Axonal Damage in Multiple Sclerosis

van der Star, B.J.

2014

document version
Publisher's PDF, also known as Version of record

CHAPTER 7

General Discussion and Future Perspectives

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) widely considered as an autoimmune disease in which autoagressive T cells attack myelin. As well as demyelination, neurodegeneration is a major pathological feature that plays a crucial role in the clinical disability of people with MS (1). That neuronal and axonal damage contributes to the accumulation of neurological deficits, particularly in progressive forms of MS, has redirected attention to the cause of this damage as well as whether neurodegeneration is a cause or a consequence of demyelination. Recent studies have suggested that in addition to an autoimmune attack on myelin, autoimmunity to neuronal proteins present in people with MS, is a key mechanism leading to progressive neurodegeneration and axonal damage. One of the possible targets for the (auto)immune response is the neuron-specific cytoskeleton protein neurofilament light (NF-L) (2-6). However, whether these immune responses present in MS are merely biomarkers of axonal and neuronal damage or whether they play a pathogenic role and contribute to axonal damage in people with MS is the key question of this thesis.

Insights in the mechanisms contributing to neurodegeneration are crucial for the development of new drug targets to prevent neuronal damage and progressive neurological deficits. Therefore the goal of this thesis is to elucidate the role of immune responses to neuronal antigens, in particular NF-L. As depicted in 'Chapter 1', the thesis had several aims to test the hypothesis: **'Autoimmunity to NF-L contributes to axonal damage in MS'**. Each aim is discussed below in perspective of the results and recent knowledge. We conclude by discussing whether therapies targeting autoimmunity to neuronal antigens might be relevant for treating disease progression in MS.

Major questions:

1. *How can autoimmunity to neuronal antigens arise in MS?*
2. *What are the mechanism(s) by which NF-L-specific T cells cause axonal damage?*
3. *Can antibodies to NF-L serve as biomarkers of disease and/or do they contribute to axonal damage in MS?*
4. *Targeting autoimmunity to neuronal antigens; is this relevant for disease progression in MS?*

How can autoimmunity to neuronal antigens arise in MS?

Accumulating evidence indicates that. as well as myelin antigens, autoimmunity to neuronal antigens are present in MS and other neurodegenerative diseases. However, how these responses arise is unclear. One key feature for the generation of immune responses is antigen presentation. This could take place after neuronal or axonal damage, but also when micro-organism antigens show similarity with self-antigens (so-called 'molecular mimicry') (7). Molecular mimicry is demonstrated in experimental models for viral particles that share homology with myelin proteins, including proteolipid protein (PLP) (8), myelin basic protein (MBP) (9) and myelin oligodendrocyte glycoprotein (MOG) (10). Bacterial antigens are also shown to activate myelin-specific T cells, resulting in exacerbation of experimental autoimmune encephalomyelitis (EAE) (11). In humans, an example of axonal pathology following bacterial infections is Guillain-Barré Syndrome (GBS), although

mainly the peripheral nerves are affected (12). However, homologies between neurofilaments and micro-organisms have yet to be reported. Therefore, a more likely key event in the development of autoimmunity to neuronal antigens in MS is phagocytosis of neuronal debris. Several studies indicate a strong association between neuronal damage and immune cell activation in MS lesions. In **chapters 2 and 3** we demonstrated phagocytosis of neuronal antigens in the CNS of people with MS. Although engulfment of neuronal proteins was not as extensive as myelin proteins in active white matter lesions, phagocytosis of neuronal debris correlated with the extent of damaged axons. Thus, people with MS who have extensive neuronal damage could have a higher chance of autoimmunity to neuronal antigens including NF-L. On the contrary, phagocytosis of neuronal debris might lead to tolerance, since T cells and antibodies to myelin and neuronal antigens are also present in the serum of healthy controls (13). Tolerance following phagocytosis of debris is also supported by a Danish study that does not find a correlation between head injury and development of MS (14). More support for a tolerogenic effect after axonal damage is provided by Mutlu and colleagues who show that experimental brain injury significantly reduces the clinical signs of EAE, considered an animal model of MS (15). Nevertheless, uptake, degradation and presentation of neuronal antigens remain crucial steps for activation of autoreactive T cells and autoantibody production. We observed neuronal-antigen containing human leukocyte antigen (HLA)-DR^+ cells in the cerebrospinal fluid (CSF) more frequently than in non-inflammatory disorders **(chapter 2)**. A recent study by Schutzer and colleagues has identified neuronal proteins, rather than myelin proteins, in people with clinically isolated syndrome (CIS) suggesting involvement of grey matter early in MS development (16). More so, neuronal protein-containing cells are shown in cervical lymph nodes of people with MS expressing the co-stimulatory molecule cluster of differentiation-40 (CD40) (17). This is in line with our hypothesis that phagocytosis of NF-L might be the first step in the development of autoimmune responses to NF-L in MS. In addition, together with MBP and neurofilament medium, NF-L is eluted from major histocompatibility complex (MHC) class II molecules from people with MS, indicating presentation of these antigens (18). Whether NF-L antigen presentation induces an autoimmune response is still a subject of investigation. A preliminary experiment with monocyte-derived macrophages incubated with human white matter debris failed to demonstrate a proliferative effect on NF-L-specific T cells, although this experiment needs to be repeated (collaborative experiment with D.Y.S. Vogel, VUmc, