by SDS-PAGE and Coomassie Brilliant Blue staining. As positive controls, 25 Lfu/ml tetanus toxoid (TT; Chiron Behring, Marburg, Germany) and 10 μg/ml phytohaemagglutinin (PHA-P) (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) were used. For the last 18 h, 1 μCi/well [^{3}H]thymidine (Amersham Biosciences, Roosendaal, The Netherlands) was added and cells were

collected on glass-fiber filters using a TomTec Mach IIIM harvester (Hamden, CT). Incorporated radioactivity was measured using a beta-plate liquid scintillation counter (PerkinElmer, Groningen, The Netherlands). Data were expressed as mean counts per minute (cpm) of triplicate wells. Stimulation indices were calculated by dividing the mean cpm of antigen-stimulated cells by the mean cpm of control cells without antigens.

To assess whether T-cell proliferation was dependent on antigen presentation via HLA class II molecules, cells were incubated with antigens in the presence of HLA class II blocking antibodies (anti-HLA class II, clone PDV5.2; anti-HLA-DP, clone B7.21; anti-HLA-DQ, clone SPV-L3 or anti-HLA-DR, clone B8.11.2; generously provided by Prof. I.I.N. Doxiadis, Leiden University Medical Center, Leiden, The Netherlands).

To characterize antigen-specific T cells, proliferation was also assessed using carboxyfluorescein succinimidyl ester (CFSE; Fluka Biochemika, Buchs, Switzerland). Cells were incubated with CFSE for 5 min at room temperature (20 °C). Before washing, heat-inactivated FBS was added until a final concentration of 10% and cells were cultured as described above. After six days, half of the medium was replaced with fresh medium containing recombinant human IL-2 (final concentration 10 IU/ml; Proleukin, Chiron BV, Amsterdam, The Netherlands) and the cells were allowed to proliferate for three more days before staining of surface markers and flow cytometric analysis.

Flow cytometry

Cells were washed with PBS and incubated for 30 min with violet-fluorescent reactive dye using the live/dead fixable dead cell staining kit (Molecular Probes [Invitrogen], Breda, The Netherlands). Cells were washed with 1% bovine serum albumin in PBS and incubated for 15 min with anti-CD4-PE (clone MT310; DakoCytomation, Glostrup, Denmark), anti-CD3-APC (clone UCHT1), anti-CD8-PerCP (clone SK1, both from BD Biosciences, Alphen aan den Rijn, The Netherlands) and anti-CD45RO-ECD (clone UCHL1; Beckman Coulter BV, Mijdrecht, The Netherlands). Non-binding antibodies were removed by washing three times with PBS/ 1% bovine serum albumin. Cells were fixed with 1% paraformaldehyde in PBS, measured on the LSRII cytometer (BD Biosciences, Alphen aan den Rijn, The Netherlands) and data were analyzed with BD FacsDiva software.

Cytokine analysis

Culture supernatants from the T-cell proliferation assays were collected on day 6 and stored at -30 °C until use. The cytokines IL-2, IL-4, IL-5, IL12p70, IFN-γ and TNF-α were measured using a cytometric bead array (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's protocol. Beads were measured on a FacsCanto cytometer (BD Biosciences, Alphen aan den Rijn, The Netherlands) and analysis was performed using the software provided by Bender MedSystems. IL-17A was measured by ELISA (Biosource, Etten-Leur, The Netherlands).

Statistical analysis

Data were analyzed using GraphPad Prism software and expressed as mean ± standard error. Statistical analysis was performed using either analysis of variance followed by Bonferroni's multiple comparison test or the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Correlations were performed with Spearman's rank test. $p < 0.05$ was regarded as statistically significant.

RESULTS

T-cell responses against NF-L are present both in MS and control subjects.

To determine whether T-cell responses to the axonal protein NF-L are present in patients with MS and to assess whether this response is different from healthy donors, we performed a thymidine incorporation assay. As a comparison, T-cell responses to the myelin protein MOG, an irrelevant protein OVA, the recall antigen TT and the mitogen PHA were measured.

We first examined the basal T-cell proliferation as determined by thymidine incorporation and observed a higher basal T-cell response in MS patients compared with responses in controls (5846 ± 689 cpm vs. 2790 ± 405 in controls; $p < 0.05$, Figure 1A). However no differences were observed in the responses to the positive control antigens

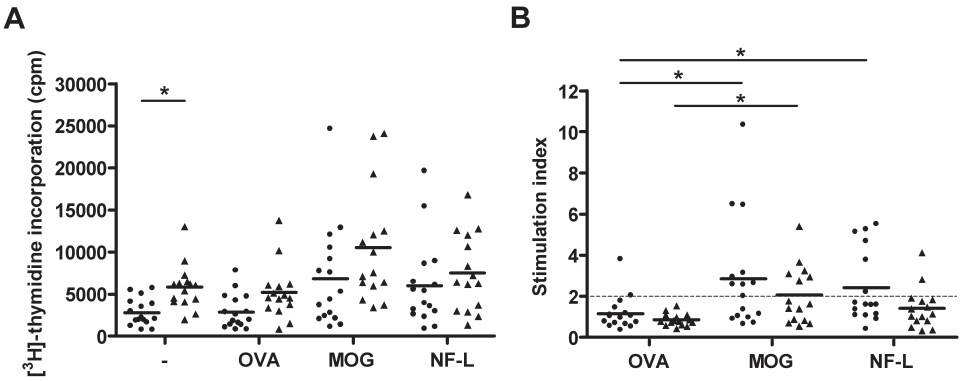

Figure 1. Similar proliferative responses to myelin oligodendrocyte glycoprotein (MOG) and neurofilament light (NF-L) in MS patients and control subjects. Peripheral blood mononuclear cells isolated from MS patients ($n = 15$) and healthy donors ($n = 16$) were cultured in the presence of 10 μg/ml ovalbumin (OVA), MOG or NF-L, and [^{3}H]thymidine incorporation was measured and expressed as counts per minutes (cpm, **A**). The average stimulation index (SI) for MOG and NF-L in control subjects was significantly higher compared with OVA (*, $p < 0.05$; Kruskal-Wallis test followed by Dunn's multiple comparison test), whereas in MS patients, only the SI for MOG was elevated compared with OVA (**B**). No significant differences in proliferative responses were observed between patients and controls.

TT and PHA (TT: mean 59652 ± 8163 cpm in controls vs. 45715 ± 7599 cpm in MS patients; PHA: mean 22670 ± 3172 cpm in controls vs. 32181 ± 3392 cpm in MS patients). One patient did not respond to either TT or PHA (SI < 2) and was therefore excluded from further analysis.

When the individual baseline proliferation of each subject was taken into account and data were expressed as stimulation indices, proliferative responses were observed to both MOG and NF-L. In control subjects, T-cell responses to MOG (defined by SI > 2) were observed in 9 out of 16 donors (mean SI = 2.86 ± 0.67; $p < 0.05$ compared with OVA; Figure 1B). Positive responses to NF-L were observed in 6 out of 16 donors (mean SI = 2.43 ± 0.44; $p < 0.05$ compared with OVA). In contrast, T-cell responses to the control antigen OVA were present in only 2 out of 16 healthy donors (mean SI = 1.16 ± 0.21). Patients with MS also showed T-cell responses to MOG (in 6 out of 15 patients; mean SI = 2.07 ± 0.36; $p < 0.05$ compared with OVA) and to a lesser extent to NF-L (in 2 out of 15 patients; mean SI = 1.42 ± 0.26), but not to OVA (mean SI = 0.86 ± 0.08). No significant differences were observed when MOG- or NF-L-responses of patients with MS were compared to those from control subjects. Of note, all MS patients and controls who responded to NF-L, also responded to MOG.

To investigate whether T-cell proliferation against NF-L required antigen presentation via HLA class II molecules, cells were incubated with NF-L in the presence of HLA class II blocking antibodies. No inhibition of T-cell proliferation was observed in the presence of blocking antibodies against the individual HLA class II molecules (either HLA-DP, -DQ or -DR). In contrast, blocking all HLA class II molecules reduced T-cell proliferation against NF-L with 61% from 1548 ± 262 cpm to 562 ± 17, which was almost near background proliferation (376 ± 70 cpm; data not shown).

In summary, T-cell responses to both the myelin protein MOG and the axonal antigen NF-L were detected at similar levels in patients with MS and healthy controls.

T-cell responses to NF-L in controls and MS patients are diverse

To further characterize the T-cell responses to MOG and NF-L, a CFSE-based proliferation assay was performed with PBMC from MS patients ($n = 11$) and control subjects ($n = 13$). Only a subgroup of patients and controls could be analyzed due to the availability of cells.

Different patterns of T-cell reactivity were observed and representative example flow cytometry plots from controls and patients illustrating these patterns are shown in Figure 2. In 4 out of 13 control subjects, a dominant response against NF-L was observed (Figure 2A), while none showed a dominant response to MOG. A further 4 of the 13 control subjects displayed a mixed reaction, both to MOG and NF-L (Figure 2B) while the remaining 5 were unresponsive to both MOG and NF-L. In contrast, only one of the 11 MS patients had a dominant response to NF-L while 4 out of 11 showed a dominant

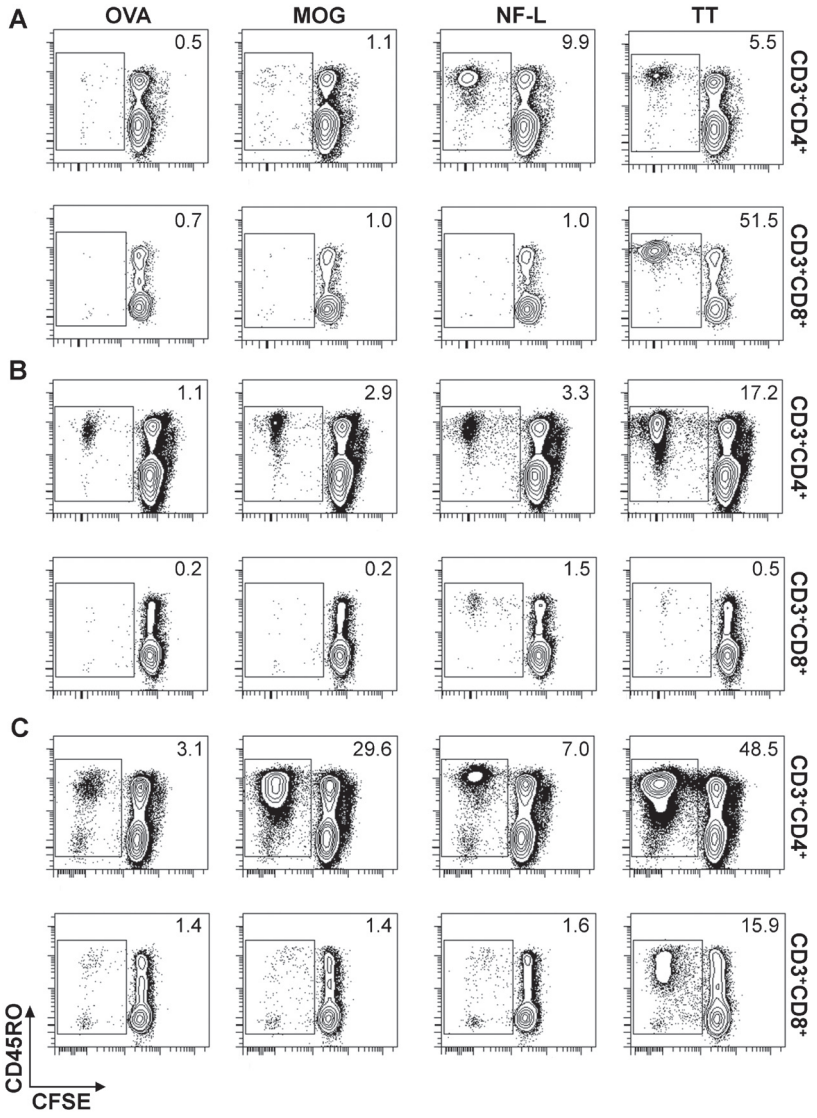

Figure 2. Carboxyfluorescein succinimidyl ester (CFSE)-proliferation reveals different patterns of T-cell responses to myelin oligodendrocyte glycoprotein (MOG) and neurofilament light (NF-L). **A-C** show representative flow cytometry plots of CFSE-labeled T cells, stimulated with ovalbumin (OVA), MOG, NF-L or tetanus toxoid (TT). Proliferation of $CD3^+CD4^+$ T cells is observed in response to NF-L, but not MOG in control subject 3 (**A**). Control donor 14 developed similar $CD3^+CD4^+$ responses to MOG and NF-L as well as a $CD3^+CD8^+$ T cell response to NF-L (**B**). In contrast, MS patient 8 showed a $CD3^+CD4^+$ T-cell response to MOG and to a lesser extent to NF-L, whereas no $CD3^+CD8^+$ T-cell response was observed against these antigens (**C**). The majority of the divided T cells expressed CD45RO, indicative of a memory phenotype.

response to MOG (Figure 2C) and also one patient responded to a similar extent to MOG and NF-L. Three patients were unresponsive to both MOG and NF-L. Two patients were tested for only MOG or NF-L, one showed a response to MOG, while the other did not show a response to NF-L.

CFSE analysis revealed that responses to MOG in both control and MS patients were largely restricted to $CD3^+CD4^+$ T cells, only one control subject showed a marginal $CD3^+CD8^+$ T-cell response to MOG. In contrast, NF-L-reactive cells in MS patients and controls were found in both the $CD3^+CD4^+$ as well as in the $CD3^+CD8^+$ population of T cells. In all subjects, the proliferating cells were expressing CD45RO indicative of a memory phenotype (Figure 2).

Comparison of the thymidine incorporation assays with the CFSE assay revealed that of the 7 controls who responded to MOG in the thymidine incorporation assay, only 3 showed a positive response using CFSE-based assay (Table 2). The responses to NF-L were confirmed in 4 out of 6 control donors. In contrast all MS patients reactive to MOG or NF-L in the thymidine assay also showed proliferation of $CD4^+$ T cells in the CFSE-based assay (Table 3). The patients that responded to NF-L in both assays had the primary progressive (PP) subtype of MS, while patients that reacted to MOG had either PP- or RR-MS.

In general, MS patients showed a significantly higher proliferation of $CD4^+$ T cells to MOG, but not NF-L, compared with OVA ($p < 0.01$; Table 3). Healthy controls showed a significant $CD4^+$ T-cell response to NF-L compared with OVA ($p < 0.05$; Table 2). No significant differences were observed between the responses of MS patients and controls.

Of note, a significant correlation was found between the proliferation of $CD4^+$ T cells to MOG (as measured in the CFSE-dilution assay) and the proliferation of unfractionated cells to MOG (expressed as SI) as measured by the thymidine incorporation test (Spearman $r = 0.42$, $p < 0.05$). Also, the percentage of divided $CD4^+$ T cells to NF-L correlated with the NF-L-response (SI) in the thymidine assay (Spearman $r = 0.55$, $p < 0.01$; data not shown). No such correlation was found between the divided $CD8^+$ T cells and SI-responses to either MOG or NF-L.

Mixed Th1/Th2 cytokine profile in response to NF-L

To characterize the cytokine profiles of the MOG or NF-L-reactive cells we analyzed the supernatants on day 6 following antigen stimulation.

In control subjects MOG-stimulated PBMC produced IFN-γ (293.7 ± 97.8 pg/ml) in contrast to stimulation with OVA in which no IFN-γ could be detected ($p < 0.01$; Figure 3A). Similarly IFN-γ levels were also significantly higher in MOG-stimulated cells of MS patients compared with OVA stimulation (204.6 ± 59.2 pg/ml for MOG vs. 12.7 ± 12.7 pg/ml for OVA; $p < 0.05$). Although stimulation with NF-L also resulted in the

Table 2. T-cell responses to OVA, MOG and NF-L in healthy controls.

	OVA			MOG			NF-L		
Control	**SI[#]**	**CD4[‡]**	**CD8[‡]**	**SI[#]**	**CD4[‡]**	**CD8[‡]**	**SI[#]**	**CD4[‡]**	**CD8[‡]**
1	1.02	0.2	0.4	0.71	***0.9***	0.5	1.68	***1.4***	0.7
2	0.76	n.d	n.d	1.35	n.d	n.d	1.12	n.d	n.d
3	1.10	0.5	0.7	***6.48***	***1.1***	1.0	***5.51***	***9.9***	1.0
4	1.04	n.d	n.d	0.64	n.d	n.d	1.06	n.d	n.d
5	0.36	1.6	1.6	2.00	1.7	1.9	0.41	1.6	1.7
6	1.44	1.1	1.1	***2.58***	1.4	1.2	***2.20***	***2.4***	***3.2***
7	0.69	0.4	0.4	1.04	0.6	0.3	1.35	***1.7***	0.7
8	0.94	n.d	n.d	***2.56***	n.d	n.d	1.07	n.d	n.d
9	0.56	1.0	1.1	1.13	1.0	0.8	1.59	1.2	1.0
10	***2.03***	7.2	6.9	***6.43***	14.3	7.5	***5.12***	12.3	7.1
11	0.64	0.3	0.5	0.90	0.6	0.4	0.89	***2.1***	0.8
12	1.77	2.2	1.5	***3.13***	***17.8***	2.6	***4.67***	***17.4***	2.3
13	0.73	13.2	1.3	***2.63***	17.2	1.5	1.59	17.4	***4.4***
14	0.52	1.1	0.2	0.96	2.9	0.2	1.57	***3.3***	***1.5***
15	1.16	1.5	0.6	***2.93***	***10.9***	1.5	***3.76***	***9.0***	1.2
16	***3.79***	9.0	2.2	***10.35***	9.1	0.8	***5.25***	9.9	3.6
mean	1.16	3.0	1.4	2.86	6.1	1.6	2.43	5.9	2.3
Sem	0.21	1.1	0.5	0.67	1.9	0.5	0.44	1.5	0.5
P	n.a.	n.a.	n.a.	*	n.s.	n.s.	*	*	n.s.

[#] stimulation index (SI) from thymidine incorporation assay, numbers in bold refer to positive responses (SI > 2); [‡] percentage $CD4^+$ or $CD8^+$ T cells that divided in response to antigen as measured by CFSE-dilution, numbers in bold refer to > 2-fold increase in T-cell proliferation compared with OVA; n.a., not applicable; n.d., not determined; n.s., not significant; *, $p < 0.05$ (compared with OVA, Kruskal-Wallis test followed by Dunn's multiple comparison test).

production of IFN-γ (172.2 $\pm$ 69.4 pg/ml in controls and 165.2 $\pm$ 46.5 pg/ml in MS patients), this was not significantly different from OVA-stimulation. No relationship was observed between the level of IFN-γ and the degree of response to MOG or NF-L as determined by the SI in the thymidine incorporation assay (Spearman $r = 0.14$, $p = 0.69$ for MOG; $r = 0.54$, $p = 0.09$ for NF-L).

TNF-α levels were significantly elevated in response to NF-L in controls and MS patients (Figure 3B) and while IL-4 was elevated in control and MS patients, levels were not significantly different from responses to OVA (Figure 3C). Likewise IL-2 levels in MOG or NF-L stimulated cultures were similar to OVA stimulation (Figure 3D). The level of IL-5 was below the detection limit in all cultures and IL-12p70 could only be detected

Table 3. T-cell responses to OVA, MOG and NF-L in patients with MS.

	OVA			MOG			NF-L		
Patient	SI[#]	CD4[‡]	CD8[‡]	SI[#]	CD4[‡]	CD8[‡]	SI[#]	CD4[‡]	CD8[‡]
1	0.42	1.6	1.5	***3.26***	***4.3***	1.7	1.47	14.3	***11.7***
2	0.98	n.d.	n.d.	***3.11***	n.d.	n.d.	1.03	n.d.	n.d.
3	0.78	8.0	1.0	0.55	11.6	1.7	0.82	1.5	1.9
4	0.73	1.4	1.8	0.83	2.3	2.5	0.32	2.6	2.8
5	1.54	0.6	1.4	0.67	***2.7***	1.8	0.81	***2.3***	1.9
6	0.63	n.d.	n.d.	0.71	n.d.	n.d.	0.50	n.d.	n.d.
7	0.85	0.4	0.6	0.88	***10.2***	0.8	1.76	***1.6***	1.0
8	0.54	3.1	1.4	1.77	***29.6***	1.4	1.91	***7.0***	1.6
9	0.58	n.d.	n.d.	1.42	n.d.	n.d.	1.40	n.d.	n.d.
10	1.31	n.d.	n.d.	***2.76***	n.d.	n.d.	1.84	n.d.	n.d.
11	1.09	3.0	1.5	***5.41***	***31.1***	1.2	***2.83***	***7.5***	1.9
12	1.09	1.0	2.1	***2.95***	***7.8***	1.8	***4.12***	***2.5***	2.0
13	0.79	0.3	n.d.	***3.65***	***13.7***	n.d.	0.36	n.d.	n.d.
14	0.75	1.0	0.6	1.63	0.8	0.7	1.02	1.3	1.0
15	0.83	1.1	1.1	1.40	n.d.	n.d.	1.14	1.5	1.2
mean	0.86	1.9	1.2	2.07	10.1	1.6	1.42	3.9	2.5
sem	0.08	0.6	0.2	0.36	3.0	0.2	0.26	1.2	0.9
p	n.a.	n.a.	n.a.	*	**	n.s.	n.s.	n.s.	n.s.

[#] stimulation index (SI) from thymidine incorporation assay, numbers in bold refer to positive responses (SI > 2); [‡] percentage $CD4^+$ or $CD8^+$ T cells that divided in response to antigen as measured by CFSE-dilution, numbers in bold refer to > 2-fold increase in T-cell proliferation compared with OVA; n.a., not applicable; n.d., not determined; n.s., not significant; *, $p < 0.05$; **, $p < 0.01$ (compared with OVA, Kruskal-Wallis test followed by Dunn's multiple comparison test).

in NF-L-stimulated cultures in 3 out of 5 control subjects and in 4 out of 6 MS patients, but levels were very low (< 20 pg/ml; data not shown). Finally we determined whether IL-17A was induced in response to MOG or NF-L and observed that elevated IL-17A levels as compared with OVA-stimulation, could only be detected in MOG-stimulated cultures of one MS patient as well as one control (31.0 pg/ml and 34.5 pg/ml respectively).

In summary, MOG-stimulated cells predominantly produced IFN-γ in contrast to NF-L-stimulation which besides IFN-γ also resulted in the production of TNF-α and IL-4. No differences in cytokine profile could be observed between MS patients and healthy controls.

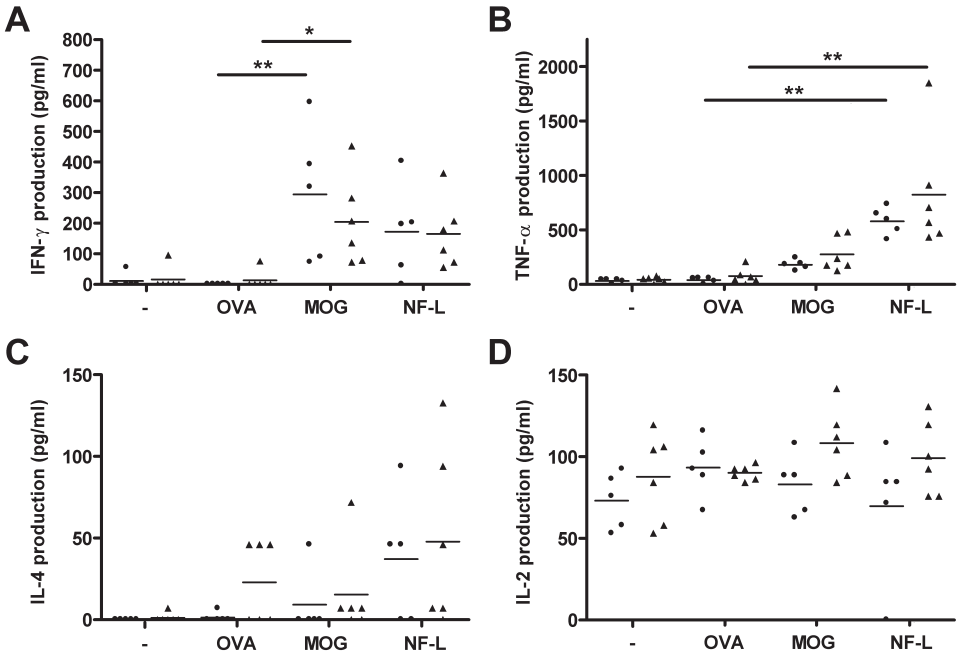

Figure 3. Cells reactive to myelin oligodendrocyte glycoprotein (MOG) produce mainly interferon-γ in contrast to neurofilament light (NF-L)-stimulated cells. Culture supernatant of proliferation assays was analyzed on day 6 for the production of interferon (IFN)-γ (**A**), tumor necrosis factor (TNF)-α (**B**), interleukin (IL)-4 (**C**) and IL-2 (**D**). MOG-stimulated cultures produced significantly more IFN-γ than ovalbumin (OVA)-stimulated cultures, whereas NF-L-stimulated cultures also produced more TNF-α compared with OVA. IL-4 was also elevated in response to NF-L, although this was not significantly different from OVA. IL-2 levels were similar in all cultures (*, $p < 0.05$; **, $p < 0.01$, Kruskal-Wallis test followed by Dunn's multiple comparison test).

DISCUSSION

Axonal degeneration is a major feature of MS by contributing to irreversible symptoms observed in progressive neurological disease (Trapp *et al.*, 1999). Unraveling the mechanisms of axonal degeneration is crucial for the development of novel therapies that target neurodegeneration. We, among others, have hypothesized that autoimmunity to neuronal and axonal antigens may play a role in inducing axonal degeneration (DeVries, 2004, Huizinga *et al.*, 2008). Although several studies have described the presence of antibodies against the axonal cytoskeletal protein NF-L (Eikelenboom *et al.*, 2003, Silber *et al.*, 2002), which suggests that T-cell responses to NF-L are present in MS patients, definite proof for this condition is lacking. In this study we therefore

investigated whether MS patients as well as healthy controls have T-cell responses to NF-L. MOG was used as a control autoantigen, since it is considered to be a dominant myelin autoantigen in MS (Diaz-Villoslada *et al.*, 1999, Kerlero de Rosbo *et al.*, 1993, Sun *et al.*, 1991). Our results indicate that T-cell responses to NF-L are present in MS patients and that they do not differ from the responses in controls. While in our study the T-cell response to MOG was predominantly found in the $CD4^+$ population along with IFN-γ secretion, the response to NF-L was more heterogeneous and observed in both the $CD4^+$ as well as in the $CD8^+$ T-cell population. The finding that T-cell proliferation to NF-L occurs not only in patients but also in healthy controls is in line with studies describing that T-cell responses to myelin antigens are present in both patients and controls (Goebels *et al.*, 2000, Hellings *et al.*, 2001, Martin *et al.*, 1990, Van Noort *et al.*, 1995).

A strong point of this study is that the classical thymidine incorporation assay was performed in parallel with a CFSE-based assay to detect proliferative responses to MOG and NF-L. In this way we were able to confirm the observed T-cell responses to MOG and NF-L in all patients and in the majority of controls. Also, we could demonstrate that the majority of cells that proliferated to MOG were $CD4^+$. NF-L-reactive cells were found both in the $CD4^+$ as well as in the $CD8^+$ T-cell population. Despite differences between the thymidine incorporation and CFSE-based proliferation assays, we did find a significant correlation between divided MOG or NF-L-reactive $CD4^+$ T cells and proliferation in the thymidine assays.

The low incidence of $CD8^+$ T-cell responses to MOG in the individuals studied is somewhat unexpected as Zafranskaya *et al.* (2007) reported significant proliferation of $CD8^+$ T cells in response to MOG. Both their methods to detect proliferation and the sample size were very similar to those used in our study, suggesting that this controversy must be explained by other factors, such as differences in HLA haplotype (Weissert *et al.*, 2002). Alternatively, differences in the disease status, duration or clinical severity between the patient cohorts could account for the observed differences. For example, myelin-reactive T cells, especially those directed to immunodominant epitopes, are known to be expanded during clinical exacerbation (Tejada-Simon *et al.*, 2000). In this respect, it would also be valuable to perform longitudinal studies of the NF-L T-cell response in MS patients.

MS is often associated with the upregulation of the proinflammatory cytokines IFN-γ, TNF-α and IL-12. In agreement with this we also show that responses to MOG were directed towards a Th1-phenotype as evidenced by selective production of IFN-γ. The production of IFN-γ by Th1 cells is mediated by IL-12 (Magram *et al.*, 1996), however in this study only marginal levels of IL-12p70 could be detected. NF-L stimulation resulted in significant levels of IFN-γ and TNF-α. In addition, also IL-4 was produced in some donors, suggesting that NF-L-stimulation leads to a mixed Th1/Th2 profile in contrast to MOG-stimulation.

Although IL-17 is reported to play a role in $CD4^+$ T-cell mediated EAE, its role in MS is still under debate. In our study IL-17A production was found in only one control donor and one MS patient, suggesting that Th17 cells can in fact be induced following stimulation with MOG, but the low levels of IL-17A suggest a minor role for these cells.

The relevance of NF-L as an autoantigen is illustrated by the reports that patients with progressive MS have elevated levels of autoantibodies against NF-L (Ehling *et al.*, 2004, Silber *et al.*, 2002) and that these levels correlate with the degree of cerebral atrophy (Eikelenboom *et al.*, 2003). Although the number of patients in our study is too low to draw definite conclusions, it is still striking that the two patients that reacted to NF-L in both proliferation assays had the PP subtype of MS. A large cohort of PP-MS patients would be required to corroborate these findings. That autoimmunity to NF-L is potentially pathogenic is shown by the finding that immunization with NF-L induces spastic paresis and axonal degeneration in mice (Huizinga *et al.*, 2007).

How antineuronal T-cell responses arise and what their exact role is in disease is debatable. As described for myelin antigens (De Vos *et al.*, 2002), it is likely that neuronal and axonal antigens are released following brain damage and provoke an immune response in the cervical lymph nodes. In the CSF of MS patients elevated levels of NF and tubulin proteins have been detected (Norgren *et al.*, 2003, Semra *et al.*, 2002). Following drainage to lymph nodes, an autoimmune response could be induced as seen in patients with brain trauma, who develop antibodies to neuron-specific tubulin. This study illustrates that at least antibodies to neuronal antigens can be induced after brain damage (Skoda *et al.*, 2006). Similar findings have been reported after experimental brain contusion in rats (Rudehill *et al.*, 2006). That antibodies to neuronal antigens are pathogenic is clearly observed in paraneoplastic disorders (Sommer *et al.*, 2005) and Guillain-Barré syndrome (Halstead *et al.*, 2004) although whether NF-L-specific T cells described in this study are pathogenic remains to be determined. The presence of peripheral T-cell responses to NF-L in both MS patients and in controls, as observed for myelin antigens, would suggest that this is not the case. However, like suggested for myelin antigens an additional trigger may be required for these autoreactive T cells to infiltrate the CNS and become pathogenic. Activated T cells can induce cell death of neurons both *in vitro* and *in vivo* (Giuliani *et al.*, 2003, Zhu *et al.*, 2006) although it is also clear that the CNS exhibits several mechanisms to inhibit these otherwise potential damaging cellular inflammatory reactions. For example, neurons express high levels of FasL that induces T-cell apoptosis (Bechmann *et al.*, 1999) and ICAM-5 secretion by neurons has recently been reported to suppress T-cell activation (Tian *et al.*, 2008). Similarly, other pathways exploited by neurons to inhibit the activation of microglia (Cardona *et al.*, 2006, Mott *et al.*, 2004, Tian *et al.*, 2008) may also regulate T-cell activation indirectly.

Whether T cells outside the brain parenchyma, for example in the meninges, are also subjected to such neuronal immunosuppression is unknown. Lymphoid-like tissue

in the meninges of MS patients consists of macrophages and dendritic cells, that could interact with T cells directed to neuronal antigens, as well as clusters of B cells and plasma cells (Prineas, 1979, Serafini *et al.*, 2004). Whether this lymphoid tissue in the meninges is involved in the perpetuation of the pathogenic response in MS is as yet unclear but may be crucial to understand factors underlying chronic inflammation in the CNS.

In summary there is accumulating evidence to suggest that the pathogenesis of MS involves more than just myelin autoimmunity and destruction. Following up on our report that immunization with NF-L is pathogenic in mice, we here show that the cytoskeletal protein NF-L may also be the target of autoreactive T-cell responses in humans. Whether these responses are indeed pathogenic in MS is unknown and further investigation of the role of T-cell responses to neuronal antigens MS is warranted.

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1

DAMAGED AXONS ARE ENGULFED BY ACTIVATED PHAGOCYTES IN MULTIPLE SCLEROSIS LESIONS

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ABSTRACT

Demyelination and axonal degeneration are important features of the pathology in the central nervous system in patients with multiple sclerosis (MS). Although it is clear that myelin sheaths are phagocytosed by microglia and blood-born macrophages, the fate of degenerating axons in MS is unknown. As microglia have been reported to phagocytose neurons and axons in experimental systems of axonal degeneration, we hypothesized that these cells also play a role in the removal of damaged axons in MS lesions. The uptake of axonal antigens may result in antigen presentation and subsequent induction of an immune response directed against axons as has been described for neurofilament light (NF-L).

Using double labeling immunohistochemistry we observed that HLA class II$^+$ cells were totally engulfing amyloid precursor protein, non-phosphorylated neurofilament heavy, or NF-L positive axonal ovoids. The engulfed axons were found in brain sections of 12 out of 15 MS patients (80%) but only in 1 out of 5 (20%) control subjects. Active inflammatory areas were especially sites of axonal engulfment although also more distant from the lesions axonal antigens were observed inside HLA class II$^+$ cells, possibly indicating Wallerian degeneration. When mimicking the degradation of NF-L *in vitro* using lysosomal enzymes we observed that the breakdown of the full-length protein occurred within 2 h and that the antibody only recognized the breakdown products up till 4 h. This illustrates that the breakdown of axonal antigens in phagocytes occurs in a short time-span, complicating the visualization of the final stage of axonal phagocytosis *in situ*.

In conclusion, this study shows that damaged axons in MS lesions are engulfed by HLA class II$^+$ cells. These cells could play a role in stimulating newly infiltrated T cells in MS lesions.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The presence of antibodies directed to myelin antigens has led to the general assumption that MS is an autoimmune disease targeting myelin sheaths. Antibodies in patient serum have been shown to bind myelin *in vitro* and facilitate myelin phagocytosis (Breij *et al.*, 2006, Van der Goes *et al.*, 1999). In addition, both antibodies and complement are deposited on myelin sheaths which may not only cause lysis and oligodendrocyte death but also opsonisation promoting macrophage-mediated phagocytosis (Breij *et al.*, 2008, Storch *et al.*, 1998).

Besides inflammation and demyelination, axonal degeneration is a prominent feature in the MS lesion, starting already early in disease (Kuhlmann *et al.*, 2002). The ongoing process of axonal damage is thought to accumulate in irreversible neurological symptoms and progression of disease (Trapp *et al.*, 1999). Axonal degeneration may be caused by chronic demyelination (Griffiths *et al.*, 1998), release of toxic factors by macrophages (Smith *et al.*, 2001) or by energy imbalance in the axon (Dutta and Trapp, 2007). Alternatively, axons may be subject to autoimmune-mediated damage as has been shown in other neurodegenerative diseases. MS patients have antibodies and T-cell reactions to neuronal antigens (Forooghian *et al.*, 2007, Mathey *et al.*, 2007, Silber *et al.*, 2002) and axon-reactive B cells are present in the cerebrospinal fluid (Zhang *et al.*, 2005). Immunization with one of these axonal proteins, neurofilament light (NF-L), induced axonal degeneration and neurological disease in mice (Huizinga *et al.*, 2007). In addition, administration of antibodies directed to neurofascin-186 aggravated disease in rats with experimental autoimmune encephalomyelitis (EAE) (Mathey *et al.*, 2007). These animal studies indicate that antineuronal autoimmunity can indeed be pathogenic.

In MS, autoimmune responses to axonal proteins may be initiated or controlled by the cervical lymph nodes (CLN), which drain the CNS. Indeed myelin antigens are present in antigen presenting cells (APC) in the CLN of MS patients and animals with EAE (De Vos *et al.*, 2002). The phagocytosis of myelin antigens in the brain and the expression of CCR7 on myelin-containing cells in the CLN suggests that myelin antigens are transferred via a cellular route to the CLN (De Vos *et al.*, 2002). However, whether neuronal antigens are present in phagocytes in MS brain and gain access to the CLN has not been addressed. From animal studies it is suggested that both microglia and astrocytes are capable of phagocytosing degenerating axons after the induction of an entorhinal cortex lesion (Bechmann and Nitsch, 1997). The presence of neuronal antigens in APC is particularly important for the reactivation of newly infiltrated T cells. In EAE, T-cell reactivation by APC present in the perivascular space has been shown to be crucial for development of inflammatory lesions and clinical disease (Greter *et al.*, 2005).

Here we investigated whether damaged axons in MS lesions are subject to phagocytosis by activated macrophages or microglia using double labeling immunohistochemistry for HLA class II and axonal and neuronal antigens. We show that a fraction of damaged axons were completely engulfed by HLA class II^+ macrophages or microglia suggesting that phagocytosis of damaged axons occurs in MS brain. These cells may play an important role in the reactivation of axon or neuron-reactive T cells.

MATERIALS AND METHODS

Patients

Post-mortem brain material of 15 patients (20 blocks) with clinically definite MS, 3 patients with cerebrovascular accident and 5 controls (Table 1) was obtained through cooperation with the Netherlands Brain Bank (Amsterdam). Lesions had been identified by MRI-guided sampling of brain slices (1 cm) as described before (Bo *et al.*, 2004). Blocks containing lesions had been fixed with formalin and embedded in paraffin wax for neuropathological characterization.

Antibodies

Details of all primary antibodies used in this study are shown in Table 2. To expand the repertoire of antibodies to NF-L we produced hybridoma cell lines. Briefly, splenocytes from NF-L-immunized Biozzi ABH mice (Huizinga *et al.*, 2007) were fused with SP2/0-ag14 myeloma cells and cultured in the presence of 5% hybridoma cloning factor (Tebu-bio, Heerugowaard, The Netherlands). Positive clones were identified using ELISA for recombinant mouse NF-L, expanded, and the resultant immunoglobulin in the supernatant purified using Protein A-coupled sepharose columns (Amersham Biosciences, Roosendaal, The Netherlands). The three clones that were obtained recognized NF-L as a band of 61 kD on a western blot of human CNS white matter. The cross-reactivity with leukocyte antigens was assessed by staining human tonsil using immunohistochemistry as described below.

Secondary antibodies used in this study included horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1, HRP-conjugated goat anti-mouse IgG2a and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG2b (all from Southern Biotechnology Associates, Birmingham, AL).

In vitro degradation of neurofilament light protein

To examine whether the anti-NF-L antibodies bound to degraded NF-L we treated recombinant mouse NF-L protein (Heins *et al.*, 1993) with enzymes to mimic what may occur in macrophages following uptake of neuronal antigens. NF-L was dialysed into 50 mM sodium acetate (pH 5) and incubated with 50 µg/ml cathepsin B (12.1 U/mg) and 50 µg/ml cathepsin D (4.6 U/mg; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) at 37 ºC. Samples (10 µl) were taken at 0, 10, 20, 40, 60, 120, 180, 240 and 300 min of incubation, inactivated with 40 µl of 1M Tris-HCl pH 9 and frozen at -20 ºC. Samples were diluted in NuPage sample buffer and reducing agent. Gel electrophoresis of approximately 2 µg protein of each time point was performed for 1 hour at 200 V using a 4-12% Bis-Tris gel with MOPS as running buffer (all materials from Invitrogen, Breda, The Netherlands). Proteins were transferred onto a nitrocellulose membrane

Table 1. Clinical details of patients and controls used in this study.

	Patient number	Sex	Age	Length of disease (years)	Disease course	Lesion type
Multiple sclerosis	1A	F	48	8	SP	Preactive + active + inactive
	1B	F	48	8	SP	Active
	2A	F	53	27	SP	Active + inactive + shadow + GM
	2B	F	53	27	SP	Preactive + active + chronic active
	3A	M	43	unknown	MS	Chronic active
	3B	M	43	unknown	MS	Active + chronic active
	4	F	75	42	MS	Chronic active + shadow
	5	F	48	25	PP/SP	Preactive + active + chronic active + GM
	6	F	50	unknown	MS	Inactive
	7	F	44	8	PP	Inactive
	8	F	77	unknown	unknown	Inactive
	9	M	63	24	PP	Chronic active
	10A	M	56	27	SP	Preactive and inactive
	10B	M	56	27	SP	Active + chronic active
	11	F	69	53 y	PP	Preactive + inactive
	12	M	66	unknown	unknown	Active + inactive + shadow
	13A	F	70	40	PP (slow)	Inactive
	13B	F	70	40	PP (slow)	GM
	14	M	47	7	PP	Preactive + active + GM
	15	M	41	unknown	unknown	Chronic active
	Patient number	**Sex**	**Age**	**Cause of death**		
Stroke	1	F	67	Aortic dissection		
	2	F	72	Bronchopneumonia		
	3	F	60	Disseminated fungal infection		
Controls	1	F	89	Cardiac arrhythmia		
	2	F	45	Cardiac arrhythmia		
	3	F	99	Pneumonia		
	4	F	63	Pneumonia and pulmonary embolism		
	5	F	58	Myocardial infarct		

(Amersham Biosciences) using an Ancos semi-dry electroblotter and membranes were incubated subsequently with anti-NF-L antibodies (0.5 µg/ml) and HRP-conjugated rabbit anti-mouse immunoglobulin (1:1000; DakoCytomation, Glostrup, Denmark) for

Table 2. Antibodies used for immunohistochemistry.

Antigen	Species and isotype	Clone	Dilution / concentration	Source
APP	mIgG1	LN27	1:4000	Zymed Inc, South San Francisco, CA
HLA class II	mIgG2b	LN3	1:250	Gift from Dr Hilgers, VUMC, The Netherlands
MBP	mIgG1	26	1:4000	Gift from Prof Groome, Oxford Brookes University, UK
NF-L	mIgG2b	4F8.1	5 µg/ml	Made in-house
NF-L	mIgG1	5B4.2	5 µg/ml	Made in-house
NF-L	mIgG1	10H9	5 µg/ml	Made in-house
NF-L	mIgG1	DA2	1:100	Zymed Inc, South San Francisco, CA
Non-phospho-rylated NF-H	mIgG1	SMI-32	1:500	Sternberger Monoclonals Inc, Baltimore, MD
PLP	mIgG2a	Plpc1	1:250	Serotec, Oxford, UK
isotype control	mIgG1	MOPC-31C	1:100	BD, Alphen a/d Rijn, The Netherlands
isotype control	mIgG2a	C1.18.4	1:100	BD, Alphen a/d Rijn, The Netherlands
isotype control	mIgG2b	MPC-11	1:100	BD, Alphen a/d Rijn, The Netherlands

1 h at RT (20 °C) in TBS with 5% milk powder and 0.1% Tween. Bound antibodies were visualized on a Chemidoc XRS (Bio-Rad Laboratories, Veenendaal, The Netherlands) using ECL reagent (Amersham Biosciences). Lanes were analyzed using NIH Image J software (http://rsb.info.nih.gov/ij/index.html).

Immunohistochemistry

Sections were deparaffinized, rehydrated and endogenous peroxidase activity was blocked by incubating with 0.3% H_2O_2 in methanol for 30 min. After washing in PBS, slides were processed for heat-mediated antigen retrieval in 0.1M citric acid at pH 6. After washing with PBS/0.05% Tween sections were incubated with 10% normal goat serum (DakoCytomation) and 5% normal human AB serum (NHS; Sanquin, Leiden, The Netherlands) in PBS/0.1% BSA for 30 min. Primary antibodies (Table 2) were allowed to bind overnight at 4 °C. After washing, sections were incubated with appropriate secondary antibodies in PBS / 1% BSA / 1% NHS for 45 min at RT. Bound antibodies were visualized using Fast-Blue for AP-conjugated secondary antibodies, resulting in a blue precipitate. HRP-conjugated antibodies were visualized with 3-amino-9-ethylcarbazole (AEC) resulting in a red product. Aspecific binding was evaluated by replacing primary antibodies for isotype-matched control antibodies.

Analysis of brain sections of multiple sclerosis patients

Brain sections were viewed on an Olympus microscope and the number of HLA class II positive cells containing axonal antigens was assessed by two observers based on consensus. To assess whether uptake of axonal antigens occurred more frequently in regions of axonal damage the number of APP^+ axonal ovoids was determined as a measure of axonal degeneration.

RESULTS

Reactivity of anti-NF-L antibodies with leukocyte antigens and NF-L breakdown products

NF-L belongs to the intermediate filament protein family, a group of homologous cytoskeletal proteins that are expressed in various cell types. It is therefore crucial to determine whether the commercial or three anti-NF-L antibodies that were generated, cross-reacted with antigens expressed in leukocytes. Using immunohistochemistry on human tonsil, we observed that clones 4F8.1 and 5B4.2 stained tonsillar follicles (Table 3). A commercially available anti-NF-L antibody (clone DA2) showed a similar staining. The clone 10H9 did not show any tonsil staining apart from an axonal staining within the tonsil.

In order to investigate whether the anti-NF-L antibodies would be able to detect breakdown products of NF-L in phagocytes, we mimicked the proteolytic degradation of NF-L *in vitro* using the lysosomal enzymes cathepsin B and D. Samples were taken at various time points, subjected to gel electrophoresis and western blotting using the anti-NF-L antibodies.

Using the 10H9 clone, several breakdown products of NF-L could be detected already after 10 minutes of enzymatic treatment (Figure 1). The full-length recombinant

Table 3. Binding characteristics of anti-NF-L antibodies used in this study.

Antibody	Clone	Reactivity on brain (IHC)	Reactivity on tonsil (IHC)	Number of breakdown products recognized (WB)
NF-L	4F8.1	++	++ (follicles)	12
NF-L	5B4.2	++	+	n.d.
NF-L	10H9	++	-	8
NF-L	DA2	++	++ (follicles)	7

IHC, immunohistochemistry; WB, western blotting; n.d., not done
- no staining, + moderate staining, ++ strong staining

NF-L protein was almost completely degraded after 120 minutes. After 4 h of treatment with cathepsin enzymes NF-L breakdown products were only hardly detectable using clone 10H9 (Figure 1A). The DA2 clone recognized less breakdown products than the 10H9 clone, while the 4F8.1 clone recognized more breakdown products (Table 3). All anti-NF-L antibodies recognized a different combination of breakdown products, indicating different specificities.

In summary, the 10H9 clone did not cross-react with leukocyte antigens, but recognized breakdown products of NF-L, therefore this clone was selected for double-staining NF-L and HLA class II in brain sections.

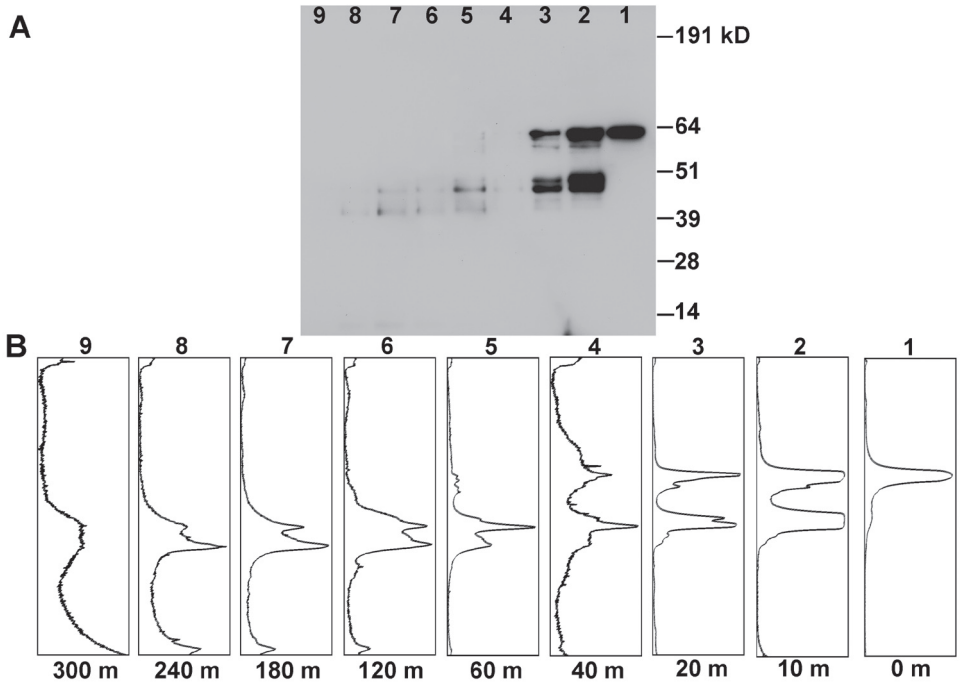

Figure 1. The anti-NF-L antibody 10H9 recognizes breakdown products of NF-L. NF-L was digested *in vitro* using cathepsin B and D enzymes. Samples were taken before adding the enzymes (lane 1) and after 10, 20, 40, 60, 120, 180, 240, and 300 minutes of enzymatic treatment (lane 2-9 respectively). After electrophoresis, protein was transferred onto a nitrocellulose membrane, which was stained with the 10H9 clone recognizing NF-L (**A**). The band density in each lane was analyzed using Image J software (**B**).

Damaged axons are engulfed by HLA class II$^+$ cells in MS brain

Axonal damage is a prominent feature in MS lesions however the fate of neuronal products released during axon degeneration is not known. We hypothesize that damaged axons are cleared from the lesion by macrophages, similar to what is suggested to occur with damaged myelin sheaths. Therefore, we examined the association of neuronal antigens with macrophages and examined whether, like myelin antigens, neuronal proteins are taken up by macrophages in the MS lesion. MS lesions were double-stained for HLA-DR as a marker for activated macrophages or microglia, and for the axonal antigens APP, non-phosphorylated NF-H (using the SMI-32 antibody) or NF-L. In addition, double-stainings for HLA-DR and the myelin proteins PLP and MBP were performed to confirm phagocytosis of myelin antigens and to compare the level of myelin uptake with uptake of neuronal antigens.

Staining for myelin antigens revealed lesions with increased numbers of HLA-DR positive cells and loss of myelin (Figure 2A). As expected, HLA class II$^+$ macrophages or microglia containing MBP were mostly present in the brain parenchyma or the perivascular space around blood vessels in active lesions or at the border of chronic active lesions. Also in blocks that contained chronic inactive lesions, HLA class II$^+$ cells containing myelin antigens were occasionally observed.

Staining with all three axonal markers revealed degeneration of axons as evidenced by the presence of large axonal swellings or ovoids positive for APP (Figure 2B) or SMI-32 (not shown). Macrophages and microglia were often observed next to these damaged axons. In addition, we observed HLA-DR positive cells that were totally surrounding axons positive for APP (Figure 2B, inset), SMI-32 (Figure 2C) or NF-L (Figure 2E and F, insets). In contrast, no staining was observed in HLA class II$^+$ cells when antibodies were replaced with isotype-matched control antibodies.

The HLA-DR$^+$ cells engulfing axons were observed in 80% (12/15) of the MS patients (13 out of 20 MS brain sections, 65%) but only in 20% (1/5) of the control donors (Table 4). In 2 out of 5 controls, HLA-DR$^+$ cells were observed surrounding the neuronal cell body. These controls also contained degenerating APP$^+$ neurons associated with activated microglia, suggesting phagocytosis of damaged neurons. In MS lesions we did not observe HLA class II$^+$ cells surrounding neuronal cell bodies despite the presence of grey matter lesions.

Ischemia due to a cerebrovascular accident is associated with both myelin and axonal/neuronal damage, therefore we also investigated whether HLA class II$^+$ cells contained myelin or axonal antigens. Myelin positive HLA class II$^+$ cells were observed in 1 out of 3 stroke patients, while HLA class II$^+$ cells containing axonal antigens were not found in any of these patients.

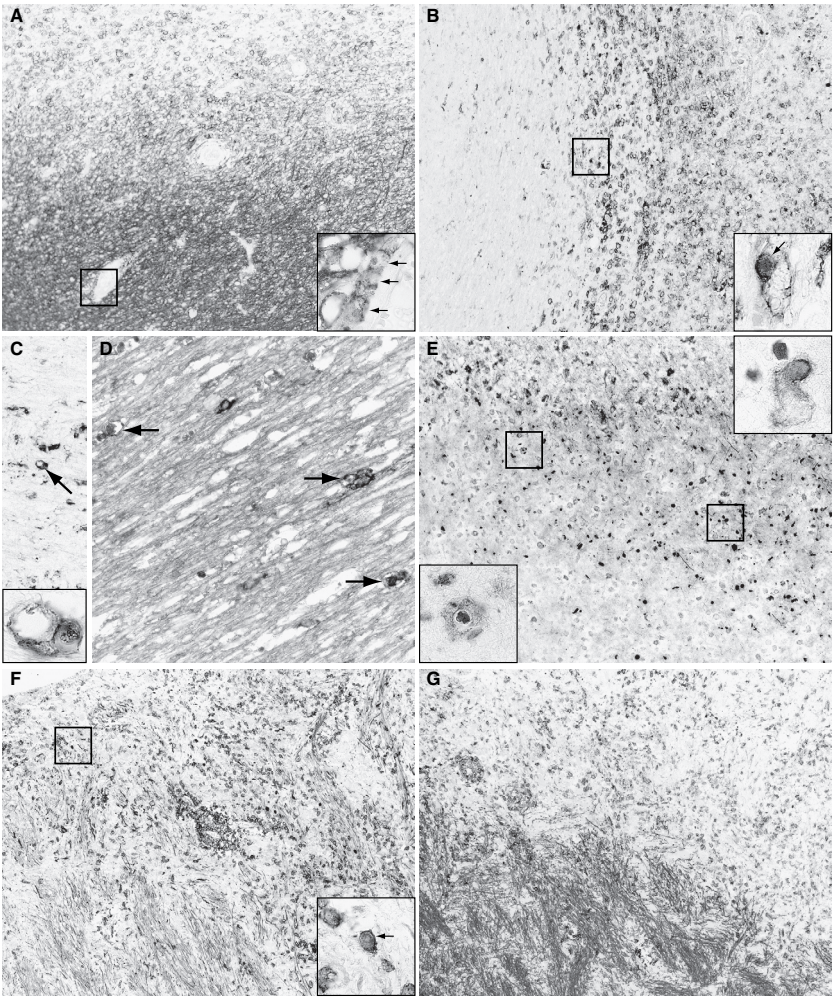

Figure 2. Axons are engulfed by HLA class II$^+$ cells in MS brain lesions. Paraffin sections were doublestained for HLA class II (blue) and myelin or axonal antigens (red). In an active lesion MBP is present in HLA class II$^+$ cells in the perivascular space of blood vessels (**A**, arrows). Axonal damage as indicated by APP$^+$ ovoids is present in the hypercellular rim containing HLA class II$^+$ cells (**B**). Some of these axons were totally engulfed by HLA class II$^+$ cells (inset in **B**). Both A and B are from case 14. HLA class II$^+$ cells also engulf SMI-32$^+$ axons (**C**, from case 12). The area in which these cells were located was outside a lesion where HLA class II$^+$ cells were also shown to contain MBP (**D**). Numerous ovoids positive for NF-L were present in case 3 (**E**) and HLA class II$^+$ cells were observed to surround these damaged axons (inset in **E**). A damaged axon positive for NF-L in case 12 was engulfed by a HLA class II$^+$ cell (**F**). The lack of space between the axon and the HLA class II$^+$ cell suggests that the myelin sheath is already degenerated, resulting in direct interaction between the axon and the HLA class II$^+$ cell. The border of myelination is shown in **G** (MBP stain). See page 205 for a full-color representation of this figure.

Table 4. Number of HLA class II$^+$ cells containing myelin or axonal antigens.

Patient/ block number	Lesion type	APP$^+$ oviods	PLP	MBP	Total of myelin antigens	SMI-32	APP	NF-L	Total of axonal antigens
10 A	P + I	15	0	1	1	3	0	0	3
11	P + I	2	3	8	11	0	0	0	0
14	P + A	445	1	13	14	6	3	0	9
1 A	P + A + I	53	2	1	3	0	0	0	0
2 B	P + A + CA	151	20	8	28	0	2	1	3
5	P + A + CA	140	5	3	8	0	2	0	2
1 B	A	> 1100	14	2	16	2	0	0	2
2 A	A + I + S	22	0	3	3	0	0	0	0
12	A + I + S	135	90	24	114	8	1	4	13
3 B	A + CA	> 3000	11	10	21	1	0	1	2
10 B	A + CA	17	0	8	8	0	0	0	0
3 A	CA	719	6	2	8	2	0	5	7
15	CA	586	0	10	10	1	6	1	8
9	CA	17	0	1	1	0	2	1	3
4	CA + S	patches	0	0	0	0	0	0	0
7	I	9	7	3	10	0	0	1	1
6	I	29	0	3	3	0	0	0	0
13 A	I	11	4	6	10	1	0	0	1
8	I	6	0	0	0	2	0	0	2
13 B	GM	9	1	0	1	0	0	0	0

P, preactive lesion; A, active lesion; CA, chronic active lesion; I, inactive lesion; S, shadow plaque; GM, grey matter lesion.

Frequency and location of axonal antigen-containing HLA class II$^+$ cells

We next investigated the frequency of axon phagocytosis compared to myelin phagocytosis. As a measure of myelin phagocytosis the total number of HLA class II$^+$ cells that contained MBP or PLP antigens was determined. Similarly, as a measure of axon phagocytosis the total number of HLA class II$^+$ cells that contained APP, SMI-32 or NF-L antigens was counted. HLA class II$^+$ cells containing MBP or PLP were present in significantly higher numbers than those containing APP, SMI-32 or NF-L ($p < 0.05$; paired t-test; Figure 3A).

Axons engulfed by HLA class II cells were observed in all types of MS lesions. Of note, sections with a high number (> 5) of axonal antigen-containing HLA class II$^+$ cells were all active or chronic active lesions, while inactive lesions generally contained

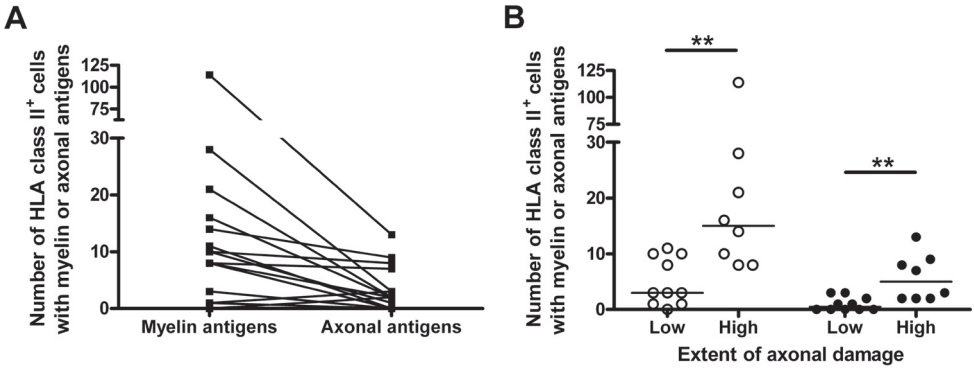

Figure 3. Phagocytosis of axonal antigens is observed less frequently than phagocytosis of myelin antigens. Brain sections of MS patients were stained for HLA class II and myelin or axonal antigens and the number of HLA class II$^+$ cells containing myelin (PLP, MBP) or axonal antigens (NF-L, APP, SMI-32) was quantified (* $p < 0.05$, paired t-test) (**A**). Axonal antigen (filled circles) or myelin-containing (open circles) HLA class II$^+$ cells were determined in areas with a high degree of axonal degeneration (i.e. > 100 APP$^+$ ovoids) and areas with a low degree of axonal degeneration (i.e. < 100 APP$^+$ ovoids) (**B**).

only few (1 or 2) HLA class II$^+$ cells engulfing axons (Table 4). In active lesions, engulfed axons were located throughout the area showing active inflammation and demyelination whereas in chronic active lesions engulfed axons were located at the hypercellular rims (as shown in Figure 2B, E and F). In contrast to myelin, HLA class II$^+$ cells containing axonal antigens were not found in perivascular spaces and also not in meningeal areas. Outside lesions HLA class II$^+$ cells were also found to engulf degenerating SMI-32$^+$ axons (Figure 2C) as well as myelin sheaths (Figure 2D), possibly as a result of Wallerian degeneration.

Sections that contained a high number of damaged axons (> 100 APP$^+$ ovoids / section) showed a higher degree of axonal engulfment and myelin phagocytosis compared with sections containing few (< 100) APP$^+$ ovoids (Figure 3B and Table 4).

DISCUSSION

Axonal damage is a major pathological feature in MS lesions and plays a crucial role in disease since it is considered to underlie the development of chronic irreversible disability. Axons may degenerate as a consequence of demyelination or due to the inflammatory environment induced by activated myeloid cells. Recent evidence suggests that also autoimmunity to axonal antigens may be important in inducing axonal degeneration as antibodies to neurofascin isolated from MS patients exacerbate axonal damage in rats with EAE (Mathey *et al.*, 2007). Similarly, antibodies and T cells

directed to axonal antigens have been described in patients with MS (Forooghian *et al.*, 2007, Silber *et al.*, 2002). Although it is well-known that degenerating myelin sheaths are phagocytosed by macrophages, the fate of degenerating axons in MS lesions is largely unclear. Therefore we studied whether axonal antigens could be detected in HLA class II$^+$ cells in MS lesions. The uptake of axonal antigens by APC may be crucial in the induction of an immune response directed against axons that may subsequently aggravate CNS damage.

By double staining the various MS lesion types i.e. active, chronic active and chronic inactive, that were observed to display varying degrees of axonal degeneration, we observed HLA class II$^+$ cells that completely surrounded damaged axons. Axonal engulfment was observed in all lesion types, was less frequent than myelin phagocytosis and occurred preferentially in areas with a high degree of axonal damage. These results not only confirm the strong association between macrophages and damaged axons in MS lesions (Bitsch *et al.*, 2000) but also suggest that macrophages play a role in the clearance of damaged axons.

Phagocytosis of neuronal antigens has been described previously in other neurodegenerative diseases as well as in several experimental models of neurodegeneration. In mice in which an entorhinal cortex lesion was induced it was shown that both astrocytes and microglia were able to phagocytose degenerated axons (Bechmann and Nitsch, 1997). Similarly, following middle cerebral artery occlusion in mice, microglia and to a much lesser extent blood-born macrophages were observed to incorporate neurons identified by the expression of NeuN, a protein present in neuronal nuclei (Schilling *et al.*, 2005). Also during physiological axonal degeneration (axon pruning in development) phagocytes are crucial for the removal of degenerating axons and neurons via a process called “engulfment-promoted cell death” (Mallat *et al.*, 2005). For example, microglia were shown to promote apoptosis by engulfing degenerating Purkinje cells and producing superoxide anions (Marin-Teva *et al.*, 2004).

A question that remains is: how do macrophages or microglia recognize damaged axons? One possibility is that the phagocytosis of apoptotic neurons is triggered by the expression of so-called ‘eat-me signals’ like phosphatidylserine on plasma membranes of apoptotic neurons (Witting *et al.*, 2000). In addition soluble mediators secreted by damaged axons may promote phagocytosis as has been described for the nucleotides UDP/UTP that bind to the $P2Y_6$ recptor on microglia causing actin rearrangements (Koizumi *et al.*, 2007). Finally opsonization by complement or antineuronal antibodies may augment phagocytosis via complement receptors or Fc receptor-mediated recognition by macrophages or microglia. One target of antibodies may be neurofascin, a protein exposed on demyelinated axons (Mathey *et al.*, 2007), or yet unidentified antigens present in axonal membranes (Lily *et al.*, 2004, Rawes *et al.*, 1997). Of note, defects in the removal of apoptotic cells have been linked to the development

of autoimmunity (Viorritto *et al.*, 2007). In this respect it is important to mention that the removal of axonal antigens in MS is not complete since axonal antigens like NF-L can be readily detected in the CSF (Semra *et al.*, 2002).

In this *in situ* study we did not find direct evidence for the degradation of damaged axons in (phago)lysosomes of macrophages both in MS and stroke patients. Although we selected antibodies to neuronal antigens that recognized the breakdown products of axonal proteins it is still possible that the final stage of phagocytosis was missed. In the *in vitro* degradation experiment using the enzymes cathepsin B and D, we observed that the full-length recombinant mouse NF-L protein was digested within 2 h and the reactivity of the antibody was abolished after 4 h of incubation with the enzymes. In other *in vitro* studies it was described that microglia readily engulf apoptotic neurons and that digestion of these neurons was completed within 2 h (Parnaik *et al.*, 2000). Thus it is probable that once the lysosomes of the macrophage fuse with the phagosome containing axonal antigens, the degradation will occur very rapidly. This rapid degradation of neuronal antigens may have prevented more obvious detection of the phagocytosed neuronal antigens. Moreover the sensitivity of the staining may be too low to detect the axonal antigens in the lysosomal vesicles.

The adequate removal of degenerating cells or tissue debris by phagocytes in the CNS is necessary for regeneration and may prevent or dampen a destructive inflammatory reaction. For example myelin phagocytosis by macrophages induces the production of anti-inflammatory cytokines (Boven *et al.*, 2006). Whether ingestion of degenerating axons also results in an anti-inflammatory cytokine profile of the macrophages is not known but may be crucial to the induction of an anti-axonal immune response.

In conclusion, this study provides evidence for phagocytic clearance of damaged axons in multiple sclerosis lesions. The processing of axonal antigens by HLA class II positive cells may be important in inducing an antineuronal immune response or in reactivating newly infiltrated T cells.

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BRAIN ANTIGENS IN FUNCTIONALLY DISTINCT ANTIGEN-PRESENTING CELL POPULATIONS IN CERVICAL LYMPH NODES IN MS AND EAE

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ABSTRACT

Drainage of central nervous system (CNS) antigens to the brain-draining cervical lymph nodes (CLN) is likely crucial in the initiation and control of autoimmune responses during multiple sclerosis (MS). We demonstrate neuronal antigens within CLN of MS patients. In monkeys and mice with experimental autoimmune encephalomyelitis (EAE), and in mouse models with non-inflammatory CNS damage, the type and extent of CNS damage was associated with the frequencies of CNS antigens within the cervical lymph nodes. In addition, CNS antigens drained to the spinal cord-draining lumbar lymph nodes. In human MS CLN, neuronal antigens were present in pro-inflammatory antigen-presenting cells (APC), whereas the majority of myelin-containing cells were anti-inflammatory. This may reflect a different origin of the cells or different drainage mechanisms. Indeed, neuronal antigen-containing cells in human CLN did not express the lymph node homing receptor CCR7, whereas myelin antigen-containing cells *in situ* and *in vitro* did. Nevertheless, CLN from EAE-affected CCR7-deficient mice contained equal amounts of myelin and neuronal antigens as wild type mice. We conclude that the type and frequencies of CNS antigens within the CLN are determined by the type and extent of CNS damage. Furthermore, the presence of myelin and neuronal antigens in functionally distinct APC populations within MS CLN suggests that different T-cell subsets may be activated.

INTRODUCTION

Irreversible neuronal damage is a major pathological feature of multiple sclerosis (MS) and ranges from mild pathology to complete axonal transection (Ferguson *et al.*, 1997, Sospedra and Martin, 2005). The cause of neuronal damage is not yet elucidated, but autoreactive B and T cells directed against neuronal antigens could conceivably be instrumental (Huizinga *et al.*, 2008). Indeed, MS patients have increased circulating antibody levels against the neuronal proteins neurofilament light (NF-L) and neurofilament heavy (NF-H) (Bartoš *et al.*, 2007a, Eikelenboom *et al.*, 2003, Silber *et al.*, 2002) in serum and against the medium subunit of neurofilament in cerebrospinal fluid (CSF) (Bartoš *et al.*, 2007b). In addition, T cells from MS patients proliferate in response to the neuronal antigens synapsin and neuron-specific enolase (NSE) (Forooghian *et al.*, 2007, Polak *et al.*, 2001). In mice, T-cell mediated autoimmunity against neuronal antigens leads to CNS inflammation and neurological symptoms (Aktas *et al.*, 2005, Furlan *et al.*, 2003, Huizinga *et al.*, 2007, Mor *et al.*, 2003, Rosenmann *et al.*, 2006).

Autoreactive lymphocytes against myelin proteins and likely also against neuronal antigens are recruited into the CNS (Sospedra and Martin, 2005). Where in the body

these lymphocytes initially are activated is still unclear. This is a critical issue, in view of possible therapeutic interventions aiming to limit activation of autoreactive T cells. Under experimental conditions, dendritic cells activate naïve $CD4^+$ transgenic T cells directed against proteolipid protein (PLP) peptide 139-151 within the CNS (McMahon *et al.*, 2005). However, the classical view on the initiation of naïve T-cell activation holds that antigens or antigen-containing antigen-presenting cells (APC) must be transferred to the brain-draining cervical lymph nodes (CLN) to effectively activate naïve T cells (Karman *et al.*, 2004, Mohindru *et al.*, 2004). CNS-resident APC subsequently reactivate these antigen-experienced T cells and allow them to exert their effector functions within the target organ (Flügel *et al.*, 2001, Greter *et al.*, 2005). A crucial role of the CLN in CNS inflammation is supported by the observation that surgical removal of the CLN reduced the number of brain lesions in cryolesion-enhanced experimental autoimmune encephalomyelitis (EAE) in the rat (Phillips *et al.*, 1997). Furthermore, myelin basic protein (MBP), PLP and neutral lipid-containing APC are present in the CLN of MS patients and EAE-affected marmoset and rhesus monkeys (de Vos *et al.*, 2002, Fabriek *et al.*, 2005). These APC stimulated myelin-specific T-cell proliferation (de Vos *et al.*, 2002), demonstrating that myelin drainage to the CLN during demyelinating disease results in the activation of autoreactive T cells.

We hypothesize that drainage of neuronal antigens from the target organ to the CLN may be similarly involved in initiating or modulating neuron-specific immune responses. We have analyzed whether brain-derived neuronal antigens are present in CLN of MS patients and of selected animal models with different degrees of CNS damage. In addition, we determined whether these antigens are present in distinct APC subsets, which may influence T-cell activation.

METHODS

MS tissues

Human jugular (deep) CLN and supraclavicular CLN were taken from MS patients with active disease at autopsy. Chronic inactive and active plaques were present in the cerebrum of these patients. The MS patients died of non-MS-related causes. In addition, CLN were taken from controls without neurological disease at autopsy by the Netherlands Brain Bank (coordinator Dr. R. Ravid). CLN were snap-frozen in liquid nitrogen and stored at -80°C until use.

EAE tissues

All animal studies in the current study followed the principles of animal care and were approved by local ethical committees based on national legislation. Tissues were

obtained from animals that were used for studies designed for other purposes, thus avoiding the sacrifice of animals for the present study only.

Deep CLN were isolated from rhesus monkeys (*Macaca mulatta*) with acute EAE (*n* = 5) which was induced as described (Kerlero de Rosbo *et al.*, 2000) and from rhesus monkeys with collagen-induced arthritis (*n* = 3) (de Bakker *et al.*, 1992). Deep CLN were also isolated from common marmoset monkeys (*Callithrix jacchus*) during chronic EAE (*n* = 5) ('t Hart *et al.*, 1998, Brok *et al.*, 2000). Control CLN were from marmosets (*n* = 2) immunized with ovalbumin in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI) (de Vos *et al.*, 2002). The monkeys were housed at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands).

Deep and superficial CLN were isolated from Biozzi ABH (H-2^{dq1}) mice, obtained from stock bred at the BPRC, at the acute phase of disease (*n* = 3) and during the first relapse (*n* = 3). EAE was induced by immunization with spinal cord homogenate or MOG8-21 in CFA as described (Amor *et al.*, 1993, Baker *et al.*, 1990). Deep and superficial CLN were also isolated from CCR7-deficient (*n* = 4) and wild type mice (*n* = 4) (Höpken *et al.*, 2004) with chronic EAE, obtained from stock bred at the animal facility of the Max Delbrück Centre for Molecular Medicine (Berlin, Germany). Chronic EAE was induced by s.c. injections of 200 µg MOG35-55 emulsified in CFA containing 400 µg of *Mycobacterium tuberculosis*. Mice received i.v. injections with 200 ng *Bordetella pertussis* toxin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) on day 0 and 2 post-immunization. The CCR7-deficient mice used in these experiments had been backcrossed to C57BL/6 mice for eight generations.

All animals were examined daily for clinical symptoms of EAE as described ('t Hart *et al.*, 1998, Amor *et al.*, 1993, de Vos *et al.*, 2002, Visser *et al.*, 2005). Tissues were snap-frozen in liquid nitrogen and stored at -80°C.

Non-inflammatory CNS damage models

Ischemia was induced in C57BL/6 mice (BgVV) by occlusion of the left middle cerebral artery (MCAO) (Prass *et al.*, 2003). The MCAO did not exceed 60 min. CLN were isolated 24 h (*n* = 5) and 72 h (*n* = 5) after MCAO. Entorhinal cortex lesion (ECL) and facial nerve axotomy (FNA) were performed as described (Rappert *et al.*, 2004) in C57BL/6 mice (*n* = 5 and *n* = 3, respectively; Charles River Laboratories, Sulzfeld, Germany). CLN were isolated seven days later.

Demyelination was induced chemically by cuprizone (Sigma-Aldrich Chemie) in SJL/J mice (*n* = 3; Janvier BioServices, Schuijk, The Netherlands) and C57BL/6 mice (*n* = 3; Harlan, Horst, The Netherlands) as described (Hiremath *et al.*, 1998). These animals were representative animals within a larger experiment designed for other purposes, consisting of 18 SJL/J mice and 18 C57BL/6 mice. Five to six weeks after start of treatment with cuprizone, superficial CLN, deep CLN and lumbar lymph nodes (LLN) were isolated. All tissues were snap-frozen and stored at -80°C.

Immunohistochemistry

Immunohistochemistry was performed as described (Claassen and Jeurissen, 1996, Laman *et al.*, 1998). Primary antibodies were polyclonal rabbit antibodies against TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA) and neurofilament light (NF-L; Abcam, Cambridge, UK), and monoclonal mouse-antibodies against neuron-specific enolase (NSE; MIG M3; Abcam), microtubule associated protein-2 (MAP-2; HSM 5; Pierce Biotechnology, Rockford, IL), neurofilament heavy (NF-H; SMI-32; Sternberger Monoclonals, Baltimore, MD), PLP (J1/06; Laman *et al.*, 2001), HLA-DP/DQ/DR (CR3/43; Dako, Glostrup, Denmark), CD40 (5D12; dr. M. de Boer), IL-1ra (A71B6D11; Bioscource, Etten-Leur, The Netherlands), TNF-α (61E71; U-CyTech, Utrecht, The Netherlands), IL-12p40/p70 (C8.6; BD, Alphen a/d Rijn, The Netherlands) and CCR7 (2H4; BD).

Primary antibodies were detected by biotinylated donkey-anti-rabbit immunoglobulin (Ig; Amersham Biosciences, Roosendaal, The Netherlands) or rabbit-anti-mouse Ig (Dako), and HRP-conjugated avidin-biotin complex (Dako). HRP activity was revealed with 3-amino-9-ethylcarbazole (Sigma-Aldrich), which resulted in a red precipitate. Brain or spinal cord sections from the same species were used as positive control tissue. As negative controls, sections were incubated with isotype-matched primary antibodies of irrelevant specificity, or the primary antibody was omitted.

Immunofluorescence

Double-labeling was performed using immunofluorescence as described (Visser *et al.*, 2006). Sections were incubated with primary antibodies for 1 h at RT, followed by FITC- or TRITC-labeled rabbit-anti-mouse Ig or TRITC-labeled swine-anti-rabbit Ig (both form Dako) for 30 min at RT. Subsequently, sections were incubated for 1 h at RT with Alexa594-labeled anti-MAP-2 (IgG1 Zenon labeling kit, Molecular Probes [Invitrogen], Breda, The Netherlands) or with anti-MOG for 1 h at RT, followed by biotinylated anti-IgG2a (Southern Biotechnology Associates, Birmingham, AL) for 30 min at RT and FITC-labeled streptavidin (Dako) for 1 h at RT. Sections were mounted in glycerol/tris/DAPI (50 µg/ml; Molecular Probes).

As controls, single stainings were performed following the procedure described above but with omission of one of the primary antibodies used in the double-staining. Human reactive tonsil or brain was used as positive control tissue. Negative controls were performed as described above.

CCR7 expression by myelin-laden human monocyte-derived macrophages

Monocytes from healthy donors were routinely purified and cultured (Boven *et al.*, 2006). For the current study, experiments were performed with cells from two individual donors that responded representatively for at least 50 donors assessed in the preceding

years. Monocyte-derived macrophages were seeded into 24-well tissue culture plates at 2 x 10^5 cells/well. After 24 h, non-adherent cells were removed and remaining cells were incubated with human myelin (Norton and Poduslo, 1973) for the indicated times. Control macrophages were obtained from the same donor.

Total mRNA was extracted from cell cultures as described (Boven *et al.*, 2006). CCR7 mRNA expression was analyzed by real-time PCR using the iCycler (Bio-Rad Laboratories, Veenendaal, The Netherlands) and the iQ SYBR Green supermix (Bio-rad). The housekeeping gene HPRT1 was used for normalization. The following primers were used: 5'-TGGTCGTGGTCTTCATAGTC-3' and 5'-CAGGTGCTACTGGTGATGTT-3' for CCR7, and 5'-TGACACTGGCAAAACAATGCA-3' and 5'-AGCTTGCTGGTGAAAAGGACC-3' for HPRT1.

CCR7 surface expression by human monocyte-derived macrophages was determined using flow cytometry. Fc-receptors were blocked using 20% Fc-block (Miltenyi Biotec) and CCR7 was stained using phycoerythrine-labeled mouse-anti CCR7 (150503; R&D Systems, Abingdon, UK) for 30 min on ice. An isotype-matched antibody of irrelevant specificity was used as negative control. Samples were analyzed on a FACSCalibur flow cytometer using CellQuest analysis software (BD).

T-cell proliferation assay

Deep CLN, superficial CLN, and LLN were isolated from C57BL/6 mice with chronic EAE and from Biozzi ABH mice with relapsing-remitting EAE. EAE in C57BL/6 mice was induced by immunization with 200 μg MOG35-55 in CFA (Visser *et al.*, 2005) and in Biozzi ABH mice as described above. Lymph nodes from five to ten EAE-affected animals were pooled per experiment to obtain enough cells for restimulation. A single cell suspension was obtained by passing the lymph nodes through a 70 μm gauze. 2 x 10^5 lymph node cells were seeded into 96-well round-bottom plates and stimulated with the indicated concentrations of MOG8-21, MOG35-55 (both from Advanced Biotechnology Centre, Imperial College London, UK), recombinant mouse NF-L (rmNF-L) (Heins *et al.*, 1993) or ovalbumin (Worthington Biochemical Corp., Lakewood, NJ). After 4 days, T-cell proliferation was determined by incorporation of [^{3}H]-thymidine for 18 h (Amersham Biosciences) as described (Visser *et al.*, 2005).

Image analysis

The area of the sections was measured using a VIDAS-RT image analysis system (Kontron Elektronic/Carl Zeiss) to obtain the number of antigen-positive cells per square millimeter. Differences in number of neuronal antigen-containing cells in CLN between treatment groups were determined by a two-tailed Mann-Whitney test using the statistical software program SPSS, version 11.0. A significance level of 0.05 was used.

RESULTS

Presence of neuronal antigens within the CLN of MS patients

To determine the presence of neuronal antigens in CLN of MS patients with active disease, cryosections were stained with antibodies specific for three neuronal antigens: microtubule associated protein-2 (MAP-2), neuron-specific enolase (NSE) and NF-H. Cells containing each of the neuronal antigens were detectable in CLN from all MS patients, which were predominantly large cells located in the medulla of the lymph node with a morphology resembling macrophages. Neuronal antigen-containing cells were also found in the paracortex, where dendritic cells and T cells are located (Figure 1A-C).

As previously described, neuronal antigen-containing cells were also present in CLN of control subjects (Figure 1D-F) (Yan *et al.*, 2005, Zhao *et al.*, 2002). To investigate whether the presence of neuronal antigens in CLN of MS patients was the result of neuronal damage in the CNS or the result of expression by cells in the CLN, neuronal antigen-containing cells were quantified and compared with the number of neuronal antigen-containing cells in CLN of control subjects without neurological disease. As the numbers of MAP-2, NSE, and NF-H positive cells in CLN of MS patients and control subjects were not statistically different (Figure 1D-F), we could not definitively conclude that increased neuronal damage within the CNS resulted in increased neuronal antigen drainage to CLN. Hence, we performed an elaborate analysis in a variety of EAE models and in animal models of non-inflammatory CNS damage (Table 1).

Presence of neuronal antigen-containing cells in CLN of EAE animals reflects the intensity of neuronal damage

It has been well established that the rhMOG-induced EAE model in common marmoset monkeys is characterized by chronic progressive disease with limited axonal destruction, whereas the same EAE model in rhesus monkeys follows a short-lasting acute disease course with extensive axonal destruction (‘t Hart *et al.*, 2005). In CLN of EAE-affected marmoset monkeys, NSE and NF-H-containing cells were observed (Figure 2A, B). Quantification revealed a higher, but not significantly different, number of neuronal antigen-containing cells in CLN of EAE-affected marmosets as compared to CFA-controls (Figure 2C, D). In contrast, the CLN of EAE-affected rhesus monkeys contained significantly more MAP-2 and NF-H-containing cells (Figure 2E, F), than the CLN of CFA-controls ($p < 0.05$; Figure 2G, H).

The neuronal antigen NF-L was detected in deep CLN from EAE-affected Biozzi ABH mice with clinical EAE, but only rarely in superficial CLN (Figure 2I). This EAE model is characterized by a first peak of acute disease with inflammation and axonal injury, followed by one or more relapses. As an example, Figure 2K shows the typical relapsing-remitting disease course in this model. CLN isolated during acute EAE as

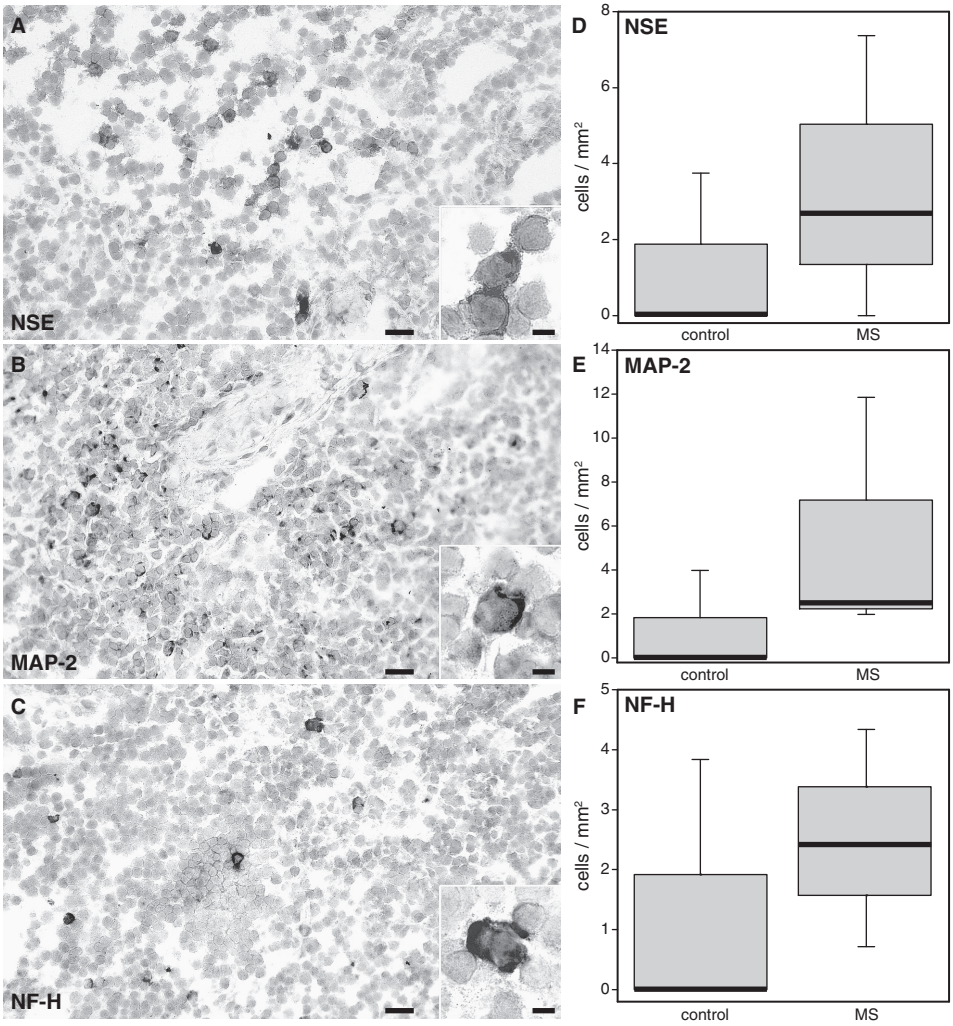

Figure 1. Neuronal antigens drain to the CLN in MS. CLN of MS patients contain cells that are positive for the neuronal antigens NSE (**A**), MAP-2 (**B**), and NF-H (**C**). Scale bars: 20 μm; insert 5 μm. Quantification of the number of MAP-2 (**D**), NSE (**E**), and NF-H (**F**) positive cells demonstrated a higher, but not significantly different, number of neuronal antigen-containing cells in CLN of three MS patients than in CLN of three controls without neurological disease. Three sections from CLN of each patient were quantified for each neuronal antigen. Results are shown as box plots with medians, twenty-fifth and seventy-fifth percentiles as boxes, tenth and ninetieth percentiles as whiskers. See page 206 for a full-color representation of this figure.

Table 1. Characteristics of MS and the animal models used in this study.

Disease	Selected characteristics for this study	Demyelination	Neuronal damage
Human MS	Myelin antigens in CLN (neutral lipids, MBP, PLP) (de Vos *et al.*, 2002)	Present (Sospedra and Martin, 2005)	Present (Sospedra and Martin, 2005)
Marmoset EAE	Chronic EAE Myelin antigens in CLN (neutral lipids, MBP, PLP) (de Vos *et al.*, 2002)	Present ('t Hart *et al.*, 2005)	Limited ('t Hart *et al.*, 2005)
Rhesus EAE	Acute EAE Myelin antigens in CLN (MBP, PLP) (de Vos *et al.*, 2002)	Dramatic destruction of myelin ('t Hart *et al.*, 2005)	Dramatic destruction of axons ('t Hart *et al.*, 2005)
Biozzi ABH EAE	Chronic relapsing EAE	Little in acute phase, abundant in relapse (Amor *et al.*, 2005)	Present in every phase of disease (Amor *et al.*, 2005)
C57BL/6 EAE	Chronic EAE	Present (Tsunoda *et al.*, 2007)	Present (Tsunoda *et al.*, 2007)
Cuprizone-treated mice	Chemically-induced demyelination without clinical disease	Extensive demyelination (Hiremath *et al.*, 1998)	Little neuronal damage (Irvine and Blakemore, 2006)
MCAO	Ischemic lesions in the cortex, striatum and hippocampus	Massive myelin loss (Prass *et al.*, 2003)	Massive neuronal loss (Prass *et al.*, 2003)
ECL	Perforant pathway is stereotactically lesioned, leading to anterograde axonal degeneration within dentate gyrus of the hippocampus (Bechmann *et al.*, 2001)	Limited to the perforant pathway	Present in hippocampus (Kwidzinski *et al.*, 2003)
FNA	Blood-brain barrier remains intact. Retraction of the motoneurons in the brainstem (Kreutzberg, 1996)	Little	Little

well as during the first relapse had significantly more NF-L-containing cells than CFA-immunized control mice ($p < 0.05$; Figure 2J). We observed similar numbers of NF-L-containing cells in the first EAE episode and the subsequent relapse.

As in CLN of MS patients, neuronal antigen-containing cells in the CLN of EAE-affected mice and monkeys were large macrophage-like cells which were located in the medulla of the lymph node, and, although in lower numbers, in the paracortex.

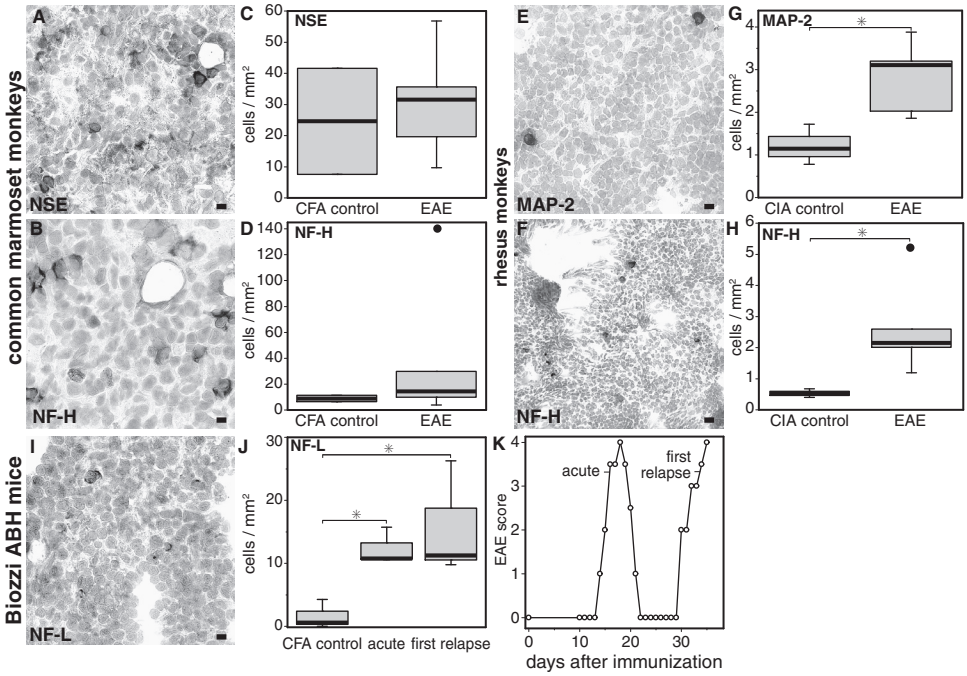

Figure 2. The presence of neuronal antigen-containing cells in the CLN during EAE reflects the extent of neuronal damage within the CNS. CLN of EAE-affected common marmoset monkeys contain NSE (**A**) and NF-H (**B**) positive cells. More NSE (**C**) and NF-H (**D**) positive cells were observed in CLN of five marmosets with EAE as compared to two CFA-immunized control marmosets, although this difference was not significant. In CLN of EAE-affected rhesus monkeys MAP-2 (**E**) and NF-H (**F**) positive cells were found. Quantification of MAP-2 (**G**) and NF-H (**H**) positive cells revealed a significantly higher number of cells in CLN of five rhesus monkeys with EAE than in CLN of three collagen-induced arthritis (CIA) control monkeys (* $p < 0.05$). NF-L-positive cells were present in the deep CLN of EAE Biozzi ABH mice (**I**). Deep CLN from three animals with acute disease as well as from three mice in the first relapse contained significantly more NF-L positive cells than CLN from three CFA-immunized control mice (**J**; * $p < 0.05$). EAE scores from one representative animal out of three are shown (**K**). Three sections from CLN of each monkey and four sections from CLN of each mouse were quantified for each neuronal antigen. Results are given as box plots as described in Figure 1 with extreme values as filled circles. Scale bars: 10 μm. See page 207 for a full-color representation of this figure.

Drainage of CNS antigens to the CNS-draining lymph nodes is reflected by the extent and type of CNS damage

To further assess whether the extent and also the type of CNS insult determines drainage of CNS compounds, and to investigate drainage routes of CNS antigens in mice, we used different mouse models with non-inflammatory CNS damage. The MCAO model is characterized by massive ischemic lesions in the cortex, striatum

and the hippocampus (Prass *et al.*, 2003). Superficial as well as deep CLN isolated 24 h after MCAO contained numerous proteolipid protein (PLP) and NF-L-containing cells (Figure 3B, C). This number was reduced after 72 h (Figure 3B, C), indicating rapid and transient drainage following CNS damage. In CLN isolated from mice with ECL and FNA, few PLP and NF-L-containing cells were observed in the CLN (Figure 3B, C), reflecting the mild CNS damage in these models (Kreutzberg, 1996, Kwidzinski *et al.*, 2003). The cuprizone model is characterized by extensive CNS demyelination induced by cuprizone, which selectively kills oligodendrocytes (Hiremath *et al.*, 1998).

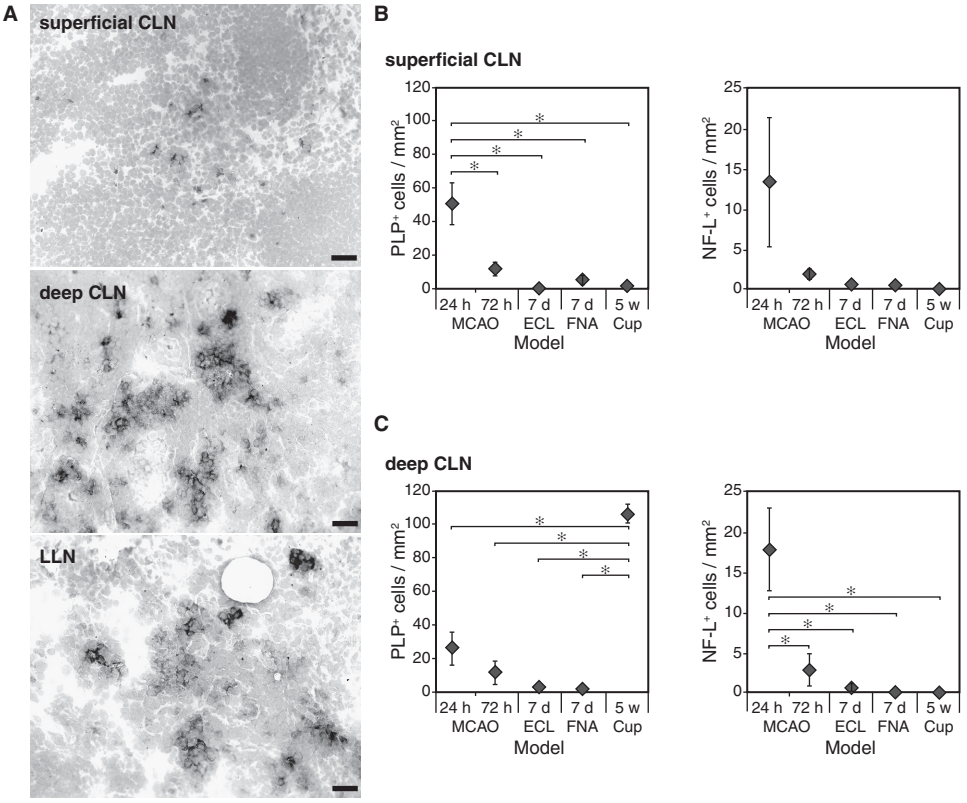

Figure 3. The type and extent of non-inflammatory CNS damage correlates with the frequencies of CNS antigens in the CNS-draining lymph nodes. PLP-containing cells in the superficial CLN, deep CLN, and the spinal cord-draining LLN of cuprizone-treated SJL/J mice. Scale bars: 50 μm (**A**). Quantification of the number of PLP- and NF-L-containing cells in the superficial CLN (**B**) and deep CLN (**C**). Tissues were isolated 24 h (n = 5) and 72 h (n = 5) after MCAO, seven days after ECL (n = 5), seven days after FNA (n = 3), and after six weeks of cuprizone treatment (Cup; n = 3). Cells were quantified in at least two sections of each tissue. Data represent the mean number of positive cells ± s.e.m. * $p < 0.05$.

Little drainage of the myelin antigen PLP to the superficial CLN was noticed (Figure 3A, B). In contrast, the deep CLN of both SJL/J mice (Figure 3A, C) as well as C57BL/6 mice (data not shown) contained significantly higher numbers of PLP-containing cells ($p < 0.001$). We also detected myelin antigens in the LLN (Figure 3A, C), reflecting drainage of antigens from the spinal cord. Only few NF-L-containing cells were observed in the CLN of cuprizone treated mice (Figure 3B, C), which is in line with the limited neuronal damage in this model (Irvine and Blakemore, 2006).

Neuronal antigen-containing cells have an APC phenotype

Although the majority of neuronal antigen-containing cells in MS CLN were located in the medulla of the lymph node, such cells were also found in the paracortex (Figure 1A-C), where APC interact with naïve T lymphocytes. We therefore investigated whether neuronal antigen-containing cells in MS CLN express APC markers. As expected in a secondary lymphoid organ, numerous cells in the CLN expressed MHC class II antigens and CD40 of which only a fraction contained the neuronal antigen MAP-2. However, almost all (90-100%) MAP-2 positive cells expressed MHC class II antigens (Figure 4A) and the costimulatory molecule CD40 (Figure 4B). This indicates that neuronal antigen-containing cells are APC, such as dendritic cells or macrophages, which may be involved in the induction of autoimmunity against neuronal antigens, or conversely in the control of autoreactivity.

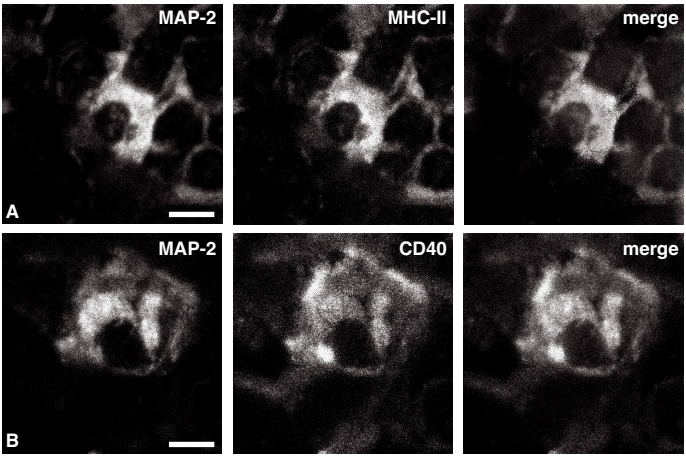

Figure 4. MAP-2 containing cells in human MS CLN are immunocompetent APC. Immunofluorescent MAP-2 staining in human MS CLN (red) and staining for the APC antigens MHC class II (green) (**A**) and CD40 (green) (**B**). Overlay shows co-expression of MAP-2 with MHC class II and CD40 antigens, which was observed in 90 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Stainings were performed on two sections of CLN from three different MS patients. Scale bars: 5 μm. See page 208 for a full-color representation of this figure.

T cells in CLN proliferate to MOG peptide, without evident epitope spreading to NF-L

The localization of neuronal antigens in APC within the CLN suggests that autoreactive T cells may be activated. This was tested in three independent experiments. Two experiments were using C57BL/6 mice in which EAE was induced by injection with MOG35-55 (*n* = 5-10 per experiment). One additional experiment was using Biozzi ABH mice (*n* = 10) in which EAE was induced by injection with MOG8-21. Figure 5 shows a representative experiment, in which we observed dose-dependent T-cell proliferation against the immunizing peptide MOG35-55 in deep CLN, superficial CLN as well as in LLN. CLN and LLN from mice immunized with MOG8-21 demonstrated quantitatively similar proliferation against MOG8-21 (data not shown). No proliferation against the irrelevant control antigen OVA was seen. We did not detect NF-L-specific T-cell proliferation in the two EAE models used for these experiments.

Differential expression of pro- and anti-inflammatory molecules by myelin-containing versus neuronal antigen-containing cells

Myelin-laden myeloid cells in MS brain with foamy appearance demonstrate a strong anti-inflammatory phenotype *in situ* and *in vitro*, implying a possible role in the resolution of local inflammation during MS (Boven *et al.*, 2006). To assess whether this also holds

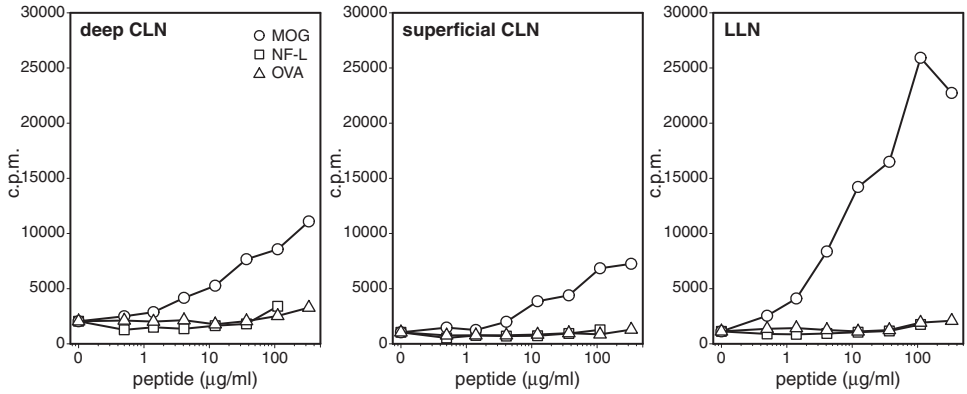

Figure 5. Drainage of neuronal antigens to CLN in MOG peptide-induced EAE does not elicit a detectable NF-L-specific T-cell response. Stimulation of lymph node cells from deep CLN, superficial CLN and LLN from EAE-affected C57BL/6 and Biozzi ABH mice with rmNF-L, MOG35-55 and ovalbumin demonstrates a dose-dependent proliferation against the myelin-derived peptide MOG, whereas no proliferation was observed against NF-L and ovalbumin. Lymph nodes of five to ten EAE-affected mice were pooled per experiment to obtain enough cells for restimulation. Results are presented as mean with the standard deviation of triplicates and are representative for three independent experiments using two different MOG peptide-induced EAE models.

true for myelin antigen-containing cells and neuronal antigen-containing cells in CLN of MS patients, we determined the expression of pro- and anti-inflammatory molecules. The majority of MOG-containing cells (90-100%) co-expressed the anti-inflammatory molecules IL-1 receptor antagonist (IL-1ra) (Figure 6A) and TGF-β (Figure 6B), whereas only a minority of the cells (2-30%) expressed the pro-inflammatory molecule IL-12p40/p70, indicating that most MOG-containing cells display an anti-inflammatory phenotype (Table 2). In contrast, none of the MAP-2-containing cells expressed IL-1ra and TGF-β, while the majority of the cells (86-100%) expressed IL-12p40/p70 (Figure 6C) and TNF-α (Figure 6D), indicating a pro-inflammatory phenotype (Table 2). These data were paralleled in the CLN of EAE-affected rhesus monkeys, where 50-95% of the MOG-containing cells expressed IL-1ra and 13-47% expressed IL-12p40/p70, whereas 75-94% of the MAP-2-containing cells expressed IL-12p40/p70 and none expressed IL-1ra.

Part of MOG-containing cells in CLN of MS patients express CCR7, whereas MAP-2-containing cells do not express CCR7

Drainage of CNS antigens to the CLN may either occur as soluble proteins or within phagocytes (Karman *et al.*, 2004, Weller, 1998). We have previously shown that myelin antigen-containing cells in rhesus monkey CLN express the chemokine receptor CCR7 (de Vos *et al.*, 2002), which mediates leukocyte homing to draining lymph nodes. Similarly, we observed that 20-42% of MOG-containing cells in human MS CLN expressed CCR7, whereas none of the MAP-2-containing cells expressed this marker (Table 2). These data were paralleled in the CLN of EAE-affected rhesus monkeys, in which 20-46% of MOG-containing cells expressed CCR7, and MAP-2-containing cells did not express CCR7.

To investigate whether CCR7 expression is induced by the uptake of myelin antigens, we incubated human monocyte-derived macrophages from healthy donors *in vitro* with human myelin and determined CCR7 mRNA expression and CCR7 surface protein expression. We observed a time-dependent increase of CCR7 mRNA expression after myelin ingestion. As compared to control macrophages, myelin-laden macrophages demonstrated a 38.4 ± 4.2 fold increase in CCR7 mRNA expression after 24 h of myelin ingestion, which was reduced to a 6.3 ± 0.9 fold increase after 7 days of myelin ingestion. In addition to mRNA, CCR7 surface protein expression was also increased after myelin ingestion (data not shown).

Finally, we assessed whether CCR7 expression affects the drainage of CNS antigens to CLN. To this end we compared the number of CNS antigen-containing cells in the deep and superficial CLN of EAE-affected CCR7-deficient and wild type mice. In our hands, CCR7-deficient mice developed mild EAE and CLN of these mice were therefore compared to CLN from wild type mice with comparable EAE symptoms. Unexpectedly, the numbers of both myelin and neuronal antigen-containing cells in CLN of EAE-affected CCR7-deficient mice were not statistically different as compared to wild type mice (Table 3).

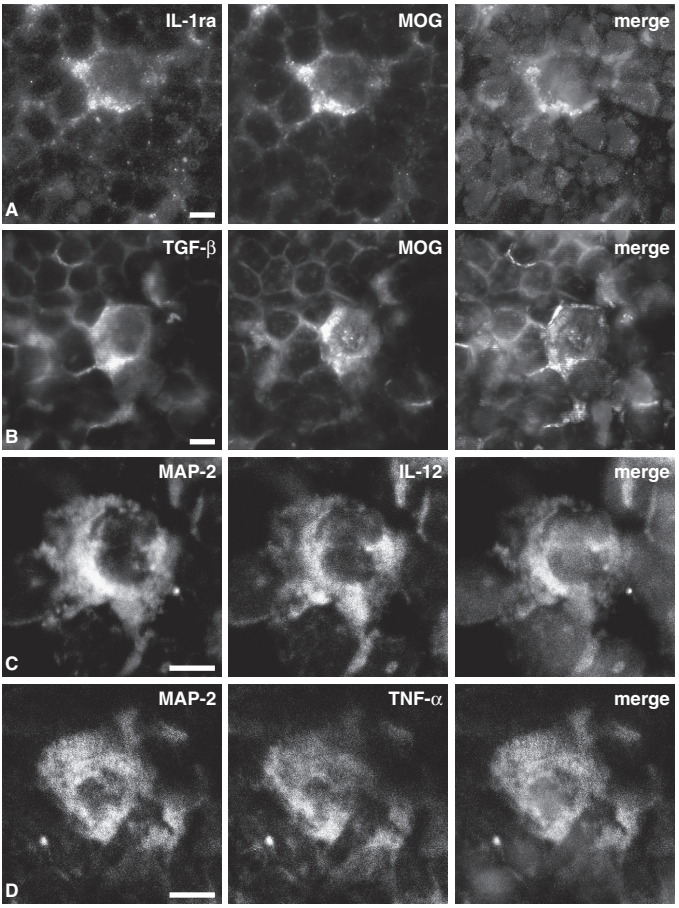

Figure 6. Differential expression of pro- and anti-inflammatory molecules by MAP-2 and MOG containing cells in human MS CLN. Immunofluorescent labeling of MOG (green) and the anti-inflammatory molecules IL-1ra (red) (**A**) and TGF-β (red) (**B**) and immunofluorescent labeling of MAP-2 (red) and of the pro-inflammatory molecules IL-12p40/p70 (green) (**C**), and TNF-α (green) (**D**) in human MS CLN. The overlays show co-localization of MOG and the anti-inflammatory molecules IL-1ra and TGF-β, which was observed in 90 to 100% of the MOG positive cells, and co-localization of MAP-2 and the pro-inflammatory molecules IL-12p40/p70 and TNF-α, which was observed in 86 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Results are representative for two sections of CLN from an MS patient for double stainings of MOG with TGF-β and TNF-α and of MAP-2 with TGF-β and for at least two sections of CLN from three different MS patients for double stainings of MAP-2 with IL-12p40/p70, TNF-α and IL-1ra and double stainings of MOG with IL-1ra and IL-12p40/p70. Scale bars: 5 μm. See page 209 for a full-color representation of this figure.

Table 2. Contrasting functional phenotype of MAP-2 versus MOG-containing cells in human MS CLN.

	Pro-inflammatory molecules		Anti-inflammatory molecules		Lymph node homing potential
	IL-12p40/p70	TNF-α	IL-1ra	TGF-β	CCR7
MAP-2	++++	++++	-	-	-
MOG	+	+/-	++++	+++	+

Quantification of double-labeling of cells in CLN sections of MS patients. – 0%, +/- 1-20%, + 21-40%, ++ 41-60%, +++ 61-80%, ++++ 81-100% of the cells co-expressing both molecules. Results are representative for two sections of CLN from an MS patient for double stainings of MOG with TGF-β and TNF-α and of MAP-2 with TGF-β and for at least two sections of CLN from three different MS patients for double stainings of MAP-2 with IL-12p40/p70, TNF-α, IL-1ra and CCR7 and double stainings of MOG with IL-1ra, IL-12p40/p70 and CCR7.

Table 3. CCR7-deficiency does not affect CNS antigen load in CLN.

	MOG	PLP	MAP-2	NF-L
WT	10.3 ± 10.6	0.8 ± 1.1	3.3 ± 4.6	1.5 ± 2.1
CCR7-deficient	29.7 ± 15.7	8.6 ± 6.1	4.7 ± 4.2	4.8 ± 7.1

Quantification of CNS antigen-containing cells in sections of deep CLN from four EAE-affected wild type mice and four EAE-affected CCR7-deficient mice. Numbers represent mean number of cells per mm^2 ± standard deviation of four sections per animal.

DISCUSSION

Interaction between the brain and the secondary lymphoid organs allows regulation of immune responses in the brain (Cserr and Knopf, 1992). This is exemplified by the crucial role of CLN in brain lesion expansion in cryolesion-enhanced EAE in rats (Phillips *et al.*, 1997), and by the presence of myelin antigens in APC in CLN of MS patients and EAE-affected rhesus monkeys and common marmoset monkeys (de Vos *et al.*, 2002, Fabriek *et al.*, 2005). The current study assessed whether neuronal antigens also drain to the CLN after neuronal damage in the CNS.

MS is both clinically and pathologically a heterogeneous disease demonstrating various degrees of neuronal damage. The obtained CLN tissues were therefore from patients at various disease stages. To further determine drainage in a more controlled setting with defined disease stages, we used a variety of EAE models and models of non-inflammatory CNS insult, representing different degrees of neuronal damage. We report here that neuronal antigens are present in CLN of MS patients and of animals after induction of EAE, MCAO, ECL and FNA. In rhesus monkeys and Biozzi ABH mice, animals in which EAE results in significant neuronal damage within the CNS, the

quantity of neuronal antigen-containing cells in the CLN reflected the extent of neuronal damage in the CNS. Furthermore, qualitative and quantitative drainage of CNS antigens followed the type and extent of CNS damage in mice after MCAO, ECL, FNA, and cuprizone treatment. Interestingly, the frequency of CNS antigens in the CLN was high 24 h after MCAO and reduced 72 h after MCAO, indicating that drainage occurs rapidly but transient following CNS damage. This transient CNS drainage might also cause the little CNS antigen-containing cells in the CLN of the ECL and FNA model, which were isolated 7 days after CNS damage induction. These data extend our previous data and demonstrate that, following CNS damage, both myelin and neuronal antigens drain to CLN.

The presence of neuronal antigen-containing cells in CLN of control subjects was not unexpected, and may be due to natural turnover of neurons in both the CNS and the periphery, innervation of the CLN (Bellinger *et al.*, 2001), expression of neuronal antigens by the cells themselves (Yan *et al.*, 2005, Zhao *et al.*, 2002), or to ageing. The frequency of neuronal antigen-containing cells in CLN from control subjects seems to differ between species, which might be due to differential neuronal turnover rates between species or differential expression levels of neuronal antigens by the cells.

To investigate drainage routes from the brain to the CLN we used different non-inflammatory CNS damage models (Hiremath *et al.*, 1998, Irvine and Blakemore, 2006, Kreutzberg, 1996, Kwidzinski *et al.*, 2003, Prass *et al.*, 2003). In cuprizone-treated animals, numerous cells containing myelin antigens were observed in the deep CLN, whereas such cells were observed only occasionally in the superficial CLN. We have found the same drainage route in EAE-affected mice, where we observed high numbers of neuronal antigen-containing cells in the deep CLN, but hardly in the superficial CLN. These data indicate that CNS antigens preferentially drain to the deep CLN. In contrast, both the deep CLN as well as the superficial CLN of MCAO-treated mice contained numerous CNS antigens. This may be caused by a different drainage route as the result of the massive CNS damage, or by the fact that this massive CNS damage has destroyed (part) of the drainage route. Myelin antigens were also observed in the LLN of cuprizone-treated mice, which are likely derived from the spinal cord, in which demyelination also takes place as is the case in MS and EAE. In fact, whereas CLN are crucial in brain lesion expansion in cryolesion-induced EAE in rats, they have no effect on lesion expansion in the lumbar part of the spinal cord (Phillips *et al.*, 1997), suggesting distinct functional relationships between CNS compartments and their local draining lymph nodes.

The nature of immune responses against CNS antigens in the CLN might be dictated by the functional phenotype of the CNS antigen-containing cells. We therefore determined the phenotype of myelin and neuronal antigen-containing cells in human MS CLN. Neuronal antigen-containing cells in MS CLN expressed the APC markers

MHC class II antigens and CD40, indicating that these cells are equipped for antigen presentation to T lymphocytes. Furthermore, neuronal antigen-containing cells were observed in the paracortex of the lymph node, where APC encounter and activate naïve T cells. Indeed, T-cell responses against neuronal antigens have been demonstrated in MS patients and EAE-affected animals (Forooghian *et al.*, 2007, Furlan *et al.*, 2003, Polak *et al.*, 2001), and T-cell mediated EAE signs can be induced by immunization with neuronal antigens (Mor *et al.*, 2003, Rosenmann *et al.*, 2006). Despite this, we did not detect T-cell proliferation against NF-L in CLN from EAE mice immunized with MOG35-55 or MOG8-21. The lack of T-cell proliferation we consistently found might be due to the absence of intermolecular spreading or to the fact that the draining antigens may act immunosuppressively on proliferation within the draining lymph nodes.

The current study shows that phagocytes containing the neuronal antigen MAP-2 in CLN of MS patients and EAE-affected rhesus monkeys had a pro-inflammatory phenotype. In contrast, the majority of phagocytes containing the myelin antigen MOG demonstrated an anti-inflammatory phenotype, suggesting a relation with the anti-inflammatory myelin-laden foamy macrophages present within MS lesions (Boven *et al.*, 2006). This differential expression of pro- and anti-inflammatory molecules likely influences the type of immune response against these antigens. The difference in functional phenotype between MAP-2 and MOG-containing cells may be the consequence of the inflammatory status of the micro-environment in which the cells have taken up their antigens. Alternatively, the nature of the phagocytosed antigen may direct the immunophenotype of the cell into a pro- or anti-inflammatory mode of action.

As both myelin and neuronal antigens are found in CLN the question arises how these antigens reach these lymph nodes. The two likely mechanisms are either as soluble antigen or by active transport within phagocytosing cells (Karman *et al.*, 2004, Mohindru *et al.*, 2004), such as the anti-inflammatory foamy macrophages within MS lesions (Boven *et al.*, 2006). Since anti-inflammatory macrophages express the lymph node homing receptor CCR7 (Martinez *et al.*, 2006), we determined whether a CCR7-dependent mechanism could be involved. This study shows that, similar to APC in MS brain as well as MOG-containing cells in EAE-affected rhesus CLN (de Vos *et al.*, 2002, Kivisakk *et al.*, 2004), myelin-containing cells in human MS CLN and *in vitro* express CCR7, but MAP-2-containing cells do not. CLN from EAE-affected CCR7-deficient mice contain slightly more myelin and neuronal antigens as compared to CLN from EAE-affected wild type mice, indicating that CCR7 is not necessarily involved. This strongly suggests that either other chemokine receptors are able to guide cell migration to the CLN, or that CNS antigens drain as soluble antigens through CSF to the CLN. This hypothesis is supported by increased free neuronal proteins in CSF of MS patients and EAE mice as compared to healthy controls (Malmestrom *et al.*, 2003, Norgren *et al.*, 2005).

In conclusion, we here report that neuronal antigen-containing cells are present in CLN during MS and in various animal models for CNS damage. The frequencies of these cells correlated with the extent of neuronal damage. In addition, neuronal antigen-containing cells in human MS CLN are present in functionally different APC subsets as compared to the majority of myelin antigen-containing APC containing. The presence of neuronal antigens in APC with a pro-inflammatory phenotype and of myelin antigens in APC with an anti-inflammatory phenotype points at a different potential to activate functionally distinct T-cell subsets.

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IMMUNIZATION WITH NEUROFILAMENT LIGHT PROTEIN INDUCES SPASTIC PARESIS AND AXONAL DEGENERATION IN BIOZZI ABH MICE

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ABSTRACT

Axonal damage is the major cause of irreversible neurological disability in patients with multiple sclerosis. Although axonal damage correlates with antibodies against neurofilament light (NF-L) protein, a major component of the axonal cytoskeleton, the possible pathogenic role of autoimmunity to axonal antigens such as NF-L has so far been ignored. Here we show that Biozzi ABH mice immunized with NF-L protein develop neurological disease characterized by spastic paresis and paralysis concomitant with axonal degeneration and inflammation primarily in the dorsal column of the spinal cord. The inflammatory central nervous system lesions were dominated by $F4/80^+$ macrophages/microglia and relatively low numbers of $CD4^+$ and $CD8^+$ T cells. In splenocyte cultures, proliferation to NF-L was observed in $CD4^+$ T cells accompanied by the production of the proinflammatory cytokine interferon-γ. Elevated levels of circulating antibodies recognizing recombinant mouse NF-L were present in the serum and immunoglobulin deposits were observed within axons in spinal cord lesions of mice exhibiting clinical disease. These data provide evidence that autoimmunity to NF-L protein induces axonal degeneration and clinical neurological disease in mice indicating that autoimmunity to axonal antigens, as described in multiple sclerosis, may be pathogenic rather than acting merely as a surrogate marker for axonal degeneration.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the brain and spinal cord characterized by demyelination and axonal pathologic changes (Ferguson *et al*., 1997, Trapp *et al*., 1998). Although it is now generally accepted that myelin damage results from a synergistic autoimmune attack of myelin-reactive T cells and antibodies to myelin oligodendrocyte protein, for example (Morris-Downes *et al*., 2002), the mechanism of axonal damage is still unclear. Elucidation of this event is crucial to preventing the irreversible neurological deficits in MS associated with axonal loss (De Stefano *et al*., 1998, Petzold *et al*., 2005, Trapp *et al*., 1999). Damage to axons may occur via several mechanisms, including a direct autoimmune attack. However, the role of autoimmunity to neuronal antigens in axonal damage is only supported by circumstantial evidence, i.e. the presence of antibodies against neurofilaments (NFs) and tubulin, in the cerebrospinal fluid (CSF) (Hughes *et al*., 2001, Silber *et al*., 2002) and serum (Almeras *et al*., 2004, Ehling *et al*., 2004, Newcombe *et al*., 1985) of patients with MS. These cytoskeletal proteins, critical for maintaining axonal caliber (Elder *et al*., 1998), are released into the CSF after axonal damage (Malmestrom *et al*., 2003, Semra *et al*., 2002) and may, as has been shown with myelin antigens, drain to the cervical lymph nodes (De Vos

et al., 2002). Subsequently, they may provoke an autoimmune response as has been described for β-tubulin (Skoda *et al*., 2006). In patients with MS, the level of antibodies against the light subunit of NF (NF-L) correlates with the degree of cerebral atrophy (Eikelenboom *et al*., 2003), suggesting that autoimmunity to neuronal antigens may indeed play a role in inducing axonal damage. Furthermore, axon-reactive B cells have been detected in lesions in the brain and isolated from the CSF of patients with MS (Zhang *et al*., 2005a, Zhang *et al*., 2005b).

Autoimmunity to neuronal antigens may also play a pathogenic role in other neurological diseases such as paraneoplastic neurological degeneration (Graus *et al*., 1986), sporadic amyotrophic lateral sclerosis (Couratier *et al*., 1998) and, more recently, Alzheimer disease, in which antibodies have been found within degenerating neurons (D'Andrea, 2003). Antibodies to NF proteins have also been detected in cases of rheumatoid arthritis complicated by peripheral neuropathy (Salih *et al*., 1998). Despite suggestive evidence in humans, animal models confirming a role for antineuronal responses in neurodegeneration are scarce. Until recently, attempts to develop these models for paraneoplastic syndromes have been unsuccessful (Sillevis Smitt *et al*., 1995). Now it is known that mice develop brain inflammation after passive transfer of paraneoplastic Ma1-reactive T cells (Pellkofer *et al*., 2004). In addition, mice immunized with the neuronal tau protein, amyloid-β or β-synuclein peptides develop experimental autoimmune encephalomyelitis (EAE) (Furlan *et al*., 2003, Mor *et al*., 2003, Rosenmann *et al*., 2006), emphasizing the pathogenic role of these antineuronal autoimmune responses.

Although various reports demonstrate the presence of autoimmunity to NF-L in neurological diseases, the pathogenic role of these responses to NF-L is unclear. To investigate the possible role of autoimmunity to NF-L, Biozzi ABH mice were immunized with recombinant mouse NF-L (rmNF-L) in adjuvant. We now show for the first time that immunization with rmNF-L protein causes not only neurological disease (i.e. spasticity and paralysis) but also axonal degeneration. The axonal damage was associated with the presence of activated macrophages, $CD4^+$ and $CD8^+$ T cells and immunoglobulin deposits. This result strongly indicates that autoimmunity to axonal proteins, rather than being merely a surrogate marker, may be a key pathogenic mechanism in neurodegeneration. This new animal model will be important in elucidating the role of autoimmunity to neuronal antigens in chronic neurological disease and may prove to be a valuable tool for the development of novel therapies for neuroprotection.

MATERIALS AND METHODS

Mice

Biozzi ABH (H-2^{dq1}) mice were bred from stock at the Biomedical Primate Research Centre (Rijswijk, The Netherlands). Male and female mice of 6 to 12 weeks of age were used for immunization. Animals were kept at standard laboratory conditions and were fed ad libitum. An animal welfare committee reviewed and approved all experiments (according to the Dutch law).

Immunization and assessment of clinical signs

Mice were immunized with rmNF-L (Heins *et al*., 1993, Herrmann *et al*., 1999) or recombinant mouse myelin oligodendrocyte glycoprotein (rmMOG) as described previously (Amor *et al*., 1993, Baker *et al*., 1990). Briefly, for each mouse, 200 μg protein was dissolved in PBS and emulsified (1:1) with complete Freund's adjuvant (CFA), prepared by adding 48 μg *Mycobacterium tuberculosis* and 6 μg *M. butyricum* to 150 μl incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI). On postsensitization days (PSD) 0 and 7, the mice were immunized subcutaneously with a 300 μl-emulsion divided over two sites on the flanks. A control group received CFA without antigens. Pertussis toxin (200 ng dissolved in PBS) derived from *Bordetella pertussis* (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) was given intraperitoneally immediately and 24 h after immunization. From PSD 10 to 50, mice were weighed daily and examined for neurological signs (i.e. flaccid paralysis and spastic paresis) by visual assessment. Scores were as follows: 1, paralysis or spasticity of the tail; 2, impaired righting reflex; 3, paralysis or spastic paresis of one limb; 4, paralysis or spastic paresis of two limbs; and 5, moribund. Animals exhibiting signs that were less severe than typically observed were scored 0.5 less than the indicated grade. Spasticity of the limbs was also monitored using a purpose-build strain gauge as has been described by Baker *et al*. (2000).

T-cell proliferation assay

T-cell responses to rmNF-L, rmMOG, Biozzi ABH spinal cord homogenate or ovalbumin (OVA) were measured in a proliferation assay. Spleens from rmNF-L-immunized animals were isolated, and a single cell suspension was obtained by passing the tissue through a 100-μm cell strainer. Mononuclear cells were isolated on a Lympholyte-M gradient (Cedarlane, Hornby, Canada) by density centrifugation. Cells were washed twice and seeded (4 x 10^5/well) in RPMI 1640 supplemented with 2% normal mouse serum (NMS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and 5 x 10^{-5} M 2-mercaptoethanol (Gibco™ [Invitrogen] Breda, The Netherlands) in the presence of 0.1, 1, 10 or 100 μg/ml rmNF-L, OVA or spinal cord homogenate. As a positive control, concanavalin A (5 μg/ml) was used.

Cells were cultured for 3 days, and proliferation was measured by incubating for the last 18 h with [^{3}H]thymidine (Amersham Biosciences, Roosendaal, The Netherlands) at 1 μCi/well. Incorporated [^{3}H]thymidine was expressed as mean counts per minute (cpm). Stimulation indices were calculated by dividing the mean cpm of antigen-stimulated cells by the mean cpm of control cells without antigens.

Fluorescence-activated cell sorter analysis of proliferating cells

Splenocytes were carboxyfluorescein succinimidyl ester (CFSE)-labeled by incubation with carboxyfluorescein diacetate succinimidyl ester (Fluka Biochemika, Buchs, Switzerland) and cultured in the absence or presence of 10 μg/ml rmNF-L or OVA for 3 days. Cells were stained with anti-CD4-PE and anti-CD8-PerCP (BD Biosciences, Alphen aan den Rijn, The Netherlands) and the percentage cells with diluted CFSE in the $CD4^+$ or $CD8^+$ compartment was determined using flow cytometry (FACSaria, BD Biosciences). Data were analyzed using FACSdiva software.

Cytokine analysis

Culture supernatants from stimulated splenocytes were collected on day three. ELISAs were performed to assess the production of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-10 (Biosource, Etten-Leur, The Netherlands) and IL-4 (BD Biosciences). The assays were performed according to the manufacturer's procedures.

ELISA for neurofilament light antibody levels

Serum was obtained from peripheral blood of normal mice and mice immunized with CFA only or with NF-L/CFA. To study the presence and the isotypes of NF-L antibodies, Microlon plates were coated overnight at 4°C with 5 μg/ml rmNF-L in PBS and subsequently blocked with 2% bovine serum albumin in PBS. Plates were incubated with sera (1:100 in PBS/1% bovine serum albumin) and, after washing, with alkaline phosphatase-conjugated secondary antibodies directed to total mouse immunoglobulins (rabbit anti-mouse Ig-alkaline phosphatase; DakoCytomation, Glostrup, Denmark), IgG1, IgG2b, IgG3, IgM (all goat anti-mouse; Serotec, Oxford, UK) or IgG2c isotypes (goat anti-mouse; Bethyl Laboratories Inc., Montgomery, TX). Bound antibodies were subsequently visualized with *p*-nitrophenyl phosphate in Tris buffer (Sigma-Aldrich Chemie) and absorbance was measured at 405 nm.

Histology and electron microscopy

Brain, spinal cord and sciatic nerve with attached muscle were removed, fixed in 5% buffered formal saline, and routinely processed for paraffin embedding. The degree of inflammation was assessed by hematoxylin and eosin stain. In addition, mice were

perfused with 4% paraformaldehyde and spinal cord blocks (1-2 mm) were fixed for 4 h in 4% paraformaldehyde in 0.15 M Sörenson's phosphate buffer, followed by 2 h in 2.5% glutaraldehyde (Agar Scientific, Stansted, UK). Tissue blocks were subsequently processed for resin embedding (Araldite CY212; Agar Scientific), semithin 1-μm sections were cut on an LKB Ultratome and stained with toluidine blue. Areas of interest were selected and ultrathin sections were cut, stained with uranyl acetate and lead citrate and viewed on an electron microscope (Jeol 1010 TEM).

Immunohistochemistry

Frozen sections (8 μm) from brains and spinal cords were fixed with acetone, blocked with 10% goat serum and, incubated with primary antibodies against F4/80 (Serotec), CD4 (clone YTS 191.1.2), or CD8 (clone YTS 169AG; ImmunoTools, Friesoythe, Germany). After washing, sections were incubated with biotinylated goat anti-rat Ig (Southern Biotech, Birmingham, AL) followed by ABC amplification (Vector Laboratories, Burlingame, CA). To stain for the presence of Ig isotypes in the spinal cord, sections were blocked with 10% goat serum, and incubated with biotinylated goat anti-mouse IgG1, IgG2a or IgG2b (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) followed by ABC amplification. In addition, unfixed spinal cord sections were extensively washed (three times for 1 hour with PBS/0.05% Tween) before fixation to wash off all antibodies that were not complexed to specific antigen. Bound antibodies were visualized using 3-amino-9-ethyl-carbazole (Sigma-Aldrich Chemie), and sections were counterstained with hematoxylin.

Statistical analysis

Data were expressed as mean ± standard error and were analyzed using either Mann-Whitney U, analysis of variance or Kruskal-Wallis test. As posttests, Newman-Keuls or Dunn's multiple comparison tests were used. $p < 0.05$ was regarded as statistically significant.

RESULTS

Immunization with rmNF-L induces neurological disease

To examine the immunogenic and pathogenic potential of NF-L protein, Biozzi ABH mice were immunized with rmNF-L in CFA. Of the 28 animals, 15 developed weight loss and neurological disease (Figure 1). Three types of neurological disease were observed: 1) flaccid paralysis of hindlimbs, as in classical EAE; 2) spastic paresis (i.e. hyperextension of the hindlimb); and 3) forelimb weakness without involvement of the hindlimbs. Animals immunized with CFA alone did not develop neurological signs (Table 1).

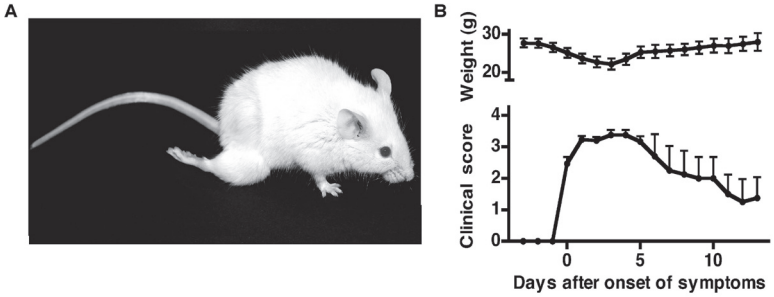

Figure 1. Immunization of Biozzi ABH mice with recombinant mouse neurofilament light (rmNF-L) causes neurological disease. Mice were immunized with rmNF-L in complete Freund's adjuvant (CFA), and clinical disease was monitored. Animals develop spastic paresis, characterized by hyperextension of the limb, and a spastic tail (**A**). The disease course in affected mice included weight loss and subsequent weakness of limbs progressing into spastic paresis or paralysis. After a week, some animals recovered, but their limbs remained weak (**B**).

Mice immunized with rmNF-L exhibiting flaccid paralysis had a tendency to develop disease earlier (PSD 25.8 ± 6.3) (Table 1) than those with spastic signs of the hindlimbs (PSD 29.3 ± 5.7) or with forelimb involvement (PSD 38.0 ± 8.4). In 4 animals the clinical spastic paresis or paralysis resolved after 5 to 6 days and did not reoccur during the observation period (14-21 days).

Table 1. Characteristics of neurological disease in Biozzi ABH mice immunized with rmNF-L in CFA or with CFA alone.

Immunogen	Clinical signs	Incidence	Onset[A]	Disease score[B]
rmNF-L	Spastic paresis	7/15	29.3 ± 5.7	3.5 ± 0.4
	Fore limb dysfunction[C]	4/15	38.0 ± 8.4	3.3 ± 0.7
	Paralysis	4/15	25.8 ± 6.3	3.5 ± 0.0
	Total	15/28 (54%)	31.7 ± 7.5	3.4 ± 0.4
CFA	None	0/6	n.a.	n.a.

Mice were immunized with recombinant mouse neurofilament light (rmNF-L) in complete Freund's adjuvant (CFA) or with CFA alone as described in materials and methods. The mice were examined for clinical signs for 50 days after immunization and the number of animals exhibiting spasticity and paralysis of the limbs recorded.

[A] Mean onset of neurological disease ± sd, in days after the first immunization.

[B] Mean maximum neurological score ± sd of animals exhibiting clinical disease.

[C] Animals with forelimb dysfunction did not display overt spasticity.

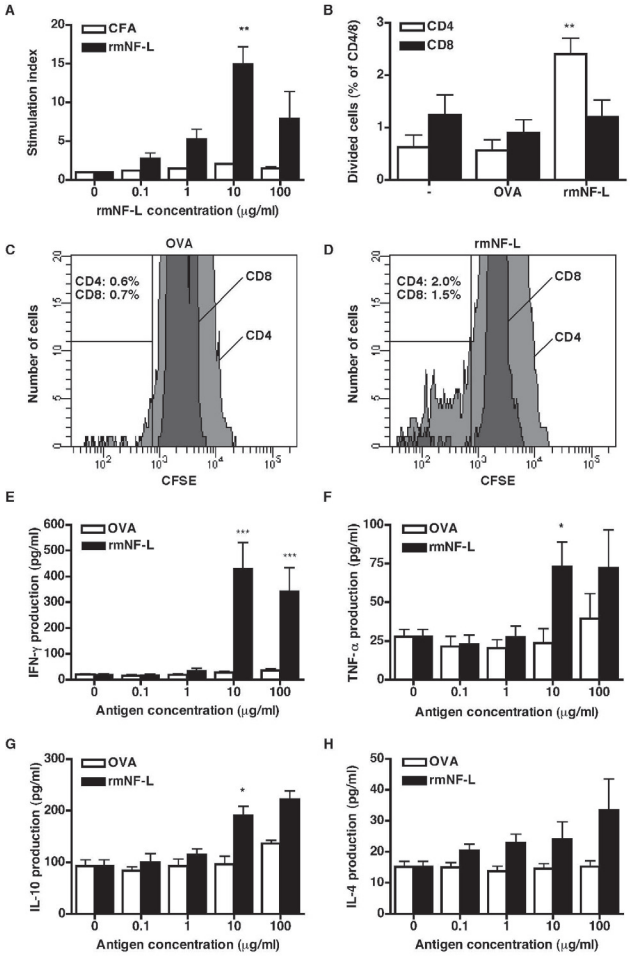

Figure 2. Immunized animals develop autoimmune T-cell responses to recombinant mouse neurofilament light (rmNF-L). Splenocyte proliferation to rmNF-L was measured by [^{3}H]thymidine incorporation. Splenocytes from rmNF-L-immunized mice (n = 7) showed a dose-dependent proliferation to rmNF-L, in contrast to splenocytes from mice immunized with CFA alone (n = 3) (**A**; **, $p < 0.01$, Mann-Whitney U test). Carboxyfluorescein succinimidyl ester (CFSE) labeling combined with CD4 and CD8 staining shows that CD4$^+$ but not CD8$^+$ cells were proliferating to rmNF-L (**B**; n = 5, **, $p < 0.01$, analysis of variance followed by Newman-Keuls multiple comparison test). Representative fluorescence-activated cell sorter plots of ovalbumin (OVA) (**C**) and rmNF-L (**D**) stimulated CFSE-labeled splenocytes of one animal show the divided CD4$^+$ T cells to rmNF-L as several peaks left to the undivided CD4$^+$ population (numbers refer to divided CD4$^+$ or CD8$^+$ cells as a percentage of the total population of CD4$^+$ or CD8$^+$ cells). Culture supernatants were collected after 3 days and assessed for interferon (IFN)-γ (**E**; n = 7), tumor necrosis factor (TNF)-α (**F**; n = 7), interleukin (IL)-10 (**G**; n = 3) and IL-4 (**H**; n = 6) production. Cytokines were secreted dose-dependently upon stimulation with rmNF-L but not OVA (*, $p < 0.05$; ***, $p < 0.001$ compared with OVA, Mann-Whitney U test).

To quantitatively measure the spasticity we determined the resistance to hindlimb flexion using a strain gauge. This system has been successfully used to measure spastic limbs in later stages of EAE, induced with spinal cord homogenate (Baker *et al*., 2000). Compared with normal mice, rmNF-L-immunized animals with spastic paresis were not significantly different in resistance to hindlimb flexion. This finding is consistent with the observation that spasticity was not always present (e.g. when mice were picked up their limbs relaxed) and that spasticity increased upon intentional movement, such as when mice attempted to walk. Mice with paralyzed limbs showed decreased resistance to hindlimb flexion compared with controls (data not shown).

rmNF-L-immunized mice develop autoimmune T-cell responses to rmNF-L

To determine whether the rmNF-L-immunized animals developed autoimmune responses to NF-L, T-cell proliferation assays were performed using splenocytes. All animals, irrespective of their clinical status, developed a dose-dependent proliferative response of splenocytes to rmNF-L (Figure 2A), with a maximum stimulation index of 14.9 ± 2.3 at 10 μg/ml of rmNF-L. In contrast, splenocytes from mice immunized with CFA alone did not proliferate to rmNF-L. The splenocytes from rmNF-L-immunized mice also proliferated to spinal cord homogenate, which contains native mouse NF-L, although these responses were not as high as those observed with rmNF-L (data not shown). No proliferation was observed at similar concentrations of OVA or a control recombinant mouse protein (i.e. rmMOG), wheras all splenocyte cultures proliferated in response to concanavalin A (data not shown).

To characterize the phenotype of the T-cell proliferative response to rmNF-L, splenocytes were labeled with CFSE and cultured in the presence or absence of rmNF-L or OVA. In response to rmNF-L, 2.4% of the total $CD4^+$ population had diluted CFSE indicative of proliferation, compared with 0.6% for OVA or when cultured without antigen ($p < 0.01$; Figure 2B-D). In contrast, in the $CD8^+$ compartment, no differences were observed between rmNF-L-stimulated splenocytes and controls. T-cell proliferation to rmNF-L was accompanied by the production of cytokines. The most abundant cytokine present in the supernatants of splenocyte cultures stimulated with rmNF-L was IFN-γ, which was produced dose-dependently. The level of IFN-γ was significantly higher in NF-L-stimulated cultures (430 ± 101 pg) compared with OVA stimulation (28 ± 6 pg) ($p < 0.001$; Figure 2E). The production of TNF-α and IL-10 was also higher in rmNF-L-stimulated splenocytes compared with OVA (both $p < 0.05$; Figure 2F, G). Although IL-4 was present in supernatant of rmNF-L-stimulated splenocytes, the difference compared with OVA-stimulated cells was not significant (Figure 2H). In summary, rmNF-L-immunized mice developed a significant $CD4^+$ T-cell response to rmNF-L concomitant with the production of high levels of IFN-γ.

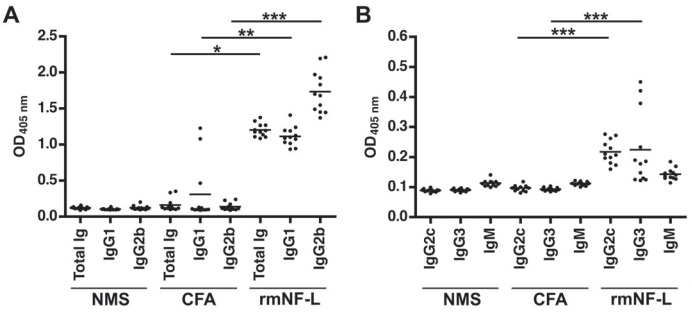

Figure 3. Immunized animals develop autoantibodies to recombinant mouse neurofilament light (rmNF-L). Antibodies to rmNF-L were determined in sera of normal mice (NMS), CFA-immunized mice and rmNF-L-immunized mice using an ELISA. Animals immunized with rmNF-L had significantly higher levels of total Ig, IgG1 and IgG2b antibodies to rmNF-L (**A**). Also, elevated levels of IgG2c and IgG3 but not IgM antibodies to rmNF-L were found (**B**; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Kruskal-Wallis test followed by Dunn's multiple comparison test).

Autoantibodies to rmNF-L are present in rmNF-L-immunized mice

We next studied whether rmNF-L-immunized animals also produced autoantibodies to NF-L. Compared with CFA-immunized controls, all mice immunized with rmNF-L developed significantly elevated levels of antibodies (total Ig) against rmNF-L ($p < 0.05$; Figure 3A). Although some animals immunized with only CFA developed IgG1 responses to rmNF-L, mice immunized with rmNF-L produced significantly higher levels of IgG1 ($p < 0.01$), IgG2b, IgG2c and IgG3 (all $p < 0.001$) antibodies (Figure 3B). The levels of IgM antibodies directed to rmNF-L in sera of mice immunized with rmNF-L were not significantly higher than those in sera of animals immunized with only CFA or in normal mouse serum. In all mice, antibodies to the irrelevant protein OVA were absent (data not shown). Although mice exhibiting neurological disease had a tendency to produce higher amounts of antibody compared with asymptomatic mice, no clear association could be found between antibody levels or isotypes and the severity and duration of disease.

Mice with clinical disease have inflammation and axonal damage

In mice exhibiting clinical neurological disease, lesions of cellular infiltration and axonal degeneration were observed in both the grey and the white matter of the spinal cord (Figure 4A-C). The lesions in the white matter were primarily observed in the dorsal column, in some mice extending to the corticospinal tract, but lesions were also observed in the lateral and ventral columns. Lesions typically showed Wallerian degeneration consisting of vacuoles in which axons had degenerated, leaving myelin sheaths containing only axonal debris (Figure 4B). In some cases the axon was absent

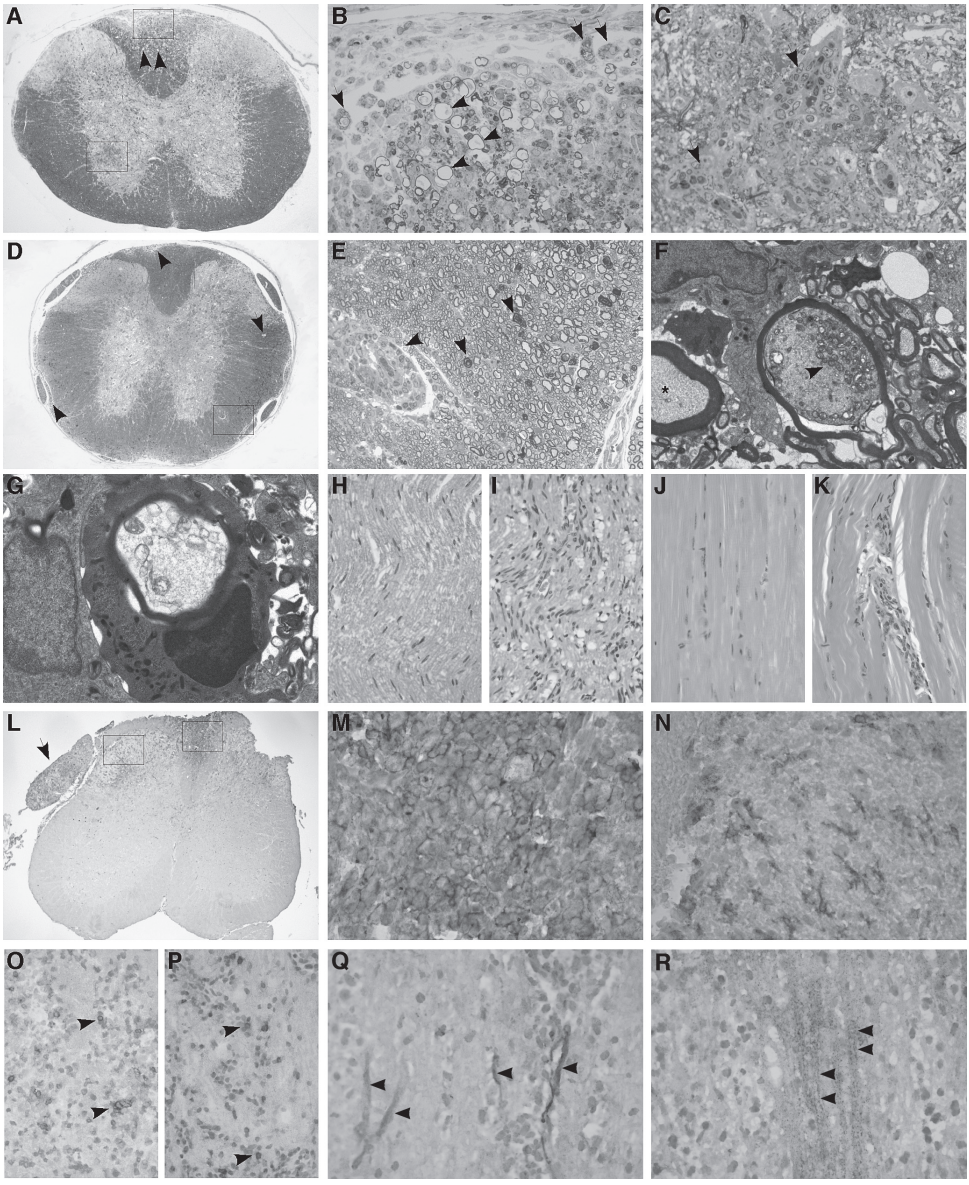
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Figure 4. Mice with neurological disease develop inflammation and axonal degeneration in the spinal cord and sciatic nerve. Semithin resin section of a mouse with spastic paresis reveals extensive vacuolation (arrowheads) in the dorsal column of the spinal cord (**A**; toluidine blue; original magnification: 25x). Axons in this area show signs of Wallerian degeneration (**B**, arrowheads, enlargement of upper rectangle in **A**; 400x); note that phagocytes contain myelin debris (arrows). In the grey matter, groups of inflammatory cells are associated with blood vessels (**C**; arrows, enlargement of lowest rectangle in **A**; 400x). Biozzi ABH mice immunized with rmMOG had inflammation in the spinal cord (**D**; arrowheads, 25x), myelin degeneration (arrows in **E**; enlargement of rectangle in **D**; 400x), but only limited axonal degeneration (arrow in **D**). Electron microscopy of the spinal cord of affected rmNF-L-immunized mice shows accumulated mitochondria in a swollen axon (**F**; arrowhead; asterisk indicates normal axon) and a phagocyte engulfing a damaged axon with its myelin sheath (**G**; 3000x). The sciatic nerve of asymptomatic mice appears normal (**H**; hematoxylin and eosin stain; 200x) whereas inflammation is present in the sciatic nerve of affected mice (**I**; 200x). Surrounding muscle tissue in mice without clinical disease appears normal (**J**; 200x), in contrast with mice exhibiting disease, in which cellular infiltrates can be seen (**K**; 200x). F4/80$^+$ macrophages are present in high numbers in the dorsal column of a mouse with neurological disease (**L**; 25x and **M**; enlargement of the right rectangle in **L**; 400x). In the grey matter, cells with a ramified morphology, resembling microglia, express the F4/80 antigen (**N**; enlargement of the left rectangle in **L**; 400x). T cells expressing the CD4 (**O**; arrowheads; 200x) and CD8 (**P**; arrowheads; 200x) antigen are present in lesions. After extensive washing of unfixed sections, IgG1 is still complexed to axons in the dorsal column of rmNF-L-immunized mice with clinical disease (**Q**; 400x), whereas IgG1 is complexed to myelin sheaths and not to axons in rmMOG-immunized mice (**R**; 400x). See page 210 for a full-color representation of this figure.

as observed from 'empty' myelin sheaths. Swollen axons containing an accumulation of mitochondria, indicative of axonal injury (Lampert, 1967), were also observed (Figure 4F). Generally lesions contained less myelin, although demyelinated axons were not observed, suggesting that the demyelination occurred simultaneously with, or later than, axonal degeneration. Phagocytes within the lesions contained myelin debris (Figure 4B) or were observed surrounding damaged axons with intact myelin sheaths (Figure 4G). Lesions in the grey matter consisted of more compact groups of inflammatory cells, often associated with blood vessels (Figure 4C). No lesions were observed in central nervous system tissues of asymptomatic mice or in brains of affected mice (data not shown). Biozzi ABH mice immunized with rmMOG with similar disease duration showed predominantly inflammation (perivascular cuffs and submeningeal inflammation) as well as myelin degeneration. However, compared with rmNF-L-immunized mice, only limited axonal degeneration in the spinal cord was observed (Figure 4D, E). This indicates that immunization with a neuronal/axonal protein induces more axonal damage than immunization with a myelin protein.

The different clinical manifestations of disease seem to associate with typical pathology. Mice in which the forelimbs were affected were noted to have lesions in the dorsal column of the cervical cord whereas those with hindlimb paresis had lesions in the dorsal column throughout the cord, but most predominantly in the lumbar regions.

Because pathologic abnormalities were observed in axons of the dorsal column, which transmit sensory information from muscle and skin to the brain stem, we also

examined the sciatic nerve and muscle. Preliminary examination of the sciatic nerve (Figure 4H, I) and surrounding muscle tissue (Figure 4J, K) revealed the presence of inflammatory cells, suggesting that the lesions of inflammation and axonal degeneration were present throughout the ascending sensory nerve tract. This is further supported by the presence of cellular infiltrates in dorsal roots (Figure 4L).

Immunohistochemistry was performed to further study the phenotype of infiltrating cells in the spinal cord. Most infiltrated cells in the spinal cord were activated F4/80$^+$ macrophages (Figure 4L, M). In addition, in the dorsal horns, F4/80$^+$ cells with a ramified morphology were present, possibly representing activated microglia (Figure 4N). Compared with the abundant macrophages in the lesions, relatively low numbers of CD4$^+$ (Figure 4O) and CD8$^+$ (Figure 4P) T cells were present in both the grey and the white matter of rmNF-L-immunized mice exhibiting neurological disease.

To investigate the role of antibodies in the pathologic condition, spinal cord sections were stained for mouse immunoglobulin isotypes. Biozzi ABH mice make high levels of IgG1 and IgG2b, but do not produce IgG2a (Amor *et al*., 2005). In the spinal cord of rmNF-L-immunized mice with neurological disease, IgG1 and IgG2b antibodies were observed not only in the inflammatory lesions, as expected due to loss of blood-brain barrier integrity but also within axons and nerve cell bodies. Such staining was not seen in asymptomatic rmNF-L-immunized or control mice (data not shown). IgG2a could not be detected in the spinal cord of affected rmNF-L-immunized mice, indicating that the immunoglobulin staining was specific. To determine whether the observed antibodies in the spinal cord were bound to their target antigen, the unfixed snap-frozen sections from rmNF-L and rmMOG-immunized mice were extensively washed before fixation, assuming that when immunoglobulin was not complexed to a specific antigen, the immunoglobulin should be washed out. After washing, IgG1 staining was reduced in neuronal cell bodies, whereas axonal profiles in lesioned dorsal column areas remained positive (Figure 4Q). In rmMOG-immunized mice, neither neuronal cell bodies nor axonal profiles were present. Instead, thin parallel lines of punctuate staining were observed near inflammatory lesions, representing immunoglobulin complexes on myelin sheaths (Figure 4R). In conclusion, these results show that antibodies complexed to axons are only observed in the lesioned dorsal column of mice immunized with rmNF-L and not in lesions of mice immunized with rmMOG.

DISCUSSION

Axonal damage has been identified as the major cause of irreversible neurological disability in patients with MS. The mechanism for the development of axonal damage is still unknown, but autoimmunity to neuronal antigens has been proposed to play

a role (DeVries, 2004). This study demonstrates that autoimmunity to the neuronal antigen NF-L is pathogenic and induces clinical neurological signs in mice that are also observed in MS. We show that 1) immunization of Biozzi ABH mice with rmNF-L induces autoimmune T- and B-cell responses to NF-L, 2) 54% of the animals immunized with rmNF-L develop spastic or paralytic disease, and 3) animals with clinical neurological disease have severe inflammation and axonal degeneration indicative of Wallerian degeneration. These results suggest that NF-L protein is not only immunogenic but that autoimmunity to NF-L could play a role in inducing axonal damage and clinical neurological symptoms. Although this report describes autoimmunity to NF-L in an experimental system, such autoimmunity to neuronal antigens may be applicable to human neurodegenerative disorders such as MS in which antibodies to NF-L have been described (Almeras *et al*., 2004, Ehling *et al*., 2004, Hughes *et al*., 2001, Newcombe *et al*., 1985, Silber *et al*., 2002).

To our knowledge, our study is the first to show that immunization with the intracellular neuronal protein NF-L induces clinical neurological disease associated with axonal degeneration and inflammation. However, neurological disease induced with neuronal antigens has been reported before, using tissue homogenates of the ventral horn of the spinal cord or purified motor neurons (Engelhardt *et al*., 1989, Engelhardt *et al*., 1990). The specific autoantigens were, however, not defined. Of note is the fact that several of the rmNF-L-immunized mice in our study showed clinical signs similar to those of guinea pigs immunized with spinal cord ventral horn homogenate, suggesting that (one of the) disease-inducing proteins in the ventral horn may be NF-L. Also, immunization with tau protein has been reported to induce neurological disease, that is, limb paralysis (Rosenmann *et al*., 2006). Compared with myelin protein-induced EAE, tau-immunized C57BL/6 mice developed disease relatively late (PSD 41-149). This result is consistent with our findings of late-onset disease in Biozzi ABH mice after NF-L immunization (PSD 21-50). However, tau is not only expressed by neurons, but also by astrocytes and oligodendrocytes (LoPresti *et al*., 1995) and thus disease may not be specifically targeted to neurons alone.

The Biozzi ABH mice immunized with rmNF-L showed axonal degeneration predominantly in the dorsal column of the spinal cord. In rodents, the dorsal column consists of ascending sensory fibers and descending corticospinal fibers (Tracey, 2004). In humans, degeneration of these tracts is known to occur in hereditary spastic paraplegia and in MS (DeLuca *et al*., 2004a, DeLuca *et al*., 2004b). Corticospinal fibers are important for the control of movement through their terminations on spinal motor neurons that innervate the trunk and limb muscles. Damage to these fibers is known to result in spasticity (Pearson, 2000), a symptom also frequently observed in hereditary spastic paraplegia and MS (Goodin, 1999). The spastic model in mice described here thus provides an ideal platform for the development of new compounds for treating spasticity in these neurological disorders.

Exactly how and where the pathogenic events result in axonal damage in the rmNF-L-immunized mice is still unclear. The observation that axons in the spinal cord show signs of Wallerian degeneration indicates that the primary lesion may be not in the spinal cord but rather in the peripheral nerve or dorsal root ganglia. In support of this, inflammation in the sciatic nerve and the dorsal roots were observed in affected animals. The axonal damage observed in the mice may be initiated by $CD4^+$ T cells, which may activate microglia and macrophages to produce cytokines and reactive oxygen species. *In vitro*, $CD4^+$ T cells proliferated and secreted high levels of IFN-γ in response to rmNF-L, pointing to a pro-inflammatory Th1-mediated disease, similar to that observed in myelin-induced EAE (Begolka *et al*., 1998).

Alternatively, axonal damage may occur due to pathogenic antibodies recognizing NF-L protein in axons. In rmNF-L-immunized mice, immunoglobulin was found complexed to axons in the lesioned dorsal column of the spinal cord. Recently, a pathogenic role has been described for antibodies against amphiphysin, a protein associated with synapses, in stiff-person syndrome. Passive transfer of high-titer antibodies isolated from a patient with this syndrome induced spasms and stiffness in rats, which had also received encephalitogenic T cells to open up the blood-brain barrier (Sommer *et al*., 2005). Like amphiphysin, NF-L is also an intracellular protein; therefore, antibodies must enter the cell first to bind their antigen. This could be achieved through retrograde axonal transport (Fabian and Petroff, 1987, Fratantoni *et al*., 1996) or by internalization via the cell body (Dalmau *et al*., 1991). Once inside axons, antibodies may interfere with the cytoskeleton, as has been described for tubulin antibodies (Johnston *et al*., 1986, Stubbs *et al*., 1998). This mechanism would explain why axonal degeneration in rmNF-L immunized mice was observed predominantly in the sensory and corticospinal fibers: these axons are the longest in the nervous system and are therefore the most reliant on an intact cytoskeleton.

Whether T cells, antibodies, or combinations of both are responsible for the clinical manifestation observed in rmNF-L-immunized mice remains to be determined. To address this issue, we performed a preliminary study in which rmNF-L-activated splenocytes from rmNF-L-immunized mice were adoptively transferred into naive mice. Although one of six mice developed very mild disease (i.e. hind limb weakness), we observed no apparent pathologic changes in the spinal cord. However, it cannot yet be firmly established that NF-L-reactive T cells alone are insufficient to induce disease, because naive Biozzi ABH mice appeared to be refractory to disease induced after adoptive transfer of proteolipid protein peptide specific T cells (S. Amor and D. Baker, unpublished data, 1995). Studies in other mouse strains may therefore be more suitable to address this issue.

With regard to establishing the pathogenicity of NF-L-reactive antibodies in disease, it is apparent that high titers of antineuronal antibodies, as described by Sommer

et al. (2005), are required to induce experimental neurological disease. To obtain sufficient antibodies as well as determine the relevance for antibodies to NF-L in human neurodegenerative diseases, our current studies are focused on purifying NF-L immunoglobulin from humans for adoptive transfer into mice.

Determining the pathogenicity of antineuronal antibodies and/or T cells will not only be important for MS, in which antineuronal antibodies are present in the serum and CSF (Eikelenboom *et al*., 2003, Newcombe *et al*., 1985) and, more critically, inside axons (Zhang *et al*., 2005b). The outcome may also be relevant for other neurological diseases, such as amyotrophic lateral sclerosis, Alzheimer disease and Parkinson disease, in which antineuronal antibodies are observed (Couratier *et al*., 1998, D'Andrea, 2003). Unraveling the role of autoimmunity to neuronal antigens in neurodegenerative disorders will crucially expand the possibility for treating these otherwise chronic disorders.

In summary, this study shows that immunization with a single neuronal protein, NF-L, induces neurological disease in Biozzi ABH mice associated with inflammation and axonal degeneration predominantly of the dorsal column. These results indicate that, in humans, autoimmunity to NF-L may not be solely a surrogate marker of axonal pathology but could also play a crucial role in the progressive neurodegeneration observed in MS and other neurological diseases.

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AXONAL LOSS AND GREY MATTER PATHOLOGY AS A DIRECT RESULT OF AUTOIMMUNITY TO NEUROFILAMENTS

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ABSTRACT

Axonal damage is considered the major cause of irreversible disability in multiple sclerosis (MS). Which mechanisms underlie the damage and whether this is secondary to myelin damage remains to be clarified. Recently, we have demonstrated that autoimmunity to the axonal/neuronal cytoskeletal protein neurofilament light (NF-L) induces axonal damage and neurological disease including spasticity - a common feature of MS. To examine the relationship between axonal damage and demyelination we have characterized the detailed neuropathology of NF-L-induced disease in Biozzi mice compared to classical experimental autoimmune encephalomyelitis (EAE) induced with myelin oligodendrocyte glycoprotein (MOG).

In NF-L-induced neurological disease the lesions were predominantly located in the dorsal column displaying extensive axonal degeneration, but were also abundant in the grey matter. In contrast, lesions in MOG-EAE were restricted to the lateral and ventral columns and displayed less axonal damage and little grey matter involvement. The differential lesion location was confirmed by quantitation of leukocyte subsets. In both diseases myelin damage was a common feature although the numerous empty myelin sheaths in NF-L-disease indicative of axonal damage suggest that myelin damage was a secondary event.

In summary, autoimmunity to NF-L induces a distinct lesion topology, axonal damage and grey matter lesions supporting the notion that axonal loss and grey matter pathology can be the direct consequence of a primary autoimmune attack against axonal antigens such as NF-L rather than merely a secondary event to myelin damage.

INTRODUCTION

Multiple sclerosis (MS) is a debilitating neurological disease affecting over 2 million people worldwide. The clinical symptoms, such as visual problems, spasticity, paralysis and sensory disturbances, are a consequence of the damage in specific locations within the central nervous system (CNS). While the aetiology of MS is unknown, the association of inflammatory cells and their mediators with degenerating myelin sheaths suggests a strong involvement of the immune system. However it is the axonal damage that is associated with the irreversible long-term neurological deficits and cognitive changes (Bjartmar *et al.*, 2000, Trapp *et al.*, 1998). Although the interest in axonal loss as part of the early stages of MS has recently grown, the pathogenic mechanisms underlying the axonal damage and neurodegeneration are largely unknown. Events secondary to myelin damage such as a loss of myelin-derived trophic support (Griffiths *et al.*, 1998, Yin *et al.*, 1998) have been implicated in the development of axonal damage in MS, as

have factors such as energy failure and $Ca2^{+}$ accumulation (Dutta and Trapp, 2007). Axonal damage during early MS is associated with inflammation (Bitsch *et al.*, 2000), suggesting that a primary autoimmune attack on axonal targets could play a role similar to the primary attack on gangliosides underlying acute motor axon neuropathy in the peripheral nervous system (Ho *et al.*, 1998).

Increasing evidence suggests that also in the CNS pathogenic autoimmunity is not restricted to myelin (Huizinga *et al.*, 2008, Mathey *et al.*, 2007). Recently we have shown that immunization of Biozzi ABH mice with neurofilament light (NF-L) protein induces spastic paresis and paralysis (Huizinga *et al.*, 2007), both debilitating symptoms of MS. These findings extend previous reports which already showed that astroglial and neuronal antigens can induce experimental neurological disease in animals and that delayed-type hypersensitivity response to Bacillus Calmette-Guérin induces myelin damage in mice (Furlan *et al.*, 2003, Kojima *et al.*, 1994, Matyszak and Perry, 1995, Mor *et al.*, 2003, Rosenmann *et al.*, 2006). That autoimmunity to such targets also play a role during MS is suggested by studies documenting the presence of antibodies directed against NF-L, NF-medium (NF-M), tubulin and neurofascin in MS patients (Bartos *et al.*, 2007, Eikelenboom *et al.*, 2003, Mathey *et al.*, 2007, Silber *et al.*, 2002).

A striking feature of pathogenic autoimmunity in the CNS is that different antigens trigger damage in different regions. In rats, myelin basic protein (MBP)-specific T cells induce lesions predominantly in the spinal cord, whereas myelin oligodendrocyte glycoprotein (MOG)-specific T cells also induce lesions in the cerebellar white matter. T cells specific for the astroglial antigens s100β and glial fibrillary acidic protein lead to severe inflammation in grey matter (Berger *et al.*, 1997). The regional preference for autoimmune attacks on axonal or neuronal antigens is unknown but may be a major factor in determining clinical consequences in MS. Especially different forms of cognitive impairment have been found to be correlated to lesions in particular areas of the brain (Charil *et al.*, 2003, Geurts *et al.*, 2007, Lazeron *et al.*, 2005). Regional distribution of axonal damage as induced by primary autoimmune attack is therefore an important parameter to help understand its impact.

The aim of this study was to compare the neuropathology of mice immunized with the neuronal protein NF-L and the myelin protein MOG. We show that immunization with NF-L preferentially induces lesions in the dorsal column of the spinal cord and in the grey matter. The finding that axonal degeneration is more pronounced in NF-L-immunized mice compared to MOG-immunized mice suggests that axons and grey matter are directly targeted for autoimmune destruction. Together our data support the notion that a primary autoimmune attack on axonal and neuronal antigens can cause selective axonal loss and grey matter pathology, preceding demyelinating events.

MATERIALS AND METHODS

Mice

Female and male Biozzi ABH mice were bred and housed under enriched environmental conditions at the Biomedical Primate Research Centre, Rijswijk, The Netherlands. All experiments were performed after approval of the ethics committee as required by the Dutch law on animal experimentation and according to local guidelines to minimize discomfort.

Immunization and assessment of clinical signs

Mice (8-12 weeks of age) were immunized with 200 μg recombinant mouse NF-L or recombinant mouse MOG (amino acids 1-125) as described previously (Huizinga *et al.*, 2007, Smith *et al.*, 2005). Briefly, proteins dissolved in PBS were emulsified 1:1 with complete Freund's adjuvant (CFA i.e. 320 μg/ml *Mycobacterium tuberculosis* and 40 μg/ml *M. butyricum* in incomplete Freund's adjuvant; Difco Laboratories, Detroit, MI). Mice were injected subcutaneously on days 0 and 7 with 300 μl emulsion divided over two spots on the flanks. A control group received CFA without antigens. Directly after immunization and 24 h later, animals were injected intraperitoneally with 200 ng pertussis toxin derived from *Bordetella pertussis* (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Mice were weighed and scored daily for neurological signs: 1) tail paralysis or spasticity, 2) impaired righting reflex, 3) paralysis or spastic paresis of one limb, 4) paralysis or spastic paresis of two limbs, 5) moribund. Clinical signs that were less severe than typically observed were scored 0.5 less than the indicated grade. When clinical scores were more than 2, wet food pellets were supplied daily in the cage to ensure easy access to food and water.

Tissue preparation and resin embedding

Animals were terminally anaesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, Ceva Sante Animale, Maassluis, The Netherlands) and perfused transcardially with PBS, followed by 4% paraformaldehyde (Agar Scientific, Stansted, UK) in PBS. The spinal cord (from C1 – S2) was dissected whole and post-fixed for 4 h in 4% paraformaldehyde in PBS. Alternate 1 and 2 mm-thick blocks were cut using a 1 mm-interval metal matrix (Agar Scientific). The 2 mm blocks were immersed in PBS / 30% sucrose until saturation and subsequently frozen in OCT using dry ice-cooled isopentane. The 1 mm blocks were further fixed overnight in 4% paraformaldehyde in PBS followed by 2.5% glutaraldehyde in 0.15 M Sörenson's phosphate buffer for 2 h. After washing with Sörenson's buffer, blocks were immersed in 1% osmium tetraoxide (Agar Scientific) for 2 h and subsequently dehydrated by successive ethanol washes ranging from 70-100%. Blocks were incubated with propylene oxide/Araldite (1:1

v/v; Agar Scientific) on a rotary shaker overnight followed by 100% Araldite for 2 h. Blocks were subsequently arranged in a metal cup with fresh Araldite and allowed to polymerize at 60°C for 24 h.

Analysis of lesion distribution and quantitation of lesion area

Semi-thin (1 µm) spinal cord sections were cut using a LKB Ultratome and stained with toluidine blue. Sections were analyzed for the presence and distribution of lesions, i.e. areas with inflammation, demyelination or axonal degeneration using an Olympus BX50 microscope. For an overview of the lesion distribution throughout the spinal cord, lesions were manually drawn onto spinal cord outlines (2 cervical, 2 thoracic and 2 at the lumbar level). Subsequently, sections at three levels of the spinal cord (cervical level: C3-5, thoracic level T4-6 and lumbar level L2-4) were selected and the lesion area quantified as described by McGavern *et al.* (1999). Digital images of spinal cord sections were taken with an Olympus C5060 camera and converted to 8-bit grey-scale using NIH ImageJ Software (http://rsb.info.nih.gov/ij/index.html). The total area of the cord and the lesion area were calculated for the different anatomical regions (i.e. the total spinal cord, the dorsal funiculus, the lateral and the ventral funiculi, and the grey matter; Figure 1). The relative contribution of the lesions in different anatomical regions to the total lesion load was calculated as the lesion area in anatomical region / total lesion area in spinal cord x 100%. In addition, the lesion load in the anatomical regions was defined as lesion area in anatomical region / total area of anatomical region x 100%.

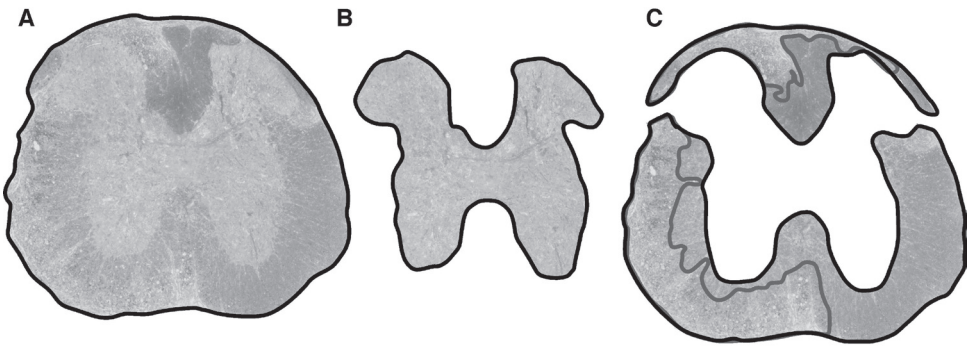


Figure 1. Method of lesion area quantitation. A spinal cord section of a Biozzi ABH mouse immunized with NF-L was stained with toluidine blue and lesion area was quantified using ImageJ software. The total spinal cord area was manually outlined (A), followed by the grey matter (B) and the dorsal column (upper part in C) and the lateral and ventral column (lower part in C). The area containing lesions was subsequently manually outlined (grey line). Finally, the pixel area of each region was measured and the percentage of affected area in that particular region was calculated (magnification x35).

To study whether inflammatory lesions were located in the brains as well as the spinal cord, fixed-frozen sections and paraffin sections of brains (including cortex, cerebellum and brain stem) of NF-L-immunized mice were stained with haematoxylin and eosin or for immune cell subsets.

Quantitation of axonal degeneration and demyelination

Quantitative analysis of the extent of axonal degeneration and demyelination was performed on toluidine blue stained semi-thin sections at three levels of the spinal cord for each animal. Axonal degeneration was quantified by counting the empty myelin sheaths, i.e. myelin sheaths where the axon had degenerated and disappeared or where only axonal debris was left within the myelin sheath. Swollen axons with accumulated organelles were counted as marker of ongoing axonal degeneration. Myelin damage was assessed by counting the number of altered myelin sheaths. The quantitation was performed blind by two observers.

Analysis and quantitation of inflammatory cells

To determine the number of macrophages, T and B cells in the spinal cord of mice immunized with NF-L or MOG, immunohistochemistry was performed on sections from fixed-frozen and additional snapfrozen spinal cord blocks. Sections were incubated for 30 min in 0.3% H_2O_2 in PBS or acetone (for snapfrozen tissue) to block endogenous peroxidase, washed in PBS and incubated overnight at 4°C with 1:1000 diluted rat antibodies against F4/80 (clone CI:A3-1), CD3 (clone CD3-12) or B220 (clone RA3/6B2, all from AbD Serotec, Oxford, UK). To stain CD3 in fixed-frozen sections, heat-mediated antigen retrieval was performed using 0.01 M citric acid at pH 6. Sections were microwaved at 900 W until boiling and subsequently allowed to simmer for 10 min at 360 W. Only snapfrozen tissue was used to detect CD4 (clone L3T4, kindly provided by Dr. P. Leenen, Erasmus MC, Rotterdam) and CD8 (clone YTS169, Bioceros, Utrecht, The Netherlands). Sections were subsequently incubated with biotinylated rabbit anti-rat immunoglobulins (1:250, DakoCytomation, Glostrup, Denmark) followed by ABComplex (1:100, DakoCytomation) and developed using 3-amino-9-ethylcarbazole (ICN Pharmaceuticals, Zoetermeer, The Netherlands) or diaminobenzidine (Merck, Schiphol-Rijk, The Netherlands). The percentage of F4/80-positive area was calculated by determining the area positive for F4/80 as well as the total area of the spinal cord section using ImageJ software as described above. CD3, CD4, CD8 and B220-positive cells were manually counted by a blinded observer. All quantitations were performed at three levels of the spinal cord.

Statistical analysis

Data, analyzed using Graphpad prism or SPSS software, were expressed as mean ± standard error unless otherwise specified. Differences between groups were analyzed with the Mann-Whitney U test and $p < 0.05$ was considered statistically significant.

RESULTS

Differential lesion distribution in NF-L vs. MOG-immunized mice

To determine whether the location or pathology of lesions in the CNS is influenced by the myelin or neuronal origin of the immunogen, mice were immunized with the axonal/neuronal antigen NF-L or the myelin antigen MOG. During clinical disease brains and spinal cords were collected from representative animals exhibiting similar severity of paralysis or spasticity (Table 1). As we have observed previously the neurological signs were significantly delayed in NF-L-immunized compared to MOG-immunized mice (p = 0.0004; Table 1).

Histopathology of the semi-thin toluidine blue-stained spinal cord sections revealed that immunization with either NF-L or MOG induced inflammation and / or demyelination and / or axonal degeneration in the lateral and / or ventral columns (Figure 2). While all MOG-immunized animals developed lesions throughout the entire length of the spinal cord, this was only observed in 2 of 6 mice immunized with NF-L (one with paralytic

Table 1. Clinical scores of mice immunized with NF-L or MOG.

Mouse number	Clinical signs	Tissue treatment	Day of onset	Day of sacrifice	Maximum score	Cumulative score
NF-L 1	paralyzed	perfused	27	28	2.5	4.5
NF-L 2	paralyzed	perfused	24	26	3.5	10
NF-L 3	paralyzed	perfused	23	24	3.5	6
NF-L 4	spastic	perfused	36	38	3	8
NF-L 5	spastic	perfused	25	29	3	15
NF-L 6	spastic	perfused	25	29	3.5	16.5
NF-L 8	paralyzed	snapfrozen	35	40	3.5	19
NF-L 7	spastic	snapfrozen	50	54	3.5	16.5
NF-L 9	spastic	snapfrozen	40	42	4.5	10.5
mean ± sd			31.7 ± 9.2	34.4 ± 9.8	3.4 ± 0.5	11.8 ± 5.2
MOG 1	paralyzed	perfused	16	18	4	8
MOG 2	paralyzed	perfused	11	14	3.5	10.5
MOG 3	paralyzed	perfused	12	19	4	15.5
MOG 4	paralyzed	snapfrozen	12	20	4	21.5
MOG 5	paralyzed	snapfrozen	16	37	3	25.5
MOG 6	paralyzed	snapfrozen	14	20	3.5	14.5
mean ± sd			13.5 ± 2.2	21.3 ± 8.0	3.7 ± 0.4	15.9 ± 6.6
***p*-value ***			0.0004	0.0076	0.31	0.20

* Mann-Whitney U test

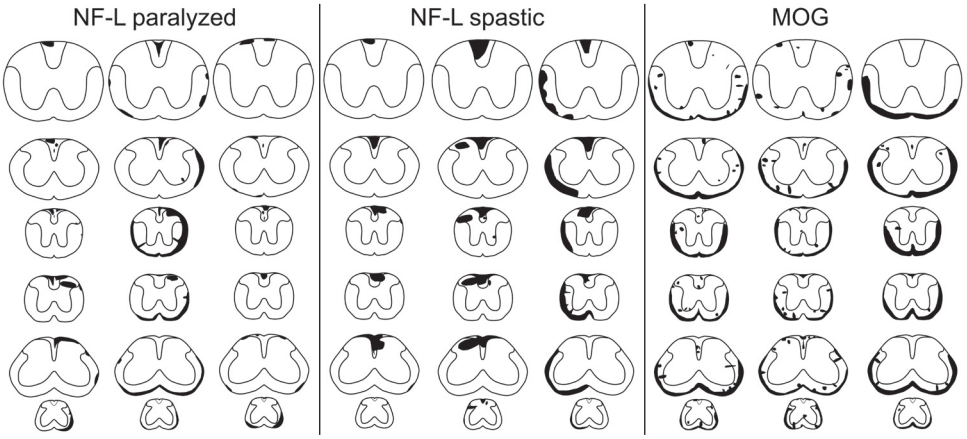


Figure 2. Lesion distribution in the spinal cord of mice immunized with NF-L or MOG. At six levels of the spinal cord, from cervical (top two panels), thoracic (middle two) to lumbar levels (bottom two), lesions were drawn into spinal cord outlines. Black filled areas represent lesions of inflammation, demyelination or axonal degeneration.

and one with spastic signs). Mice immunized with NF-L developed lesions in the dorsal funiculus at multiple levels throughout the spinal cord, whereas lesions in the dorsal column of MOG-immunized mice were observed but mainly as small perivascular cuffs.

Grey matter involvement was observed in both groups, however in 5 out of 6 NF-L-immunized mice the lesions were confined to the dorsal horn, whereas in MOG-immunized mice lesions were present in both the dorsal and ventral grey matter. In the MOG-immunized mice these lesions were often extensions of perivascular cuffs from the white matter. No clear and consistent difference in lesion location was found between spastic and paralyzed NF-L-immunized mice. As expected, animals immunized with CFA only did not develop lesions in the spinal cord.

To study whether the observed differences were statistically significant, the lesion areas in the different anatomical regions were measured at each level (Figure 1). The total lesion load in a single spinal cord section was set at 100% and the relative contribution of each anatomical area was determined (Figure 3). In NF-L-immunized mice, 69.0%, 54.0% and 32.5% of the lesion load could be attributed to lesions in the dorsal funiculus in the cervical, thoracic and lumbar spinal cord, respectively. In contrast, lesions in the dorsal column of MOG-immunized mice contributed for only 3.0%, 2.7% and 8.3% respectively (p = 0.024 at the cervical level; Figure 3A). The majority of the lesions in MOG-induced EAE were located in the lateral and ventral funiculi of the spinal cord (94.7%, 94.5% and 91.2% of lesion load; p = 0.024 at the cervical and thoracic

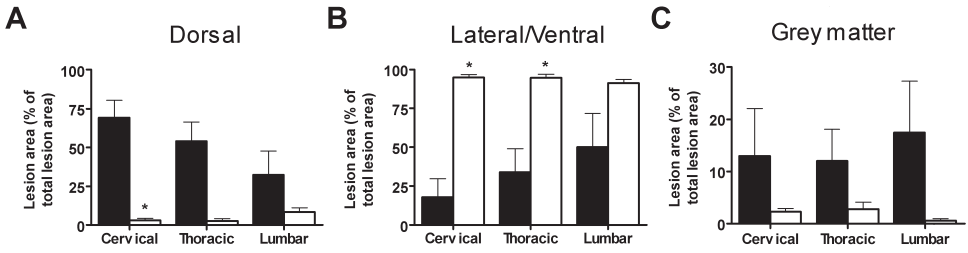


Figure 3. Differential lesion distribution in the spinal cord of mice immunized with NF-L or MOG. Lesion area was quantified in the cervical, thoracic and lumbar spinal cord in different anatomical regions, i.e. the dorsal column (**A**), lateral and ventral column (**B**) and the grey matter (**C**) (see also Figure 1). Shown are the relative contributions of the anatomical regions to the total lesion load. In NF-L-immunized mice (black bars), the dorsal column contributes significantly more to the total lesion load than in MOG-immunized mice (white bars; **A**). Almost all of the lesion area in MOG-immunized mice is located in the lateral and ventral part of the spinal cord (**B**). Grey matter involvement is higher, although not significantly, in mice immunized with NF-L compared with MOG (**C**). * $p < 0.05$, Mann-Whitney U test.

level; Figure 3B). In the mice immunized with NF-L the grey matter contributed more to the total lesion load than in MOG-immunized mice, nevertheless these differences did not reach significance (Figure 3C). No differences were detected between NF-L-immunized animals with spastic or paralytic signs (all *p*-values > 0.4).

We also determined the total lesion load at the three different levels of the spinal cord and found no differences between NF-L and MOG-immunized mice (Figure 4A). The lesion area in the dorsal column at the cervical level was however significantly larger in NF-L-immunized animals (31.5 ± 6.5%) compared to MOG-immunized animals (2.0 ± 1.0%; $p = 0.024$; Figure 4B). This difference was also apparent at the thoracic level (57.2 ± 10.4% vs. 6.0 ± 3.5% in MOG-immunized animals; $p = 0.024$) but not in the lumbar region ($p = 0.55$). Although in the ventral and lateral funiculi the lesion load was higher in MOG-immunized animals, this did not reach significance (Figure 4C). Similarly, a larger area of the grey matter was affected in NF-L-immunized mice, but was also not significantly different from MOG-immunized animals ($p = 0.55$; Figure 4D).

We next examined the brains of mice immunized with NF-L and observed inflammatory cell infiltrates in the meninges as well as in the dorsal column nuclei in the brain stem. No abnormalities were found in cortical white matter tracts, i.e. the corpus callosum and capsula interna. Also the motor cortex, hippocampus and the cerebellum were not affected (data not shown). MOG-immunized mice in contrast are known to have lesions in the white matter of the cerebellum as well as meningeal infiltrates, however brain stem nuclei are not affected (Amor *et al.*, 1996).

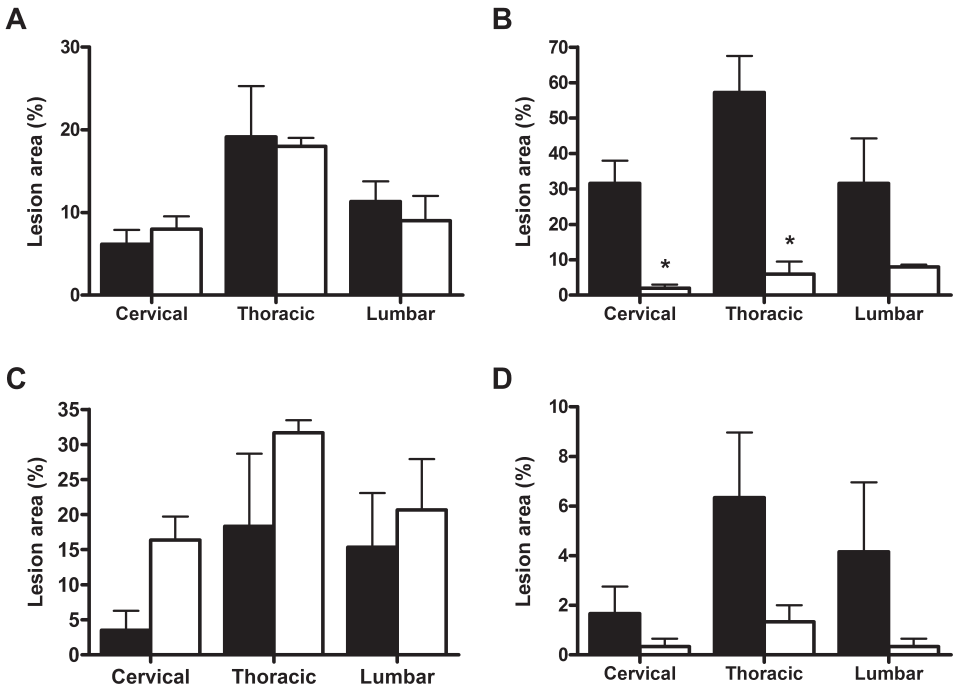


Figure 4. Differential lesion distribution in the spinal cord of Biozzi ABH mice immunized with NF-L or MOG. Lesion area was determined at the cervical, thoracic and lumbar level of the total spinal cord (**A**) as well as of its different anatomical regions, i.e. the dorsal column (**B**), the lateral and ventral column (**C**) and the grey matter (**D**). The total lesion area in the spinal cord did not differ between NF-L (black bars) and MOG-immunized animals (white bars; **A**). The lesion area in the dorsal column was significantly larger in NF-L-immunized mice compared with MOG-immunized mice (**B**), whereas the lesion area in the lateral and ventral column was larger, although not significantly, in MOG-immunized animals (**C**). NF-L-immunization resulted in a larger lesion area in the grey matter. * $p < 0.05$, Mann-Whitney U test.

In summary, Biozzi ABH mice immunized with the neuronal/axonal protein NF-L preferentially show lesions in the dorsal column of the spinal cord and the corresponding brain stem nuclei, whereas immunization with the myelin protein MOG results in lesions preferentially in the lateral and ventral columns of the spinal cord and the cerebellar white matter.

Immunization with NF-L preferentially targets axons

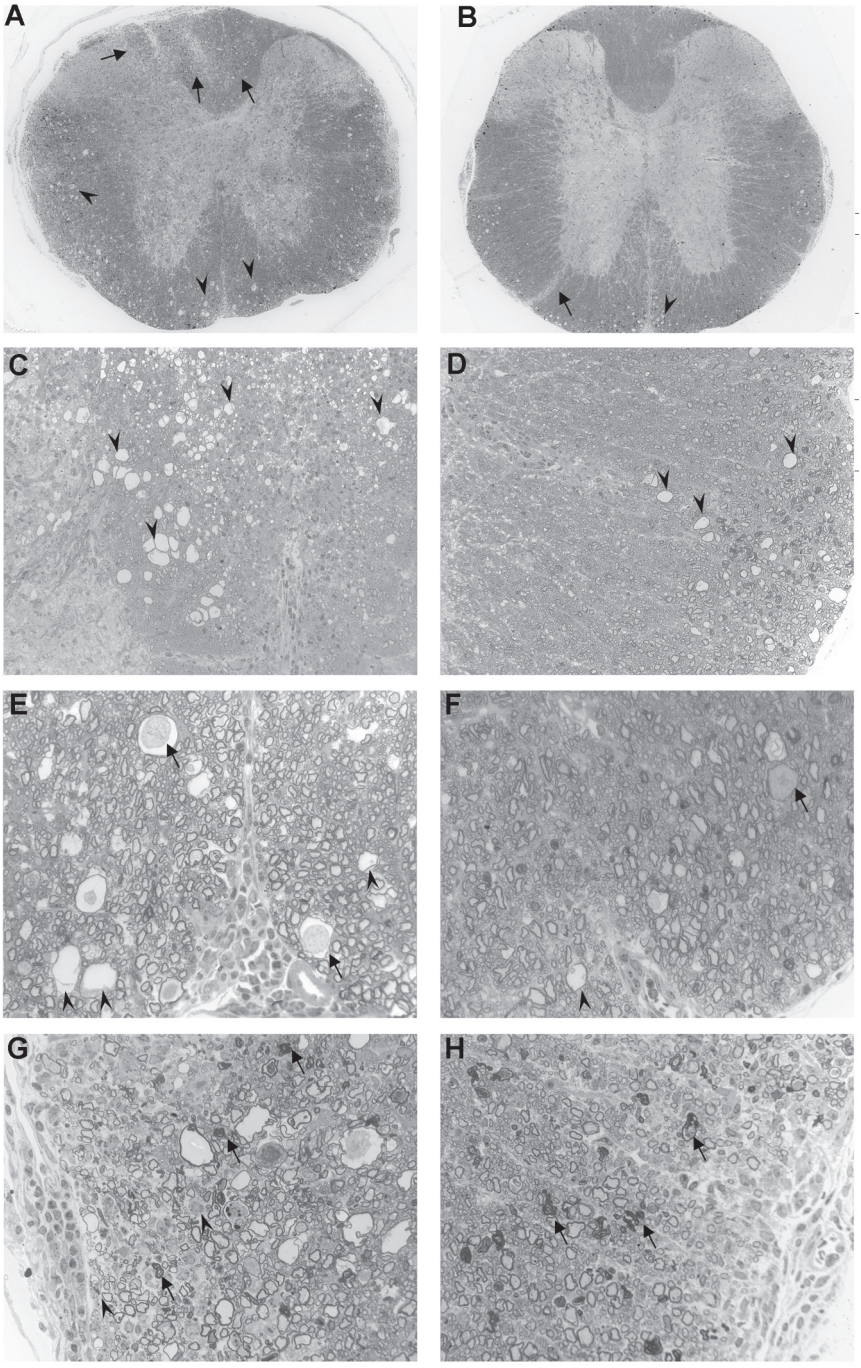
To examine whether autoimmunity to myelin or neuronal antigens induces different neuropathological patterns with respect to axonal pathology and myelin alterations, we compared the spinal cords of mice immunized with NF-L and MOG. As described

above lesions in NF-L-immunized mice were located in the dorsal as well as the ventral and lateral columns (Figure 5A) whereas in MOG-induced EAE lesions were restricted to the lateral and ventral columns (Figure 5B). Lesions in NF-L-induced disease contained numerous 'empty' myelin sheaths in which axons had degenerated or were in the process of degeneration (Figure 5C). Swollen axons which are in the process of degeneration were occasionally observed (Figure 5E). Axonal degeneration was also present in MOG-immunized animals, albeit at lower levels (Figure 5D and F). Abnormal myelin sheaths and accumulation of myelin aggregates were observed in both NF-L and MOG-induced disease (Figure 5G and H).

A more detailed assessment of the different pathological features described above was made by quantitation. As an assessment of the axonal pathology, the number of empty myelin sheaths per spinal cord section was determined. At all levels of the spinal cord we observed higher numbers of empty myelin sheaths following immunization with NF-L compared to MOG. The largest difference was observed in the lumbar area (149 ± 62 empty myelin sheaths for NF-L-immunized mice vs. 30 ± 30 for MOG-immunized animals; $p < 0.05$; Figure 6A). Consistent with the lesion location, axonal degeneration was most pronounced in the dorsal funiculus following NF-L-immunization, whereas in MOG-induced EAE axonal degeneration in this area was only rarely observed. Ongoing axonal degeneration, evidenced by swollen axons containing accumulated organelles was only observed infrequently and no significant differences between NF-L and MOG-immunized mice were detected (Figure 6B). Likewise, the extent of myelin damage as depicted by the number of disorganized or uncompacted myelin sheaths, was similar despite a trend for increased damage in the lumbar spinal cord in NF-L-immunized animals (Figure 6C).

In conclusion, compared to the myelin protein MOG, autoimmunity to the axonal protein NF-L induces more axonal degeneration at all levels of the spinal cord.

Figure 5. Neuropathological details of mice immunized with NF-L or MOG showing that NF-L-induced autoimmunity preferentially targets axons. Semi-thin (1 μm) sections of the spinal cord were stained with toluidine blue. Overview sections illustrate lesions in the dorsal column, extending into the grey matter (arrows show the lesion border) of mice immunized with NF-L (**A**; magnification x50), but not MOG (**B**; x50, arrow shows perivascular infiltrate). Axonal loss in NF-L-immunized mice is evidenced by numerous empty myelin sheaths (arrowheads in **A**, **C** [x200] and **E** [x400]) and swollen axons (arrows in **E**). Axonal degeneration is present to a lesser extent in MOG-immunized mice (arrowheads in **B**, **D** [x200] and **F** [x400], arrow in **F** shows a swollen axon). Myelin alterations are present both in NF-L-immunized mice (arrows in **G**; x400) and in MOG-immunized mice (arrows in **H**; x400). Macrophages containing myelin debris are present in the white matter parenchyma of NF-L-immunized mice (arrowheads in **G**).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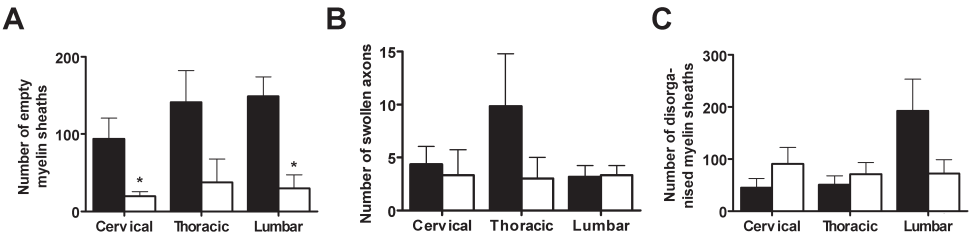


Figure 6. Quantitative analysis of neuropathological features in spinal cord lesions of NF-L or MOG-immunized mice. Axonal degeneration (empty myelin sheaths), swollen axons and disorganised myelin sheaths were counted in an entire spinal cord section at three levels in the neuraxis. NF-L-immunized mice (black bars) had significantly more axonal degeneration than MOG-immunized mice (white bars; **A**), while the number of swollen axons was similar (**B**). Abnormal myelin sheaths were comparable in both groups (**C**). * $p < 0.05$, Mann-Whitney U test.

Differential location of inflammatory cells in NF-L vs. MOG-immunized mice

To examine the relationship between infiltrating cells and CNS pathology we next characterized the inflammatory cells in the spinal cord of NF-L-immunized mice ($n = 9$) and MOG-immunized mice ($n = 6$). Fixed-frozen tissue, derived from the mice that were also used for the quantitation of lesion distribution and axonal degeneration was stained for F4/80 (macrophages and microglia), CD3 (T cells) and B220 (B cells). Additionally, since we were unable to use antibodies to CD4 or CD8 in (para)formaldehyde-fixed mouse tissue, snapfrozen tissue was used to determine the number of CD4 and CD8 T cells.

As a measure of the number of macrophages in the spinal cord of NF-L or MOG-immunized mice we determined the area staining positive for the F4/80 antigen using ImageJ software (Figure 7A-D). The area positive for F4/80 was significantly higher in the thoracic cord of NF-L-immunized mice compared with MOG-immunization (17.8 ± 3.0% vs. 11.1 ± 1.1%; $p = 0.026$; Figure 7E). In contrast, in the cervical and lumbar cord no differences could be observed in the degree of F4/80 staining.

The number of T and B cells was counted in the different areas of the spinal cord of animals immunized with MOG or NF-L. The number of T and B cells in the meninges (assessed only in the perfused animals) was significantly higher in MOG-immunized mice compared with NF-L immunized mice (Figure 8A-C and F). Conversely, in the total spinal cord tissue (excluding meninges) no difference in $CD3^+$ cells was observed between NF-L and MOG-immunized mice (Figure 8D). The number of B cells in the total spinal cord was increased in MOG-immunized mice (Figure 8E), like in the meninges. In the dorsal column of NF-L-immunized mice significantly more T and B cells were present as compared to MOG-immunized animals (Figure 8G and H) whereas $CD3^+$

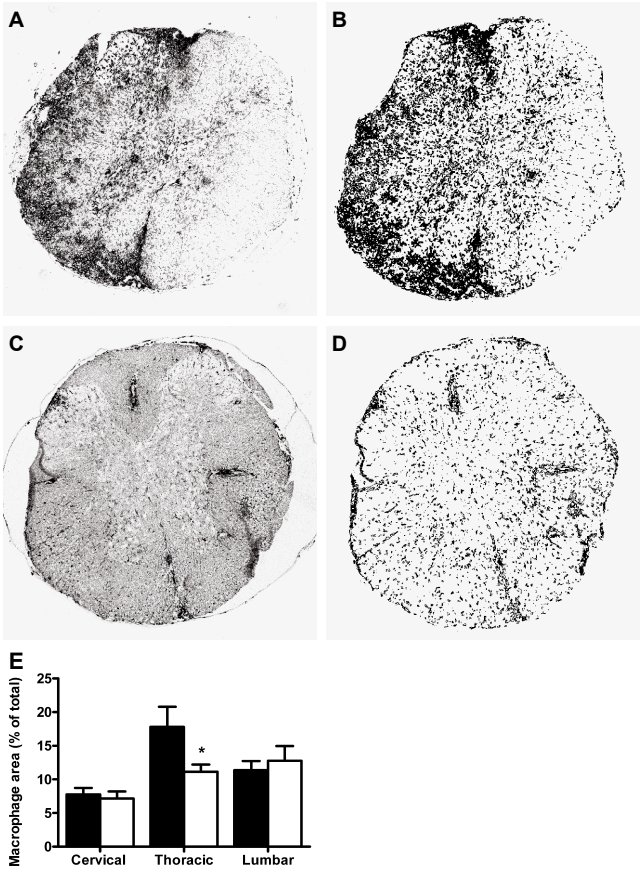
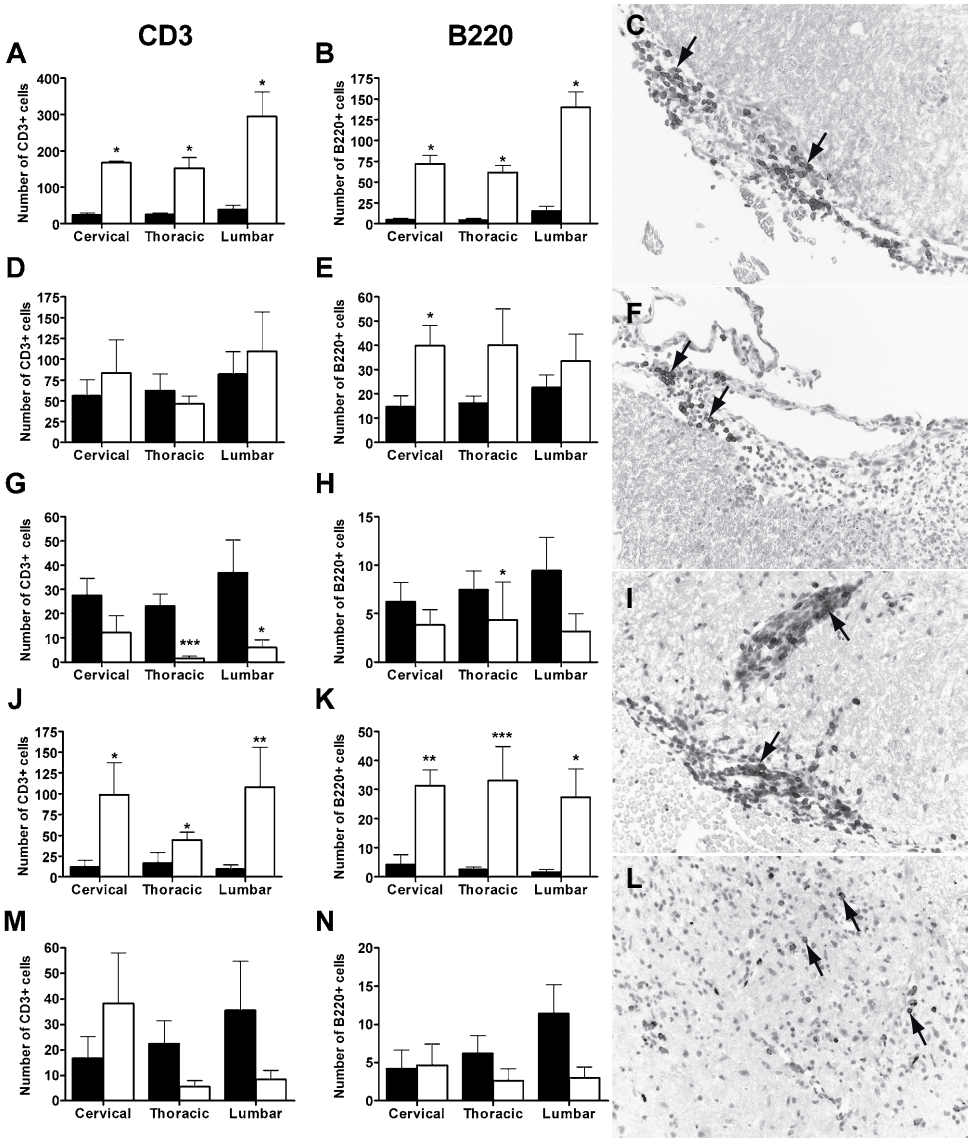


Figure 7. NF-L-immunized animals show increased macrophage/microglia staining in the thoracic spinal cord. The F4/80 antigen was stained in spinal cord sections of NF-L (**A**) and MOG-immunized (**C**) mice and images were analyzed using ImageJ software. F4/80 positive staining is converted to black (**B** and **D**) and the total pixel area was measured. Spinal nerve roots (top left corner in **A**) were excluded from analysis. NF-L-immunized mice (black bars) show increased F4/80 staining in the thoracic cord compared with MOG (white bars). F4/80 staining in the cervical and lumbar regions were comparable (**E**). * $p < 0.05$, Mann-Whitney U test. See page 212 for a full-color representation of this figure.

and B220$^+$ cells were increased in the lateral and ventral column of MOG-immunized mice (Figure 8J and K). Although the number of T and B cells in the grey matter was not significantly different between NF-L and MOG-immunized mice (Figure 8M and N), we did observe a striking difference in localization of the cells. The CD3$^+$ cells in mice immunized with MOG were mainly organized in perivascular cuffs whereas in NF-L-


CD3
B220
A
B
C
D
E
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I
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M
N
Number of CD3+ cells
Number of B220+ cells
Cervical
Thoracic
Lumbar

Figure 8. Differential distribution of T and B cells in the spinal cord of mice immunized with NF-L or MOG. Spinal cord sections of NF-L or MOG-immunized mice were stained for CD3 (T cells) or B220 (B cells) and positive cells were counted in the different anatomical regions. In the meninges, the number of $CD3^+$ cells was significantly increased in MOG-immunized mice (white bars) compared with NF-L-immunized mice (black bars; **A**). Also more $B220^+$ cells were found in MOG-immunized mice (**B** and **C**; 200x) compared with NF-L-immunized mice (**B** and **F**; 200x). In the total spinal cord parenchyma, the number of $CD3^+$ cells was similar (**D**), while the number of $B220^+$ cells was elevated in MOG-immunized mice (**E**). NF-L-immunized mice had more $CD3^+$ and $B220^+$ cells in the dorsal column (**G** and **H**) while in MOG-immunized mice cells were preferentially located in the lateral and ventral columns (**J** and **K**). NF-L-immunized mice show infiltration of $CD3^+$ cells in the grey matter parenchyma (**L**; x200) in contrast to MOG-immunized mice in which $CD3^+$ cells are mainly concentrated in perivascular cuffs (**I**; 200x, NR nerve root). * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U test.

immunized mice the $CD3^+$ cells were dispersed in the parenchyma of the dorsal horn grey matter (Figure 8I and L).

Finally we determined whether the infiltrated T cells in NF-L or MOG-immunized mice ($n = 3$ for both groups) were preferentially $CD4^+$ or $CD8^+$. At all levels of the spinal cord, the percentage of $CD4^+$ T cells was higher in MOG-immunized mice compared with NF-L-immunized mice. For example, at the thoracic level 62.9 ± 18.6% was $CD4^+$ in MOG-immunized animals compared with 41.0 ± 5.3% in NF-L-immunized mice. However, the observed differences did not reach significance ($p > 0.4$; data not shown).

Since inflammatory cells were also observed in the meninges of brains of NF-L-immunized mice, we analyzed the phenotype of these cells and compared that with MOG-immunized animals. In NF-L-immunized mice the infiltrated cells were mainly $F4/80^+$ myeloid cells. Also MOG-immunized mice showed $F4/80^+$ cells in the meninges however also a large number of B cells were present. The number of T cells was twice as high in MOG-immunized mice compared with NF-L-immunized mice (data not shown).

In summary, in the spinal cord of NF-L-immunized animals, but only at the thoracic level, $F4/80^+$ macrophages/microglia were increased compared with MOG-induced EAE. Meningeal infiltration by T and B lymphocytes was higher in MOG-immunized mice, whereas the overall number of T and B cells in the spinal cord parenchyma was similar. In NF-L-immunized mice, the leukocytes were preferentially located in the dorsal column in contrast to MOG-immunized mice in which the cells were located in the lateral and ventral column, thus confirming the differential lesion distribution between MOG and NF-L-immunized mice.

DISCUSSION

Autoimmune responses to neuronal antigens have been implicated in a variety of neurological diseases, including paraneoplastic syndromes (Vincent *et al.*, 1999). In MS,

autoantibodies against neuronal proteins e.g. to NF-L, NF-M and neurofascin (Bartos *et al.*, 2007, Mathey *et al.*, 2007, Silber *et al.*, 2002) and T-cell responses to neuron specific enolase and arrestin (Forooghian *et al.*, 2007) have been reported although the evidence that these responses are pathogenic have not been fully explored. In Biozzi ABH mice we have previously shown that autoimmunity to NF-L induces axonal degeneration and neurological disease (Huizinga *et al.*, 2007). To determine whether autoimmunity to neuronal antigens specifically damages axons or induces a particular pattern of lesion topology and pathology in the CNS, which may underlie specific neurological symptoms, we performed a detailed neuropathological study of Biozzi ABH mice immunized with either NF-L or MOG. Here we show that immunization with the axonal protein NF-L induces a distinct lesion distribution as well as preferential degeneration of axons and grey matter pathology indicating that autoimmunity to neuronal antigens could also contribute to the neurodegeneration in diseases such as MS.

Comparison with classical EAE demonstrates that the NF-L model has several advantages including the development of severe axonal damage and grey matter involvement within a few weeks after immunization. In EAE these features are only observed in chronic relapsing models after long disease duration (Pryce *et al.*, 2005), when the specific pathomechanisms of axonal damage are difficult to dissociate. Many pathomechanisms have been suggested to underlie axonal damage including the loss of trophic support by glial cells (Griffiths *et al.*, 1998, Yin *et al.*, 1998), $CD8^+$ T-cell damage, neurotoxic factors released by activated microglia (Bitsch *et al.*, 2000) and an energy imbalance (Dutta and Trapp, 2007). Here we provide further evidence that the pathological mechanisms underlying axonal damage can also include autoimmunity to neuronal antigens.

In the NF-L-model lesions were primarily observed in the dorsal column whereas in MOG-EAE the lesions were predominantly found in the lateral and ventral column and not in the dorsal column. The dorsal column in rodents consists of ascending sensory and descending corticospinal fibers (Tracey, 2004). Although only little is known about axonal loss in specific spinal cord tracts of MS patients, it is clear that the sensory and corticospinal tracts are affected. Total axon numbers in the cervical spinal cord were reduced with 24% in the sensory tract and 44% in the corticospinal tract (DeLuca *et al.*, 2004). The similarity in lesion distribution between our mouse model and in some MS patients suggests that anti-axonal immune responses could also be involved in axonal degeneration in MS. These tracts are highly dependent on axonal transport requiring an intact cytoskeleton (Perez-Olle *et al.*, 2005) which may be disrupted by antibodies to intermediate filaments such as NF-L. Alternatively the axonal loss could be a consequence of lesions in the cervical region of the spinal cord, resulting in Wallerian degeneration of axons distal to the lesion (Bjartmar *et al.*, 2001, Simon *et al.*, 2000).

That autoimmunity to MOG and NF-L results in a differential lesion topography extends the studies performed by Berger *et al.* (1997) whereby the lesion distribution in EAE is determined by the immunizing antigen. Myelin proteins, such as MOG and myelin associated glycoprotein, promote lesion formation in the periventricular and cerebellar white matter whereas astrocytic proteins such as s100β induce more lesions in the grey matter. While lesions in mice immunized with NF-L were not primarily localized in the grey matter, as is the case with astrocytic proteins, but rather in the white matter may reflect the distribution of the NF-L protein which is transported to the axon after synthesis in the cell body. The occurrence of grey matter lesions in NF-L induced disease could reflect the degeneration of axonal processes which project into the grey matter, as is the case in the dorsal horn. Although grey matter lesions were also observed in MOG-EAE these were limited to perivascular infiltrates mostly extending from the white matter.

Despite similar lesion loads in the spinal cords of NF-L and MOG-immunized mice the axonal damage in NF-L-induced disease was increased arguing for a preferential degeneration of axons due to anti-axonal autoimmunity. The higher extent of axonal degeneration in NF-L-immunized mice might be explained by a higher number of activated macrophages and microglia. These cells were suggested to play a crucial role in the induction axonal damage by secretion of toxic mediators like cytokines, reactive oxygen species, nitric oxide and glutamate (Perry and Anthony, 1999). However, we only observed a significant increase in F4/80 positive area at the thoracic spinal cord, whereas axonal degeneration is increased at all levels of the spinal cord. Therefore the increase in axonal degeneration can only be partly explained by a higher numbers of macrophages in NF-L-immunized mice. Examination of the numbers of T and B cells in the two models not only underscored the difference in lesion location between NF-L and MOG-immunized mice, but also revealed an increase of T and B cells in the meninges of animals immunized with MOG. The number of T cells in the spinal cord parenchyma excluding meninges was similar in NF-L and MOG-immunized mice. Meningeal infiltration in the spinal cord occurs early after EAE induction (day 6 after immunization of guinea pigs) before the appearance of subpial lesions, suggesting that leukocytes enter the spinal cord via the meninges (Waksman and Adams, 1962). The low number of T and B cells in the meninges of mice immunized with NF-L may suggest a different mechanism of lesion development. For example it may be possible that the primary lesion does not develop in the spinal cord itself but rather in the dorsal root ganglia or the spinal nerve roots. Subsequently, leukocytes may enter the dorsal column parenchyma via the nerve roots. In addition, axonal (Wallerian) degeneration of the dorsal column and microglia activation may result in an inflammatory milieu that alters the blood-brain barrier controlling infiltration and activation of leukocytes. Further studies are needed to identify the respective roles of neuron/axon-specific T cells or autoantibodies in inducing the primary lesion in the NF-L-model.

Although myelin damage was similarly observed in both NF-L and MOG-induced EAE we speculate that this is due to a different sequence of events. In NF-L-immunized animals, numerous empty myelin sheaths and degenerating axons with intact myelin sheaths were present suggesting that the axons degenerate and disappear prior to disintegration of the myelin sheath. In MOG-induced EAE the observation that disorganized myelin sheaths were more numerous than empty myelin sheaths i.e. degenerated axons, is suggestive of preferential damage to myelin followed by secondary axonal degeneration. The sequence of events in NF-L-induced disease corresponds to the 'inside-out' model of demyelination, as proposed by Tsunoda and Fujinami (2002). In this model, demyelination is secondary to axon damage – in this case as a result of Theiler's murine encephalomyelitis virus infection. In contrast, MOG-induced disease could match the 'outside-in' model, where myelin is the primary target and axonal damage is secondary to the initial damage. Whether the number of degenerating axons outweighs the number of disorganized myelin sheaths in the total CNS in MS is difficult to accurately assess but does give an indication of the sequence of events i.e. whether myelin damage is secondary to axonal damage or vice versa.

If autoimmunity to neuronal antigens was the only mechanism of axonal pathology in MS it would be expected that the degree of axonal pathology would exceed the degree of demyelination. That in most demyelinated MS lesions the axons are relatively spared argues against antineuronal autoimmune response as the single dominant factor inducing axonal damage in MS. However, axonal degeneration does occur independently of demyelination (Bitsch *et al.*, 2000). That axonal damage is extensive in (primary/secondary) progressive MS patients and that these patients have the highest levels of anti-NF-L antibodies (Silber *et al.*, 2002) indicates that antineuronal immune responses may be important in a subset of MS patients. Most likely, autoimmune responses to both myelin and axons occur simultaneously and may act synergistically as shown by the augmentation of demyelination in the grey matter disease by anti-MOG antibodies following immunization with the neuronal peptide β-synuclein (Escher *et al.*, 2006). Although the mechanism by which autoimmunity to neuronal antigens induces axonal damage is as yet unclear, a role for pathogenic antibodies to neuronal antigens in MS may explain the response of some MS patients to plasma exchange or intravenous immunoglobulin. The presence of grey matter lesions in the NF-L-model may be crucial to dissect mechanisms underlying grey matter pathology in MS.

In conclusion, this study shows that immunization with the axonal/neuronal protein NF-L results in a different lesion topology compared to immunization with the myelin protein MOG. Crucially, NF-L autoimmunity induces severe axonal degeneration and diffuse grey matter inflammation supporting the idea that primary immune attack on axonal antigens can cause selective axonal and grey matter pathology. These findings may have important implications for MS patients in whom autoimmune responses to

axonal and neuronal antigens are present as this may predispose them to developing earlier or more severe axonal damage and irreversible neurological deficits.

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8

GENERAL DISCUSSION

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INTRODUCTION

Elucidating the pathological mechanisms of axonal damage in MS is crucial for the development of therapies that aim to prevent or delay the occurrence of irreversible neurological deficits. The exact mechanism of axonal degeneration is however not completely understood, although it is likely that loss of trophic support from myelin sheaths and production of inflammatory mediators play an important role (Dutta and Trapp, 2007). We hypothesized that neurons and axons could also be target of autoimmune destruction as has been described for myelin antigens. In this thesis, several experiments were conducted to investigate this hypothesis. Below, the results are summarized and discussed in light of other studies that together answer the central question in this thesis: can autoimmunity to neuronal/axonal antigens be a mechanism for the induction of axonal damage in MS?

Is there evidence for antineuronal autoimmunity in multiple sclerosis?

To date, most studies focused on the pathogenicity of autoimmune T and B-cell responses directed against myelin-specific autoantigens such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). These antigens are targets for autoantibody-mediated demyelination in EAE (Morris-Downes *et al*., 2002) and are implicated in the pathogenesis of MS. By contrast, the functional significance of autoimmune responses to neuronal and axonal antigens has gone largely unexplored, despite evidence that such responses are present in MS (DeVries, 2004). Loss of immunological self-tolerance to neuronal and axonal antigens is, in fact, not a rare event and can result in overt autoaggression in several other neurological diseases. As detailed in **Chapter 2**, autoimmunity to neuronal antigens is implicated in many neurodegenerative disorders: but, what is the evidence in MS?

Reports of autoimmunity to neuronal antigens in patients with MS first appeared over two decades ago. Interest focused initially on antibodies to gangliosides, glycosphingolipids selectively enriched in neuronal membranes, that act as targets for pathogenic autoantibodies in some peripheral neuropathies such as acute motor axon neuropathy (Ho *et al*., 1999). Ganglioside-specific autoantibodies induce complement-dependent cell lysis and inhibit axonal regeneration (Lehmann *et al*., 2007). Moreover, immunization with gangliosides induces a neuropathy with axonal damage in experimental animals (Yuki *et al*., 2001). The presence of elevated titers of antibodies to the gangliosides GM4 and GD1a in MS patients suggests that they could play a similar role in the pathogenesis of this CNS disease (Endo *et al*., 1984, Sadatipour *et al*., 1998); however, as yet, this has not been demonstrated formally. Antibodies to axolemma-enriched (AXE) brain fractions are also present in the CSF and serum of MS patients (Rawes *et al*., 1997), and antibodies raised against axolemma antigens

damage neurites on mature neurons and prevent the outgrowth of neurons in spinal cord explants *in vitro*. Axon-reactive B cells are also clonally expanded within the CSF of patients with MS (Zhang *et al*., 2005a, Zhang *et al*., 2005b), the targets of which were recently identified as glycolytic enzymes triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase (Kolln *et al*., 2006). That inhibition of these enzymes induces neuronal death in culture raises the possibility that these antibodies could contribute to axonal damage and neuronal loss in MS.

Whereas most studies have concentrated on the role of antibodies in disease pathogenesis, it is pertinent to mention that these B-cell responses probably do not evolve in the absence of an antigen-specific T-cell response. T cells will play an indirect role in disease pathogenesis by providing T-cell help to the B-cell population, but they also might be pathogenic in their own right. Only few studies have described the presence of T cells specific for several neuronal antigens, including synapsin, neuron-specific enolase, retinal arrestin, and β-arrestin in MS patients (Forooghian *et al*., 2007, Polak *et al.*, 2001). In **Chapter 3** we show that $CD4^+$ and $CD8^+$ T cells of MS patients proliferated to NF-L. The fact that these responses were also observed in healthy controls may indicate that these T cells are part of the normal repertoire. The pathogenicity of these cells remains to be determined, but may also depend on the presence of additional factors, for example the recognition of the cognate antigen presented by activated APC and local reactivation in the CNS. This view is supported by recent studies showing that DC are present in perivascular cuffs of brain lesions and in inflamed meninges of MS patients (Serafini *et al*., 2006). In EAE, DC play a crucial role in the reactivation of infiltrating T cells in the brain, resulting in disease (Greter *et al*., 2005).

How can antineuronal autoimmunity develop in MS?

For the initiation of an antineuronal immune response, it is necessary that neuronal proteins are taken up by APC and are subsequently presented to autoreactive T cells, locally or in draining lymph nodes. Many previous studies have described that myelin sheaths may be phagocytosed as evidenced by the presence of myelin inside macrophages in lesions (Lampert, 1983). In addition, myelin antigens are present in APC in the cervical lymph nodes of patients with MS (De Vos *et al*., 2002, Fabriek *et al.*, 2005). Whether neuronal proteins are also phagocytosed in MS brain was unknown. We show in **Chapter 4** that damaged axons are engulfed by HLA class II^+ cells in the brain parenchyma of MS patients, suggestive of phagocytosis. Small lysosomal vesicles containing axonal proteins were however not detected in the HLA class II^+ cells, thus it is still not known whether the axonal antigens are loaded onto MHC molecules for presentation to T cells. *In vitro*, the degradation of full-length NF-L protein occurred within 2 h and breakdown products could only be detected by the antibody up to 4 h after incubation with lysosomal enzymes. Thus the degradation of degenerating axons

probably occurs in a short time span and it is possible that we have missed the final stage of axonal phagocytosis. An alternative explanation is that HLA class II$^+$ cells assist in the degeneration process of axons. For example, during development macrophages play a crucial role in axonal degeneration by engulfment-promoted cell death (Mallat et al., 2005). Macrophages may recognize degenerating axons via scavenger receptors such as the phosphatidylserine receptor, which triggers engulfment of the axon. This stimulates axons to degrade their own cytoplasmic organelles and proteins, a process called autophagy. The final remnants of the axon may subsequently be cleared by macrophages. Of note, defects in engulfment have been linked to the development of autoimmunity.

That degenerating axons in MS are not efficiently cleared, is indicated by the presence of soluble axonal proteins in the CSF, such as NF-L and NF-M (Semra *et al*., 2002). The level of NF-L in the CSF is used as a biomarker of neuronal damage in both MS and EAE (Norgren *et al*., 2005, Teunissen *et al*., 2005). It is assumed that these axonal antigens are released into the extracellular space (interstitial fluid; ISF) during axonal degeneration (Petzold, 2005). Around the ventricles neuronal proteins may directly enter the CSF since ependymal cells lining the ventricles lack tight junctions, therefore fluid can readily diffuse out of the parenchyma (Abbott, 2004). In other parts of the brain neuronal proteins in the ISF may drain along capillaries and arteries and perivascular macrophages may phagocytose these molecules (Carare *et al*., 2008). Both the CSF as the ISF draining along blood vessels may reach the cervical lymph node (CLN), although the contribution of each of these pathways and the exact route is not exactly known and may differ among species (Weller *et al*., 2008).

The pathogenic role of neuronal antigens in the CSF of MS patients remains as yet unknown, but it is possible that the presence of neuronal antigens leads to the retention and local activation of B cells secreting antineuronal antibodies as has been shown previously in rats (Knopf *et al*., 1998). In addition, release of neuronal antigens from damaged axons into the CSF/ISF and drainage to the CLN may initiate an immune response. In **Chapter 5** of this thesis we show that neuronal antigen-containing cells are indeed present in the CLN of patients with MS as well as animals with EAE. The cells containing the neuronal antigen MAP-2 expressed HLA class II molecules as well as the costimulatory molecule CD40, necessary for antigen presentation. In contrast to the myelin-containing APC, the cells containing neuronal antigens displayed a proinflammatory cytokine profile and did not express CCR7, a chemokine receptor that is involved in homing to the lymph node. Again this suggests that neuronal antigens are drained to the CLN as soluble proteins (or small insoluble particles) and subsequently ingested by APC. The presence of antibodies to neuronal antigens in the CSF could result in the formation of immune complexes which may be more effectively taken up via Fc-receptor (FcR)-mediated phagocytosis. The precise drainage of neuronal proteins

could be further investigated using transgenic mice expressing fluorescent neuronal proteins, such as NF-H-GFP (Letournel *et al*., 2006). This not only allows monitoring of axonal degeneration but also easy tracing of released neuronal proteins in for example the CSF, and uptake by APC in the CNS or CLN.

The uptake of neuronal antigens by APC may not automatically lead to a pathogenic immune response, since insufficiently activated APC favor T-cell tolerance. Nevertheless such tolerance could be overcome via activation of Toll-like receptors (TLR), either by endogenous ligands such as heat shock proteins or exogenous ligands for example peptidoglycan which is present in MS lesions (Visser *et al*., 2006). Recently also Epstein-Barr virus has been identified in B cells in MS brain (Serafini *et al*., 2007). The presence of viral antigens could not only lead to activation of APC but also the EBV could immortalize B cells leading to the production of antineuronal antibodies (Pender, 2003).

That drainage of neuronal proteins to lymph nodes results in an autoimmune response is supported by a number of studies. In the case of the CLN, the immune response seems to be directed towards a humoral response (Galea *et al*., 2007), since injection of antigen in the brain resulted in an antibody but not a cellular response (Harling-Berg *et al*., 1999). Similarly, experimental CNS injury induces anti-brain antibodies but not anti-myelin T cells (Ankeny *et al*., 2006, Rudehill *et al*., 2006). In humans, antibody formation against βIII-tubulin has been reported to develop within 3 weeks after brain trauma (Skoda *et al*., 2006).

In summary, it is now clear that neuronal antigens are not only present as free soluble protein in the CSF but also that damaged axons are engulfed by HLA class II^+ cells in lesions of MS patients. Axonal antigens are present in APC in the draining lymph nodes. APC containing axonal antigens could play an important role in the activation of autoimmune T cells and the subsequent production of autoreactive antibodies.

Is antineuronal autoimmunity pathogenic?

Despite the fact that animals with experimental brain injury developed antineuronal antibodies, no autoimmune disease was observed (Ankeny *et al*., 2006, Rudehill *et al*., 2006). Likewise, brain trauma does not increase the risk of developing MS (Goodin *et al*., 1999), arguing against a pathogenic role for antineuronal autoimmunity. In fact, autoimmunity to neuronal antigens could even be beneficial for example, antibodies directed against neuronal or axonal antigens could help to clear dying neurons (Stein *et al*., 2002). In the case of NF-L, autoantibodies could simply be needed to scavenge extracellular NF-L protein to prevent the formation of protein aggregates, since NF-L is able to form filaments on its own.

Nevertheless, in humans autoimmunity to neuronal antigens does lead to diseases such as paraneoplastic syndrome and Guillain-Barré syndrome. The pathogenicity is

difficult to reproduce in experimental animal studies. Attempts to develop an animal model for paraneoplastic syndrome by immunizing animals with Hu protein were for a long time unsuccessful (Sillevis Smitt *et al*., 1995). Also adoptive transfer of T cells specific for the onconeuronal antigen PNMA1 (paraneoplastic antigen ma1) or immunization with peptides from the cannabinoid receptor type 1 did not induce clinical disease although encephalitis was observed (Pellkofer *et al*., 2004, Proescholdt *et al*., 2002). However, recent studies showed that tolerance to neuronal antigens can be broken in animals by using strong adjuvants. Immunization with β-synuclein induced clinical disease in the rat (Mor *et al*., 2003), whereas amyloid-β 1-42 peptide and tau protein were pathogenic in mice (Furlan *et al*., 2003, Rosenmann *et al*., 2006). **Chapter 6** of this thesis now supplements these data by showing that also immunization with NF-L induced neurological disease and axonal degeneration in mice. This novel animal model presents distinctly from classical EAE induced with myelin antigens since different clinical signs were observed. Animals immunized with NF-L also showed spastic paresis, i.e. hyperextension of the hind limbs. Examination of the spinal cord revealed lesions with axonal degeneration predominantly located in the dorsal column, which in rodents also contains the corticospinal tract (Tracey, 2004). This tract is important for the fine control of movement by negatively innervating motor neurons that control voluntary movement, like locomotion. The spastic signs in the NF-L-immunized mice could be explained by degeneration of the inhibitory corticospinal tract axons which leads to increased activity of motor neurons resulting in spasticity. Whether degeneration of the corticospinal axons is indeed key to the development of spasticity in this model should be further investigated for example by injecting tracer dyes into the motor cortex or by using transgenic mice in which the corticospinal tract is fluorescently labeled (Bareyre *et al*., 2005). A complicating factor is the presence of lesions in the ventral spinal cord and sciatic nerve, which could involve motor neurons. If this would be the case, the effect of a lesion in the corticospinal tract could be masked due to motor neuron or axon damage resulting in flaccid paralysis.

In addition to a higher lesion load in the dorsal column, immunization with NF-L also induced a greater lesion load in the grey matter as well as increased axonal degeneration compared with MOG-immunization (**Chapter 7**). In contrast immunization with NF-L induced a similar degree of myelin damage as MOG-immunization. These findings indicate that NF-L autoimmunity primarily targets axons for destruction, subsequently resulting in disintegration of myelin sheaths. MOG autoimmunity on the other hand may cause primary myelin damage with axonal degeneration as a secondary event. These two different models of axon and myelin degeneration were initially proposed by (Tsunoda and Fujinami, 2002) after studying axonal degeneration and demyelination in mice infected with TMEV versus mice with EAE. Our studies show that the ‘inside-out model of demyelination’ can also be the result of autoimmunity to an axonal protein.

Where the lesions in the NF-L-immunized mice initially develop requires further study. The dorsal root ganglia (DRG) may be involved, since it contains the neuronal cell bodies of the peripheral sensory axons as well as the dorsal column axons, which are both severely affected in the NF-L-immunized mice. It is noteworthy that there is a less strict blood-nerve-barrier in the DRG (Abram *et al*., 2006, Hirakawa *et al*., 2004), suggesting that NF-L-reactive T cells and antibodies may have access to their target antigen more easily. In addition, DRG-neurons may be target for $CD8^+$ T cells through upregulation of MHC class I in response to IFN-γ (Fujimaki *et al*., 1996). An alternative explanation for the preferential degeneration of this sensory tract may be because these axons are very long and therefore highly dependent on an intact cytoskeleton and efficient axonal transport. Such a mechanism has also been proposed in the human disease hereditary spastic paraplegia in which mutations in the gene encoding spastin or kinesin cause a dying-back axonopathy of the sensory and corticospinal axons (Holzbaur, 2004). Interestingly, mice with a mutation in the kinesin KIF5A protein show a similar degeneration of sensory neurons due to impaired slow axonal transport of neurofilaments (Xia *et al*., 2003). In addition the clinical signs of these mice appear remarkably similar to the NF-L-immunized mice, suggesting that defective axonal transport could play a role in both mouse models.

In summary, recent studies as well as our own results support the concept that autoimmunity to neuronal antigens can directly cause axonal degeneration leading to neurological disease.

How do antineuronal T cells cause axonal damage?

In order to investigate whether NF-L-reactive T cells or antibodies mediate disease in NF-L immunized mice, several transfer experiments were performed. However neither the T cells nor a monoclonal antibody reactive to NF-L induced disease or aggravated MOG-induced disease, respectively. It must be noted that disease induction after T-cell transfer has never been reported in Biozzi ABH mice and attempts to transfer disease with myelin-reactive T cells have only been partially successful in Biozzi/SCID mice (S. Amor, unpublished observations). Therefore other mouse strains may be more suitable to address the question whether NF-L-reactive T cells are indeed encephalitogenic, for example C57BL/6 mice in which active immunization with NF-L also induced disease albeit at very low frequency (data not shown).

Autoimmunity may induce direct axonal degeneration by means of neuron-specific T cells ($CD4^+$ or $CD8^+$) or antibodies. A role for cytotoxic T cells in axonal damage was suggested after the observation that the number of $CD8^+$ T cells correlated with the number of APP-positive damaged axons in MS lesions (Bitsch *et al*., 2000). To become pathogenic, $CD8^+$ T cells require interaction with peptide-loaded MHC class I, which is expressed on neurons at a low level under normal conditions but can be upregulated

during inflammation (Hoftberger *et al*., 2004, Redwine *et al*., 2001). It appears that in viral models such as TMEV-infection, MHC class I and $CD8^+$ T cells are indeed crucial in mediating axonal degeneration (Howe *et al*., 2007, Rivera-Quinones *et al*., 1998). In contrast, for the induction of axonal degeneration in EAE neither MHC class I expression on neurons nor $CD8^+$ T cells were required (Linker *et al*., 2005). These studies indicate that neurons are able to process and present peptides on MHC class I molecules, however, the induction of axonal damage by $CD8^+$ T cells can only take place when the viral or neuronal peptide that is being presented is recognized. Therefore $CD8^+$ T cells do not play a role in inducing axonal damage in myelin-induced EAE, but may in NF-L-induced disease or indeed in viral models in which neurons are infected. Although $CD8^+$ T cells against neuronal antigens have been reported in humans (**Chapter 3**; Albert *et al*., 1998), there is no direct proof that killing of neurons occurs by these neuron-specific T cells.

Direct neurotoxic effects of $CD4^+$ T cells have been described in primary neuronal cultures and organotypic brain slice cultures, however these effects were independent of antigen specificity and MHC class II (Giuliani *et al*., 2003, Nitsch *et al*., 2004). Alternatively, $CD4^+$ T cells could activate macrophages and microglia to secrete neurotoxic mediators (Gimsa *et al*., 2000), which is consistent with the close association of macrophages with axonal degeneration in both MS and EAE (Bitsch *et al*., 2000, Ferguson *et al*., 1997, Papadopoulos *et al*., 2006). Finally, $CD4^+$ T cells could indirectly cause axonal damage by stimulating B cells to produce antineuronal antibodies.

In summary, both $CD4^+$ and $CD8^+$ T cells are capable of inducing axonal degeneration, either directly via MHC class I-restricted killing or indirectly via activating macrophages. Whether these cells play a role in NF-L-induced disease still requires further study.

How do antineuronal antibodies cause axonal damage?

There are several ways in which autoantibodies directed to neuronal proteins could mediate tissue damage (Figure 1). Clearly, antibodies binding to surface antigens might activate complement or provide targets for macrophage or microglial FcR, and although neurons express complement regulators as a means of resisting attack, this is not absolute (Storstein *et al*., 2004, Van Beek *et al*., 2005). Alternatively, antibodies binding to cell-surface receptors might act as agonists or antagonists, as in Rasmussen's encephalitis, in which antibodies target glutamate receptor-3 (GluR3) (He *et al*., 1998, Levite *et al*., 1999), or in neuropsychiatric lupus, in which antibodies binding to the NMDA (N-methyl-D-aspartic acid) receptor (Kowal *et al*., 2006) lead to excitotoxic neuronal death.

Yet, we are still left with the crucial question of whether we can demonstrate that axon-specific autoantibodies actually mediate axonal injury in patients with MS. A recent study argues strongly that this is indeed the case. One potential target for

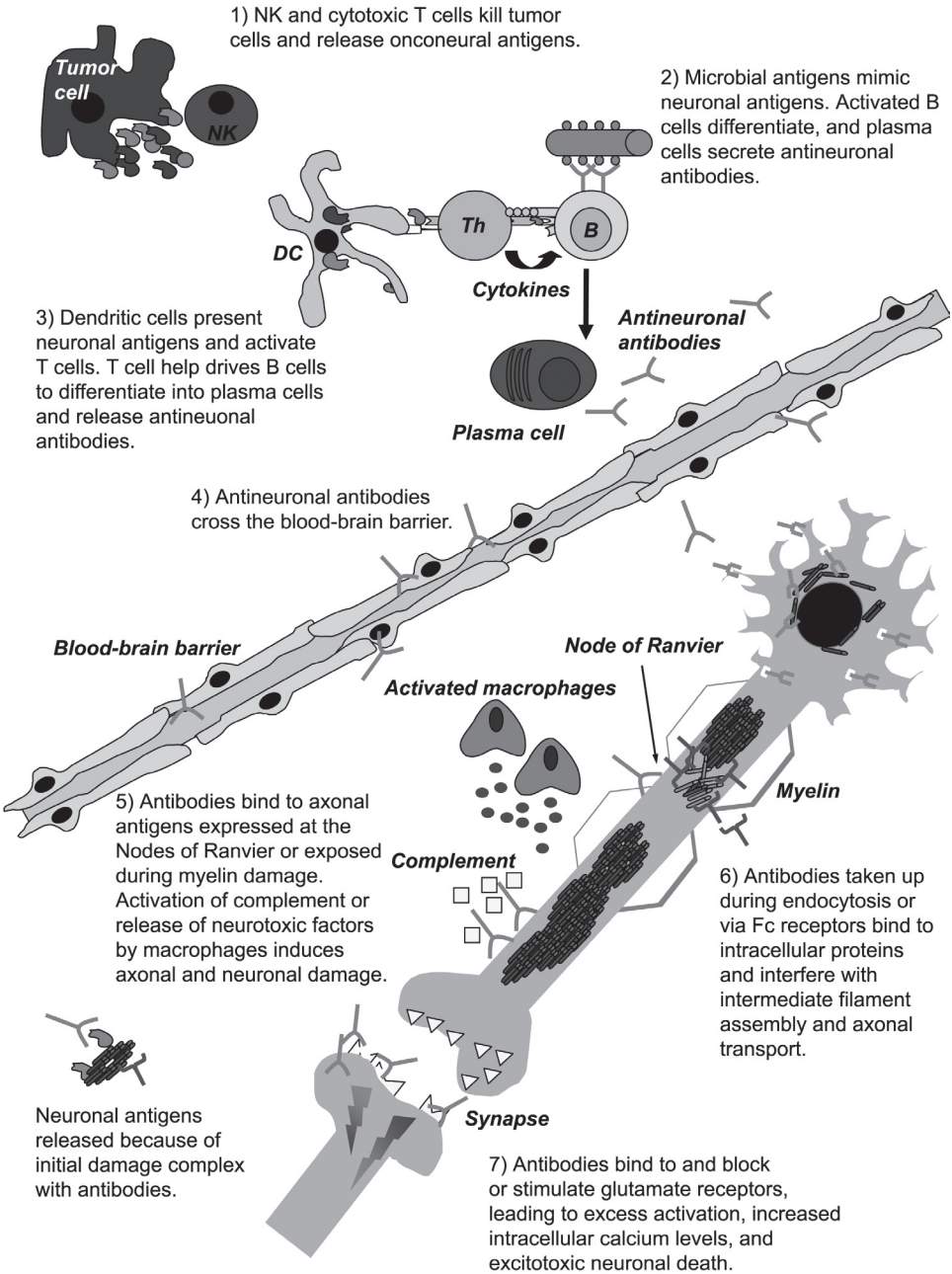
1) NK and cytotoxic T cells kill tumor cells and release onconeural antigens.
Tumor cell
NK
2) Microbial antigens mimic neuronal antigens. Activated B cells differentiate, and plasma cells secrete antineuronal antibodies.
DC
Th
B
Cytokines
3) Dendritic cells present neuronal antigens and activate T cells. T cell help drives B cells to differentiate into plasma cells and release antineuonal antibodies.
Antineuronal antibodies
Plasma cell
4) Antineuronal antibodies cross the blood-brain barrier.
Blood-brain barrier
Node of Ranvier
Activated macrophages
Myelin
5) Antibodies bind to axonal antigens expressed at the Nodes of Ranvier or exposed during myelin damage. Activation of complement or release of neurotoxic factors by macrophages induces axonal and neuronal damage.
Complement
6) Antibodies taken up during endocytosis or via Fc receptors bind to intracellular proteins and interfere with intermediate filament assembly and axonal transport.
Neuronal antigens released because of initial damage complex with antibodies.
Synapse
7) Antibodies bind to and block or stimulate glutamate receptors, leading to excess activation, increased intracellular calcium levels, and excitotoxic neuronal death.

Figure 1. Proposed pathogenic mechanisms of antibody-mediated neurodegeneration. (1) Tumor cells expressing neuronal antigens in paraneoplastic disorders are killed by NK cells and cytotoxic T cells, leading to the release of onconeuronal antigens. (2) Alternatively, antigens expressed by both microbes and neurons, e.g. gangliosides in Guillain-Barré syndrome, are recognized by the immune system as foreign (molecular mimicry). (3) In both cases, dendritic cells present neuronal antigens, which stimulates T helper cells to provide help for B cells. (4) Plasma cells produce antibodies to neuronal antigens that cross the blood brain barrier and bind to axons at nodes of Ranvier or to axonal antigens exposed after myelin damage. (5) Bound antibodies activate complement or stimulate macrophages to release cytokines and reactive oxygen species, which trigger neuronal death. (6) Antibodies to intracellular antigens are taken up by endocytosis or via FcR and bind to cytoskeletal proteins, e.g. neurofilaments. Antigen-antibody complexes cause aggregation of intermediate filaments or interfere with neurofilament assembly, therefore altering axonal transport and the function of neurons. (7) Antibodies bind to glutamate receptors, leading to excess stimulation, increased intracellular calcium, and excitotoxicity.

antibody-mediated axonal injury is neurofascin-186 (NF-186), a cell-adhesion molecule present at nodes of Ranvier, i.e. gaps in the myelin sheath where the underlying axonal membrane is exposed to the extracellular fluid, and the axonal initial segment of myelinated fibers. Neurofascin-specific autoantibodies are elevated in SP-MS and bind to the extracellular domain of NF-186 when the antigen is expressed at the surface of transfected cell lines. Adoptive transfer studies demonstrate that antibodies with this specificity exacerbate disease and axonal injury in animal models of MS by binding to NF-186 exposed at the node of Ranvier (Mathey *et al*., 2007). In this case, the target antigen is located on the axonal surface, where it is accessible to autoantibodies and acute axonal injury is complement dependent, but what about autoantibodies that recognize intracellular antigens such as neurofilaments? Traditionally, the pathogenic significance of antibodies to intracellular antigens is generally discounted, because they are considered to be unable to access their target antigens. Yet, it was recently reported that antibodies to amphiphysin, an intracellular synaptic protein, isolated from patients with stiff-person syndrome induced stiffness and spasms when transferred into rats with mild EAE (Sommer *et al*., 2005). Thus it appears that, in addition to such potential extracellular effects, antibodies to intracellular antigens do gain access to the interior of cells. Antibodies could be internalized by endocytosis, via clathrin-associated vesicles, after binding to FcR (Mohamed *et al*., 2002), or through as yet unknown pathways, and are present within neuronal cell bodies and axons in both classical EAE (Slavin *et al*., 1996) as well as disease induced after immunization with NF-L (**Chapter 6**). Similar findings have been reported in amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease (Engelhardt *et al*., 2005, Orr *et al*., 2005, Tampellini *et al*., 2007). That this internalization is pathogenic is illustrated by microinjection of IgG from Parkinson's patients, which was shown to induce neuronal loss in mice concomitant with the presence of immunoglobulin in substantia nigra neurons (He *et al*., 2002).

Studies are in progress to determine if, like antibodies to NF-186, antibodies to NF-L found in MS patients are also pathogenic. That autoimmunity to intracellular antigens

might play a role in neurological disorders is supported by the finding that antibodies to kinesin and dynein, intracellular proteins that participate in axonal transport, impair neuronal functions (Theiss *et al*., 2005).

Can neurons avoid immune-mediated damage?

The CNS has developed protection to avoid damage during inflammation, a phenomenon referred to as "immune privilege" (Galea *et al*., 2007). Neurons exploit a variety of mechanisms to help maintain this privileged state, including the immuneregulatory capacity of neuropeptides and transmitters, as well as by expressing molecules such as CD22, CD47, CD200, CX3CL1 (fractalkine) and soluble ICAM-5, all of which are involved in regulation of inflammation (Cardona *et al*., 2006, Mott *et al*., 2004, Tian *et al*., 2008). To minimize cytotoxic attack of T cells, neurons express low levels of MHC molecules (Rall *et al*., 1995), while actively promoting T-cell apoptosis via the Fas-Fas ligand pathway (CD95-CD95L) (Bechmann *et al*., 1999). It is intriguing to speculate that this immune regulation by neurons might explain the relative lack of inflammation in gray matter lesions in MS.

In contrast, the protection against antibody-mediated neuronal damage is less strict for a number of reasons. First, in MS, it is not necessary for either B cells or antibody-producing plasma cells to be in the CNS in order for antibodies to mediate tissue damage, as the BBB is damaged, allowing serum protein access to the CNS parenchyma, although whether B-cell activation and survival are influenced by neurons in the same way as T cells deserves investigation. Second, once in the CNS, antibodies do not necessarily need any other immunological cofactor or mechanism to modulate neuronal function and differentiation or to induce apoptosis once they have bound to their extracellular or intracellular target. Finally, because of their poor capacity to control complement activation neurons are particularly vulnerable to antibody-mediated complement attack.

In summary, neurons have a plethora of mechanisms that help protect them against the effects of both direct and indirect T-cell mediated damage, but this does not appear to be the case with respect to antibody-mediated pathomechanisms.

CONCLUSIONS AND FUTURE PERSPECTIVES

The research described in this thesis has lead to several conclusions which are listed in Box 1. Most importantly, our studies show that autoimmunity to neuronal antigens, like NF-L, can induce primary axonal degeneration. Therefore antineuronal autoimmunity should not be regarded solely as a marker of neurodegeneration, but rather as a potential mechanism for inducing axonal damage in MS. This mechanism

Box 1. Conclusions of the studies performed in this thesis.

- Autoimmunity to neuronal antigens leads to neuronal damage in many neurological disorders (**Chapter 2**).
- Besides antibody responses, also T-cell responses to NF-L occur in patients with MS as well as in healthy controls (**Chapter 3**).
- Damaged axons are engulfed by APC in brain parenchyma of MS patients (**Chapter 4**).
- Neuronal proteins are present in APC with a proinflammatory cytokine profile in MS patients and animals with EAE (**Chapter 5**).
- Autoimmunity to NF-L leads to axonal degeneration and neurological disease in mice (**Chapter 6**).
- Compared with autoimmunity to MOG, autoimmunity to NF-L results in a distinct lesion location in the spinal cord and increased axonal degeneration (**Chapter 7**).

is most probably applicable to a subset of MS patients and acts in concert with other mechanisms of axonal degeneration. Future research should not only focus on identifying other neuronal targets, but also on unravelling the pathogenic mechanism of antineuronal autoimmunity (other outstanding questions and suggestions for future research are listed in Box 2). Especially, the individual role of antineuronal T cells and antibodies in inducing axonal degeneration must be clarified. This could be achieved by transfer studies in C57BL/6 mice or by depletion of T-cell subsets, B cells, or complement factors. In addition, human organotypic brain slice cultures may be essential to determine the pathogenic effect on human neurons and axons.

When antineuronal autoimmunity is proven to be pathogenic for human neurons and axons, this may have important clinical implications for patients. The presence of antineuronal autoimmunity could be used as a prognostic factor, as axonal degeneration is key to the development of irreversible neuronal deficits. In the case of antibodies against neuronal antigens, a multiplex approach may be useful to identify patients with a high level of antineuronal antibodies, who may benefit from treatment with intravenous immunoglobulin or plasmapheresis.

The novel animal model described in this thesis will be key to study the pathogenic mechanisms involved in axonal damage and neurodegeneration particularly those involving pathogenic autoimmune responses. Moreover, the model will be useful to develop new therapeutic strategies and test novel compounds that treat spasticity, a common and debilitating symptom in MS patients. The extensive axonal degeneration

Box 2. Outstanding questions and suggestions for future research.

- Does the T cell and antibody response to NF-L change over time in MS patients and are responses correlated with clinical exacerbations?
- Experimentally inducing an autoimmune response to NF-L in mice is pathogenic, but are the NF-L-reactive T cells or antibodies isolated from MS patients also pathogenic? And those isolated from controls?
- If autoimmunity to NF-L is pathogenic in MS patients, it is likely that lesions also develop in the peripheral nervous system, in which NF-L is also expressed. Therefore, are lesions in MS patients really confined to the CNS?
- Why do not all mice immunized with NF-L develop disease despite the presence of NF-L-specific T cells and antibodies? Is it just a matter of threshold levels (e.g. for antibodies) or are other factors also needed for development of disease? Transgenic mice with a NF-L-specific BCR on B cells or with a NF-L-specific TCR on $CD4^+$ or $CD8^+$ T cells may be useful to study whether spontaneous disease occurs and whether exogenous triggers (e.g. an infection) can accelerate development of disease. Also it would be interesting to study whether a low degree of axonal/neuronal degeneration can trigger proliferation of NF-L-specific leukocytes resulting in disease. Similarly, is drainage of soluble NF-L to the CLN sufficient to induce such proliferation?
- What is the clinical result of inducing a combined autoimmune response to well-characterized myelin and neuronal antigens, like MOG and NF-L? Is there even more axonal degeneration as compared with autoimmunity to a neuronal protein only?
- During EAE, neurofilaments are carbonylated as a consequence of oxidative stress (Smerjac and Bizzozero, 2008). Do MS patients have autoimmune responses to this modified NF? Is it more immunogenic?

observed in this mouse model of inflammatory neurodegeneration is ideal for testing the impact of anti-inflammatory approaches as well neuroprotective drugs that aim to slow down or prevent disease progression due to accumulation of axonal degeneration.

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SUMMARY

Multiple sclerosis (MS) is a neuroinflammatory disease, characterized by the appearance of sclerotic lesions in the central nervous system (CNS). The lesions are sites of extensive inflammation, demyelination and axonal damage. Accumulation of axonal damage is responsible for the development of irreversible neurological deficits. Identifying the mechanisms of axonal damage is therefore key to the development of novel therapies that prevent the progression of neurological disease. We hypothesized that autoimmunity to neurons and axons could be a mechanism of axonal damage in MS. Such a mechanism has been described in other neurological diseases like Guillain-Barré syndrome and paraneoplastic disorders.

First, the evidence for a pathogenic role of antineuronal immune responses in neurological disorders as well as the evidence for the presence of antineuronal autoimmunity in MS was reviewed (**Chapter 2**). Although previous studies have described the presence of antibodies against the neuronal proteins neurofilament light (NF-L), neurofilament medium, tubulin and neurofascin, relatively little was known about the presence of T-cell responses directed against these neuronal proteins. In **Chapter 3** we explored the antineuronal T-cell response to NF-L, a cytoskeletal protein important for maintaining axonal caliber. We show that MS patients as well as healthy controls have peripheral T-cell responses to neurofilament light (NF-L) and that both $CD4^+$ and $CD8^+$ T cells proliferated in response to NF-L. The finding that anti-NF-L T-cell responses were not different from those in healthy controls suggests that T-cell responses to NF-L are part of the normal immune repertoire.

As immune responses are initiated and modulated by antigen-presenting cells (APC), we next investigated whether neuronal antigens could be detected in APC in the brain lesions of patients with MS (**Chapter 4**). In 80% of MS brain sections, HLA class II^+ cells were observed to completely surround damaged axons, compared with only 20% of control brain sections. Active inflammatory areas were especially sites of axonal engulfment. In **Chapter 5** it was subsequently shown that both in patients with MS and in animals with experimental autoimmune encephalomyelitis (EAE) - the experimental model of MS - neuronal antigens were present in CLN. Neuronal antigens were observed in HLA class II^+ and $CD40^+$ cells that had a pro-inflammatory cytokine profile as indicated by the expression of IL-12 and TNF-α. In contrast, myelin-containing APC expressed the anti-inflammatory cytokines IL-1ra and TGF-β. Expression of the lymph node homing receptor CCR7 on myelin-containing cells but not neuronal antigen-containing cells suggests a differential drainage routes of neuronal and myelin antigens from the brain to the CLN.

To examine the functional impact of autoimmunity to NF-L we studied the pathogenicity of autoimmunity to the axonal antigen NF-L in animals (**Chapter 6**).

Autoimmune responses to NF-L were induced in Biozzi ABH mice by immunization with NF-L protein in complete Freund's adjuvant. NF-L-immunized mice developed late-onset neurological disease characterized by paralysis and spastic paresis. In the spinal cord of affected animals activated macrophages and microglia were observed and numerous myelin sheaths with degenerating axons were present. How the disease is initiated is as yet unknown, however the dominant $CD4^+$ T-cell response concomitant with IFN-γ production and the presence of NF-L-specific IgG2b antibodies in the periphery suggests that disease is Th1-mediated.

To examine the relationship between demyelination and axonal damage we characterized the neuropathology of mice immunized with the axonal protein NF-L and compared this to the pathology observed in mice immunized with the myelin protein MOG (**Chapter 7**). MOG-immunization has previously been shown to induce extensive demyelination in Biozzi mice and here we show that the lesions are primarily localized in the lateral and ventral column of the spinal cord. In contrast, NF-L-immunized animals showed lesions that were preferentially located in the dorsal column of the spinal cord as well as in the grey matter. The differential lesion location was confirmed by immunohistochemical studies quantifying the number of T and B cells in the various anatomical areas of the spinal cord. Axonal damage was significantly higher in NF-L-immunized animals, while the extent of myelin damage was similar to MOG-immunized animals, suggesting that in NF-L-induced disease axonal degeneration is a primary event followed by secondary demyelination. Axonal loss can therefore be a direct consequence of a primary autoimmune attack against axonal antigens such as NF-L.

In conclusion, the studies described in this thesis show that 1) In addition to humoral autoimmunity, T-cell responses to NF-L are observed in MS patients as well as healthy controls; 2) Damaged axons are engulfed by APC in MS lesions and neuronal antigens are present in APC in cervical lymph nodes, which may be crucial in initiating, modulating or perpetuating the antineuronal immune response; and 3) Autoimmunity to NF-L can result in neurological disease and primary axonal degeneration. Therefore, in humans, autoimmunity to neuronal antigens such as NF-L may not solely be a surrogate marker of axonal pathology but could also play a crucial role in inducing axonal degeneration resulting in irreversible neurological symptoms and progression of disease.

SAMENVATTING

Multiple sclerose (MS) is een neurologische aandoening, waarbij ontstekingshaarden (laesies) in de hersenen en het ruggenmerg leiden tot uitvalsverschijnselen en problemen bij het zien en voelen. In de leasies bevinden zich ontstekingscellen van het afweersysteem die myeline aantasten, een vettige laag rondom zenuwuitlopers die zorgt voor snelle zenuwgeleiding. Door het verdwijnen van deze myelineschede worden zenuwimpulsen niet meer doorgegeven waardoor de uitvalsverschijnselen ontstaan. Niet alleen het myeline wordt aangetast maar ook de zenuwuitloper (axon) zelf kan beschadigd raken en juist deze axonale schade is essentieel in het ziekteproces van patiënten met MS. Aangezien het herstel van zenuwen in het centrale zenuwstelsel uiterst gering is, zal de schade namelijk leiden tot onherstelbare neurologische klachten, die kenmerkend zijn voor de progressieve fase van de ziekte. Hoe de schade veroorzaakt wordt, is nog niet precies bekend. In dit proefschrift wordt onderzocht of axonale schade veroorzaakt kan worden door een reactie van ontstekingscellen tegen componenten van axonen of zenuwcellen (neuronen). Deze antineuronale reacties komen ook voor bij andere neurologische aandoeningen zoals het Guillain-Barré syndroom.

Als eerste is in de literatuur gezocht naar bewijs voor schadelijke antineuronale reacties in verschillende aandoeningen van het perifere en centrale zenuwstelsel en naar bewijs voor het bestaan van antineuronale reacties in MS (**Hoofdstuk 2**). Verschillende studies hebben beschreven dat MS-patiënten antistoffen in het bloed hebben tegen axonale componenten zoals de lichte keten van neurofilament (NF-L), een eiwit dat belangrijk is voor de vorm en stevigheid van de zenuwuitlopers. Weinig was echter bekend over de cellulaire reacties tegen axonale componenten, daarom is in **Hoofdstuk 3** de cellulaire reactie tegen NF-L in kaart gebracht. De witte bloedcellen van MS-patiënten reageerden inderdaad tegen NF-L middels celdeling. De celdeling vond plaats door T cellen van het CD4 type en van het CD8 type. Aangezien de reacties van patiënten niet verschilden van die van gezonde controles, kan aangenomen worden dat de cellulaire reacties tegen NF-L behoren tot het normale repertoire van immuunreacties.

Antigen-presenterende cellen (APC) spelen een belangrijke rol bij het tot stand komen en het moduleren van een immuunreactie. Deze cellen zijn in staat om bacteriën en celresten op te nemen en deze in stukjes te ‘presenteren’ aan de T cellen die vervolgens de verdere afweerreactie in gang zetten. In **Hoofdstuk 4** is onderzocht of ook beschadigde axonen in de hersenen van MS-patiënten worden opgenomen door APC. In 80% van de onderzochte MS-hersenweefsels konden beschadigde axonen worden aangetoond die helemaal omgeven waren door een APC. Bij controle proefpersonen kon dit slechts in 20% van de hersenweefsels worden aangetoond. Dit suggereert dat in hersenweefsel van MS-patiënten de beschadigde axonen kunnen worden opgenomen door APC. De APC kunnen migreren naar de lymfeklier om daar een afweerreactie op

gang te brengen. Daarnaast kunnen APC in de hersenen een belangrijke rol spelen bij het (re-)activeren van binnengetreden T cellen.

In **Hoofdstuk 5** is onderzocht of axonale componenten ook aanwezig waren in de lymfeklieren in de hals van patiënten met MS en van dieren met experimentele autoimmuun encefalomyelitis (EAE) – een diermodel voor MS. De axonale componenten bleken aanwezig in cellen die ook humane leukocyten antigenen en CD40 moleculen maken. Dit suggereert dat deze cellen als APC kunnen functioneren. De cellen met axonale componenten maakten veel ontstekingsbevorderende stoffen aan, terwijl de cellen met myeline bestanddelen juist ontstekingsremmende factoren aanmaakten.

In **Hoofdstuk 6** is onderzocht of een immuunreactie tegen de axonale component NF-L ook kan leiden tot schade aan axonen en neurologische ziekte. Door muizen in te spuiten met een mengsel van NF-L en olie met daarin bacteriën, werd een immuunreactie opgewekt tegen NF-L. De muizen ontwikkelden verlammingen en spasticiteit, verschijnselen die ook vaak voorkomen bij MS-patiënten. Het ruggenmerg van aangedane dieren bevatte veel myelineschedes waar axonen beschadigd of zelfs geheel verdwenen waren. Hoe de ziekte precies ontstaat is nog niet duidelijk, mogelijk spelen T cellen van het CD4 type die de ontstekingsbevorderende stof interferon-γ maken, een belangrijke rol.

Tot slot is onderzocht of een immuunreactie tegen een axonale component ook leidt tot meer schade aan axonen (**Hoofdstuk 7**). De lokaties van de ontstekingshaarden als ook de hoeveelheid myeline en axonale schade werd bepaald in muizen die neurologische verschijnselen hadden ontwikkeld door een NF-L-immuunreactie of door een immuunreactie tegen een myeline component. De muizen met een immuunreactie tegen NF-L hadden vooral laesies in de achterstreng en de grijze stof van het ruggenmerg en hadden significant meer schade aan axonen dan muizen met een immuunreactie tegen myeline die juist meer laesies hadden in de voor- en zijstreng van het ruggenmerg. De schade aan myeline was vergelijkbaar in beide groepen. Waarschijnlijk zorgt de immuunreactie tegen NF-L voor primaire schade aan axonen en is de schade aan myeline een secundair gevolg.

Samenvattend zijn de conclusies van het onderzoek beschreven in dit proefschrift: 1) Naast antistoffen tegen NF-L komen ook cellulaire reacties voor bij zowel patiënten met MS als controle proefpersonen; 2) Beschadigde axonen worden omgeven door APC in MS laesies en neuronale componenten zijn aanwezig in APC in lymfeklieren in de hals van MS-patiënten en dieren met EAE. Deze cellen zijn mogelijk betrokken bij het op gang brengen en het in stand houden van de antineuronale immuunreactie; 3) Immuunreacties tegen axonale componenten zoals NF-L kunnen leiden tot neurologische ziekteverschijnselen en primaire axonale schade. Dit betekent dat, in mensen, immuunreacties tegen axonale componenten niet slechts beschouwd moeten worden als maat voor de hoeveelheid axonale schade, maar dat deze reacties juist cruciaal kunnen zijn in het induceren van axonale schade die leidt tot onherstelbare neurologische klachten en progressie van ziekte.

ABBREVIATIONS

ABH	Antibody high
AP	Alkaline phosphatase
APC	Antigen-presenting cell
APP	Amyloid precursor protein
ATP	Adenosine tri-phosphate
BBB	Blood-brain barrier
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CLN	Cervical lymph node
CNS	Central nervous system
CREAE	Chronic relapsing experimental autoimmune encephalomyelitis
CSF	Cerebrospinal fluid
DC	Dendritic cell
DRG	Dorsal root ganglia
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECL	Entorhinal cortex lesion
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FNA	Facial nerve axotomy
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LLN	Lumbar lymph nodes
MAG	Myelin associated glycoprotein
MAP-2	Microtubule associated protein-2
MBP	Myelin basic protein
MCAO	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte protein
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
NAA	N-acetyl aspartate

NAWM	Normal-appearing white matter
NF-H	Neurofilament heavy
NF-L	Neurofilament light
NF-M	Neurofilament medium
NMDA	N-methyl-D-aspartic acid
NP-NF	Non-phosphorylated neurofilament
NSE	Neuron-specific enolase
NO	Nitric oxide
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
PLP	Proteolipid protein
PND	Paraneoplastic disorders
PNS	Peripheral nervous system
PP-MS	Primary progressive multiple sclerosis
PSD	Postsensitization day
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RR-MS	Relapsing remitting multiple sclerosis
SCID	Severe combined immunodeficiency syndrome
SP-MS	Secondary progressive multiple sclerosis
TCR	T-cell receptor
TGF	Transforming growth factor
TMEV	Theiler’s murine encephalomyelitis virus
TNF	Tumour necrosis factor
TT	Tetanus toxoid
VLA-4	Very late antigen-4

DANKWOORD

Eindelijk, het is af! Gelukkig heb ik al het werk niet alleen hoeven doen, maar was er een groot aantal mensen die hebben geholpen bij het tot stand komen van dit boekje. Bij deze wil ik dan ook iedereen heel hartelijk bedanken! Een aantal mensen wil ik nog graag bij name noemen.

Als eerste natuurlijk Sandra, jij was vanaf de eerste dag mijn begeleidster en bent dat altijd gebleven. Ondanks dat het project al door jou geschreven was, was er volop ruimte om mijn eigen ideeën te volgen. Dankjewel voor die vrijheid! Je bent als wetenschapper en als mens in veel opzichten een groot voorbeeld voor me. Jouw motto: “Always think positive”, heb je zeker waargemaakt! Ik hoop dat we ook in de toekomst nog veel samen blijven werken.

Prof. Benner, beste Rob, in de beginjaren volgde je mijn ontwikkelingen vooral vanaf de zijlijn, in het laatste jaar van meer dichtbij. Hartelijk dank voor de mogelijkheid om mijn promotieonderzoek af te kunnen ronden op de afdeling. Jon, jij nam vervolgens het stokje over en hebt mij vakkundig door de laatste fasen van het promotietraject geloodst. Dank voor je adviezen en het doornemen van mijn teksten.

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Het schrijven van wetenschappelijke artikelen is een heel proces. In dit verband wil ik dan ook graag Bert bedanken. Jouw kritische blik heeft mijn manuscripten zeker verbeterd. Hans, ook jij was altijd bereid om mijn stukken onder de loep te nemen en van deskundig commentaar te voorzien. Samen met Richard heb je verder veel hersenmateriaal gefractioneerd en gevriesdroogd. Het blijft toch jammer dat we daarin geen nieuwe neuronale targets konden identificeren.

The study on NF-L autoimmunity all started with the generous gift of NF-L protein from Prof. Harald Herrmann. Thank you for all your advice and for introducing us into the world of intermediate filaments. The neuropathological studies would have been totally different if I had not learned to process tissue for plastic embedding. Steve, thank you for teaching me all the ins-and-outs. Wouter, door jou waren we in staat om de leukocyten in het ruggenmerg goed te kunnen kwantificeren. Het is ongekend hoe jij de histologietechnieken beheerst, veel succes met de moleculaire! Ook de overige VUMC’ers wil ik bedanken voor hun bijdrage. Christine en Ed, fijn dat we met jullie expertise een aantal monoclonalen hebben kunnen maken. Eveneens dank aan Charlotte voor het testen van de monoclonalen, ik hoop dat we met de fagocytose experimenten een mooi paper kunnen vormen.

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De collega's van lab 802 wil ik bedanken voor de steun bij het afronden van mijn onderzoek in het laatste jaar. Door jullie voelde ik me snel thuis in Rotterdam. Ik heb dan ook veel zin om met jullie het onderzoek naar MS en GBS voort te zetten. Marloes, we zijn tegelijk begonnen als AIO in het MS-veld en we hebben veel dingen samen gedaan. Het lymfklier-weg-stuk is heel mooi terecht gekomen en met die andere moet het ook goed komen. Succes met de laatste loodjes! Daarna moeten we echt snel een nieuw restaurantje testen! Karin, Manon, Jeltje en Jan-Piet, jullie gingen me voor en de praktische tips die jullie gaven, waren zeer bruikbaar! De overige (ex-)AIO's wil ik bedanken voor de gezellige weekenden waarbij het nuttige met het aangename werd verenigd.

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CURRICULUM VITAE

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2002 - 2003	Diplôme d’Études en Langue Française (2nd degree), Université de Perpignan
2003 - 2008	PhD-research “Autoimmunity to neuronal antigens in multiple sclerosis” (supervised by Dr. S. Amor), Department of Immunobiology, Biomedical Primate Research Centre, Rijswijk and Department of Immunology, Erasmus MC, University Medical Center, Rotterdam (promotor Prof. Dr. J.D. Laman)

Attended courses

- Laboratory Animal Science (Article 9, 2004)
- Molecular Immunology (2004, Postgraduate School of Molecular Medicine)
- English biomedical writing and communication (2005)
- Classical methods of data-analysis (2005, NIHES)
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- Plastic techniques for histology (supervised by S. Gschmeissner), EM Unit, Cancer Research UK, London
- Monoclonal antibody technology (supervised by Prof. Dr. C.D. Dijkstra and E. Döpp), Department of Cell Biology and Immunology, VU University Medical Center, Amsterdam

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- Supervision of trainees from middle and higher laboratory education

2008 - Postdoctoral research “Mechanisms of pathogenic B-cell responses in Guillain-Barré syndrome”, supervised by Dr. B.C. Jacobs and Prof. Dr. J.D. Laman, Department of Immunology, Erasmus MC, Rotterdam

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1. Van der Goes A, Wouters D, Van der Pol SMA, Huizinga R, Ronken E, Adamson P, Greenwood J, Dijkstra CD and De Vries HE. (2001). Reactive oxygen species enhance the migration of monocytes across the blood-brain barrier in vitro. *FASEB J* 15: 1852-1854.
2. Huizinga R, Heijmans N, Schubert P, Gschmeissner S, 't Hart BA, Herrmann H and Amor S. (2007). Immunization with neurofilament light protein induces spastic paresis and axonal degeneration in Biozzi ABH mice. *J Neuropathol Exp Neurol* 66: 295-304.
3. Huizinga R, Linington C and Amor S. (2008). Resistance is futile: antineuronal autoimmunity in multiple sclerosis. *Trends Immunol.* 29: 54-60.
4. Huizinga R and Amor S. (2008) Autoimmunity to neuronal proteins in neurological disorders. Book chapter in: New Immunology Research Developments, Müller GV (Ed), Nova Science Publishers, Hauppauge, NY.
5. Huizinga R, Gerritsen W, Heijmans N and Amor S. (2008). Axonal loss and grey matter pathology as a direct result of autoimmunity to neurofilaments. *Neurobiol Dis.* doi: 10.1016/j.nbd.2008.08.009.
6. Van Zwam M, Huizinga R, Heijmans N, Van Meurs M, Wierenga-Wolf AF, Melief M-J, Hintzen RQ, 't Hart BA, Amor S, Boven LA and Laman JD. (2008). Depletion of central nervous system-draining lymph nodes reduces relapse severity in EAE. *J Pathol, accepted for publication.*
7. Huizinga R, Hintzen RQ, Assink K, Van Meurs M and Amor S. T-cell responses to neurofilament light protein are part of the normal immune repertoire. *Submitted for publication.*
8. Van Zwam M, Huizinga R, Melief MJ, Wierenga-Wolf AF, Van Meurs M, Voerman JS, Biber KPH, Boddeke HWGM, Höpken UE, Meisel C, Meisel A, Bechmann I, Hintzen RQ, 't Hart BA, Amor S, Laman JD and Boven LA. Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. *Submitted for publication.*
9. Huizinga R, Jong R, Gerritsen W, Van Driel N, Döpp EA, Dijkstra CD and Amor S. Damaged axons are engulfed by activated phagocytes in multiple sclerosis lesions. *Manuscript in preparation.*

Chapter 4: Figure 2

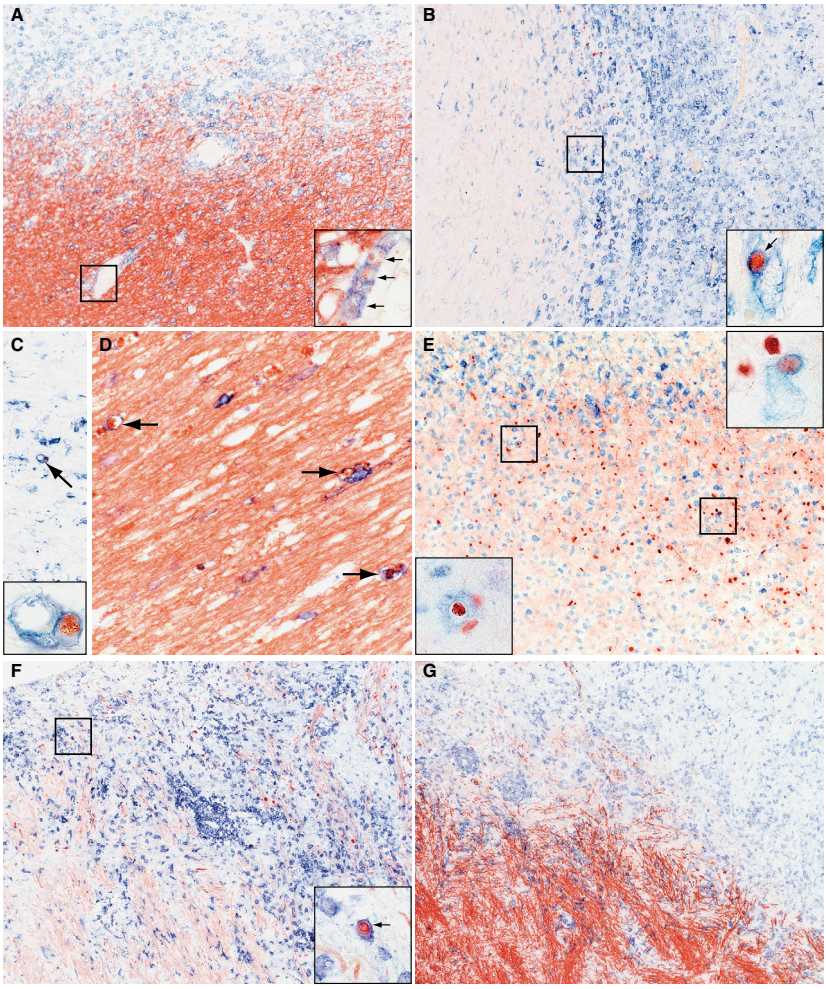

Figure 2. Axons are engulfed by HLA class II$^+$ cells in MS brain lesions. Paraffin sections were doublestained for HLA class II (blue) and myelin or axonal antigens (red). In an active lesion MBP is present in HLA class II$^+$ cells in the perivascular space of blood vessels (**A**, arrows). Axonal damage as indicated by APP$^+$ ovoids is present in the hypercellular rim containing HLA class II$^+$ cells (**B**). Some of these axons were totally engulfed by HLA class II$^+$ cells (inset in **B**). Both A and B are from case 14. HLA class II$^+$ cells also engulf SMI-32$^+$ axons (**C**, from case 12). The area in which these cells were located was outside a lesion where HLA class II$^+$ cells were also shown to contain MBP (**D**). Numerous ovoids positive for NF-L were present in case 3 (**E**) and HLA class II$^+$ cells were observed to surround these damaged axons (inset in **E**). A damaged axon positive for NF-L in case 12 was engulfed by a HLA class II$^+$ cell (**F**). The lack of space between the axon and the HLA class II$^+$ cell suggests that the myelin sheath is already degenerated, resulting in direct interaction between the axon and the HLA class II$^+$ cell. The border of myelination is shown in **G** (MBP stain).

Chapter 5: Figure 1

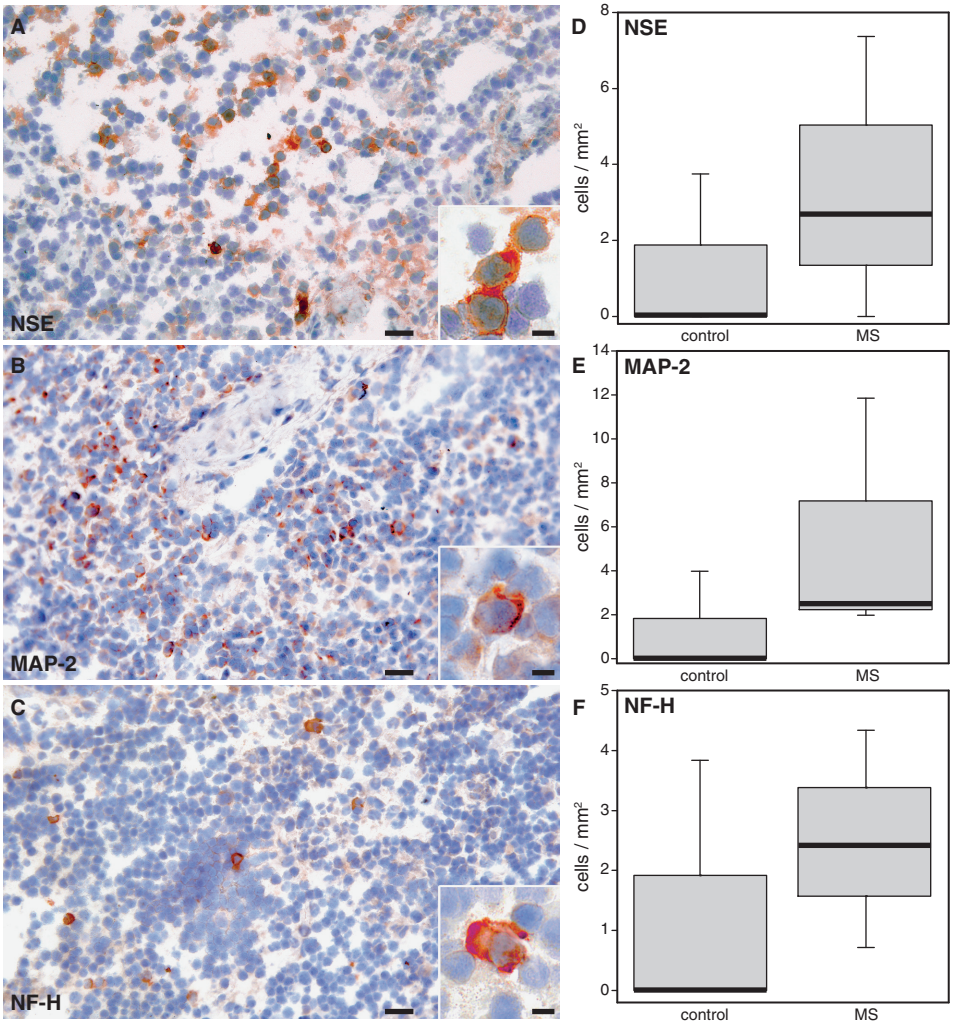

Figure 1. Neuronal antigens drain to the CLN in MS. CLN of MS patients contain cells that are positive for the neuronal antigens NSE (**A**), MAP-2 (**B**), and NF-H (**C**). Scale bars: 20 μm; insert 5 μm. Quantification of the number of MAP-2 (**D**), NSE (**E**), and NF-H (**F**) positive cells demonstrated a higher, but not significantly different, number of neuronal antigen-containing cells in CLN of three MS patients than in CLN of three controls without neurological disease. Three sections from CLN of each patient were quantified for each neuronal antigen. Results are shown as box plots with medians, twenty-fifth and seventy-fifth percentiles as boxes, tenth and ninetieth percentiles as whiskers.

Chapter 5: Figure 2

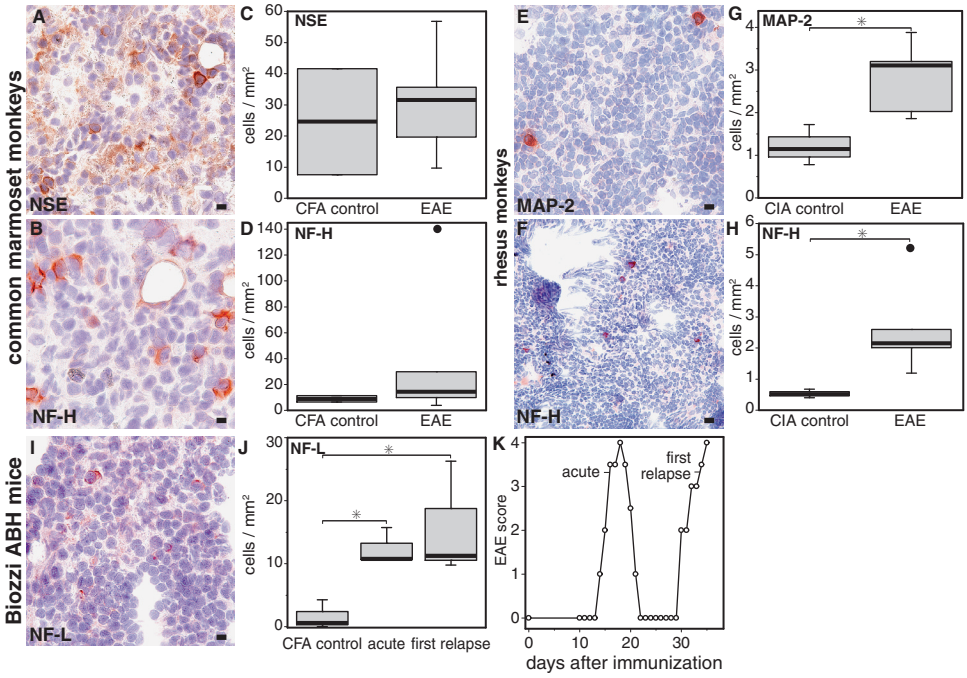

Figure 2. The presence of neuronal antigen-containing cells in during EAE reflects the extent of neuronal damage within the CNS. CLN of EAE-affected common marmoset monkeys contain NSE (**A**) and NF-H (**B**) positive cells. More NSE (**C**) and NF-H (**D**) positive cells were observed in CLN of five marmosets with EAE as compared to two CFA-immunized control marmosets, although this difference was not significant. In CLN of EAE-affected rhesus monkeys MAP-2 (**E**) and NF-H (**F**) positive cells were found. Quantification of MAP-2 (**G**) and NF-H (**H**) positive cells revealed a significantly higher number of cells in CLN of five rhesus monkeys with EAE than in CLN of three collagen-induced arthritis (CIA) control monkeys (* $p < 0.05$). NF-L-positive cells were present in the deep CLN of EAE Biozzi ABH mice (**I**). Deep CLN from three animals with acute disease as well as from three mice in the first relapse contained significantly more NF-L positive cells than CLN from three CFA-immunized control mice (**J**; * $p < 0.05$). EAE scores from one representative animal out of three are shown (**K**). Three sections from CLN of each monkey and four sections from CLN of each mouse were quantified for each neuronal antigen. Results are given as box plots as described in Figure 1 with extreme values as filled circles. Scale bars: 10 μm.

Chapter 5: Figure 4

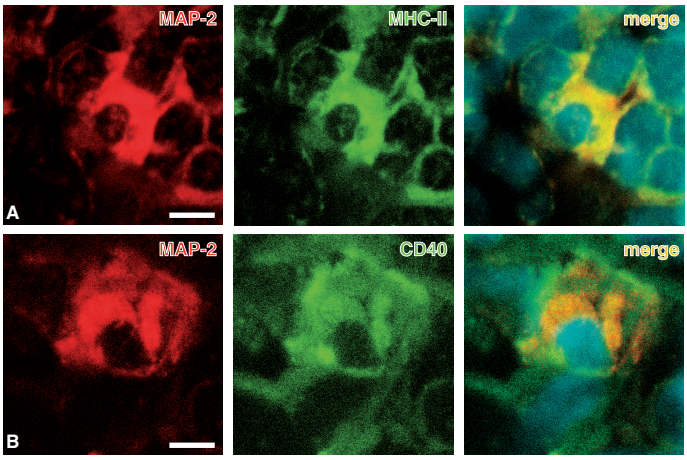

Figure 4. MAP-2 containing cells in human MS CLN are immunocompetent APC. Immunofluorescent MAP-2 staining in human MS CLN (red) and staining for the APC antigens MHC class II (green) (**A**) and CD40 (green) (**B**). Overlay shows co-expression of MAP-2 with MHC class II and CD40 antigens, which was observed in 90 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Stainings were performed on two sections of CLN from three different MS patients. Scale bars: 5 μm.

Chapter 5: Figure 6

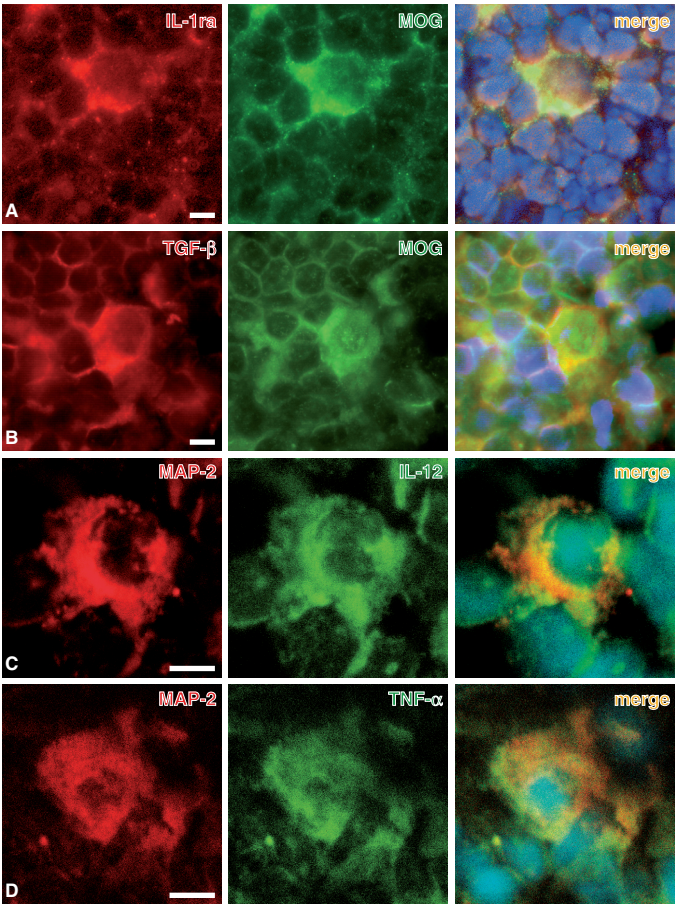

Figure 6. Differential expression of pro- and anti-inflammatory molecules by MAP-2 and MOG containing cells in human MS CLN. Immunofluorescent labeling of MOG (green) and the anti-inflammatory molecules IL-1ra (red) (**A**) and TGF-β (red) (**B**) and immunofluorescent labeling of MAP-2 (red) and of the pro-inflammatory molecules IL-12p40/p70 (green) (**C**), and TNF-α (green) (**D**) in human MS CLN. The overlays show co-localization of MOG and the anti-inflammatory molecules IL-1ra and TGF-β, which was observed in 90 to 100% of the MOG positive cells, and co-localization of MAP-2 and the pro-inflammatory molecules IL-12p40/p70 and TNF-α, which was observed in 86 to 100% of the MAP-2 positive cells. Nuclei are stained with DAPI (blue). Results are representative for two sections of CLN from an MS patient for double stainings of MOG with TGF-β and TNF-α and of MAP-2 with TGF-β and for at least two sections of CLN from three different MS patients for double stainings of MAP-2 with IL-12p40/p70, TNF-α and IL-1ra and double stainings of MOG with IL-1ra and IL-12p40/p70. Scale bars: 5 μm.

Chapter 6: Figure 4

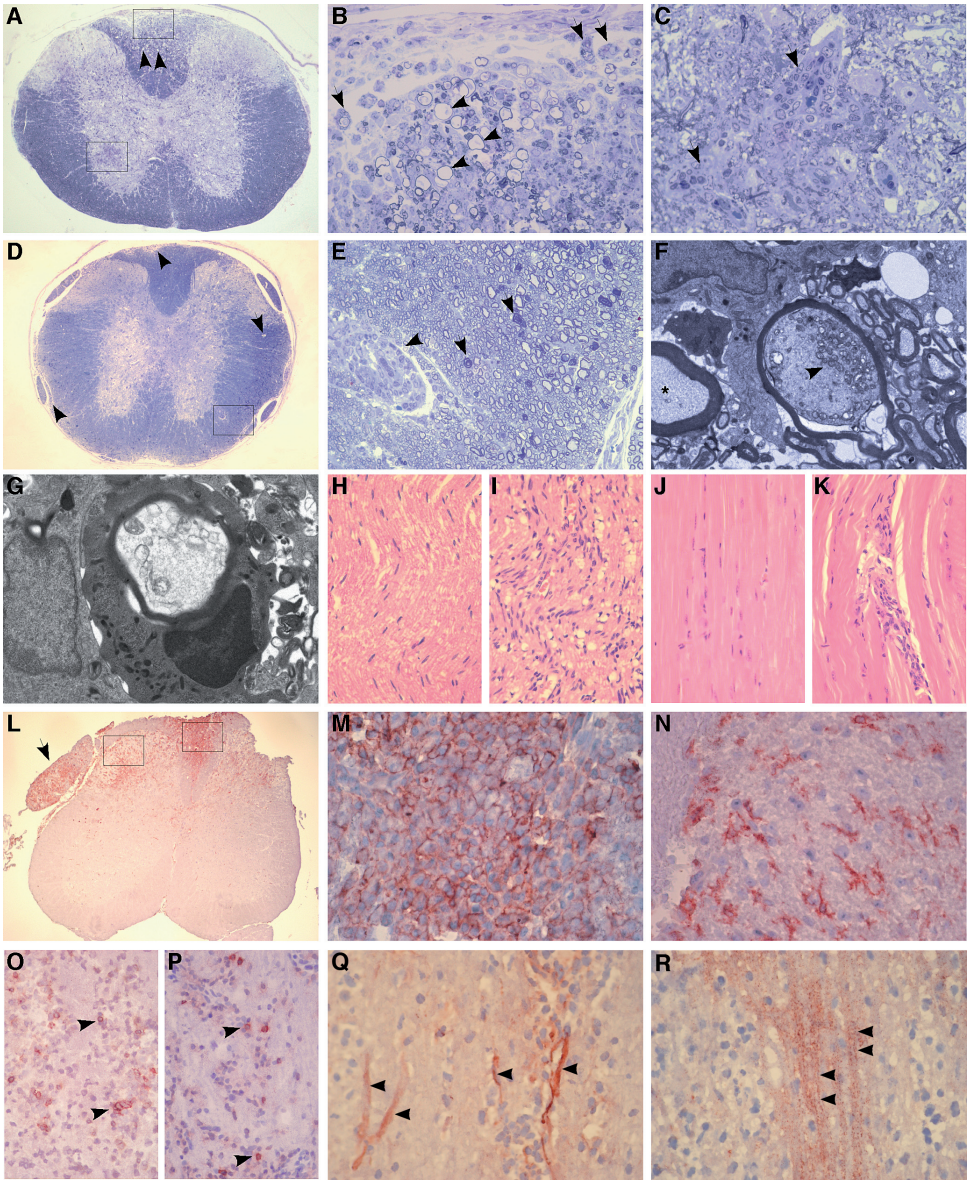

Figure 4. Mice with neurological disease develop inflammation and axonal degeneration in the spinal cord and sciatic nerve. Semithin resin section of a mouse with spastic paresis reveals extensive vacuolation (arrowheads) in the dorsal column of the spinal cord (**A**; toluidine blue; original magnification: 25x). Axons in this area show signs of Wallerian degeneration (**B**, arrowheads, enlargement of upper rectangle in **A**; 400x); note that phagocytes contain myelin debris (arrows). In the grey matter, groups of inflammatory cells are associated with blood vessels (**C**; arrows, enlargement of lowest rectangle in **A**; 400x). Biozzi ABH mice immunized with rmMOG had inflammation in the spinal cord (**D**; arrowheads, 25x), myelin degeneration (arrows in **E**; enlargement of rectangle in **D**; 400x), but only limited axonal degeneration (arrow in **D**). Electron microscopy of the spinal cord of affected rmNF-L-immunized mice shows accumulated mitochondria in a swollen axon (**F**; arrowhead; asterisk indicates normal axon) and a phagocyte engulfing a damaged axon with its myelin sheath (**G**; 3000x). The sciatic nerve of asymptomatic mice appears normal (**H**; hematoxylin and eosin stain; 200x) whereas inflammation is present in the sciatic nerve of affected mice (**I**; 200x). Surrounding muscle tissue in mice without clinical disease appears normal (**J**; 200x), in contrast with mice exhibiting disease, in which cellular infiltrates can be seen (**K**; 200x). F4/80^{+} macrophages are present in high numbers in the dorsal column of a mouse with neurological disease (**L**; 25x and **M**; enlargement of the right rectangle in **L**; 400x). In the grey matter, cells with a ramified morphology, resembling microglia, express the F4/80 antigen (**N**; enlargement of the left rectangle in **L**; 400x). T cells expressing the CD4 (**O**; arrowheads; 200x) and CD8 (**P**; arrowheads; 200x) antigen are present in lesions. After extensive washing of unfixed sections, IgG1 is still complexed to axons in the dorsal column of rmNF-L-immunized mice with clinical disease (**Q**; 400x), whereas IgG1 is complexed to myelin sheaths and not to axons in rmMOG-immunized mice (**R**; 400x).

Chapter 7: Figure 7

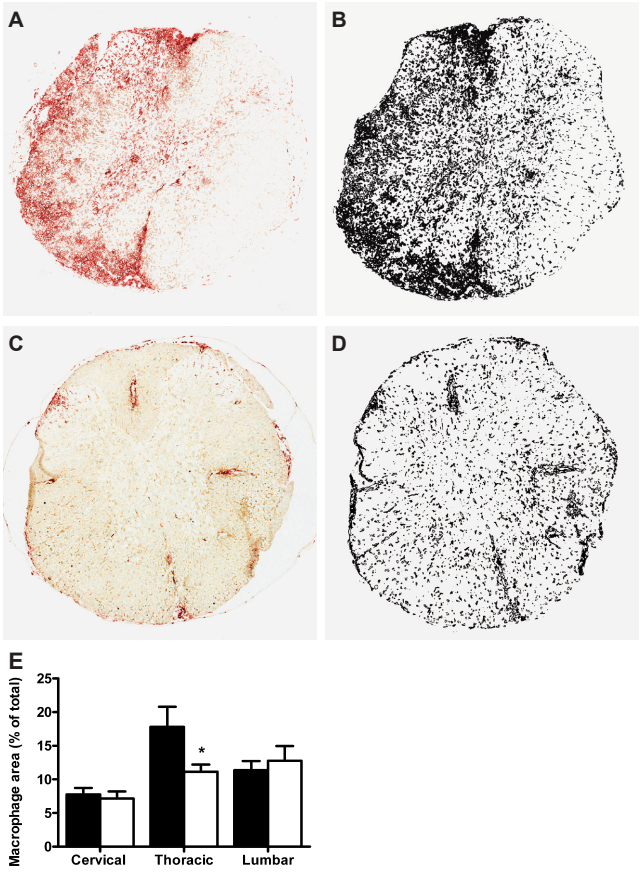

Figure 7. NF-L-immunized animals show increased macrophage/microglia staining in the thoracic spinal cord. The F4/80 antigen was stained in spinal cord sections of NF-L (**A**) and MOG-immunized (**C**) mice and images were analyzed using ImageJ software. F4/80 positive staining is converted to black (**B** and **D**) and the total pixel area was measured. Spinal nerve roots (top left corner in **A**) were excluded from analysis. NF-L-immunized mice (black bars) show increased F4/80 staining in the thoracic cord compared with MOG (white bars). F4/80 staining in the cervical and lumbar regions were comparable (**E**). * $p < 0.05$, Mann-Whitney U test.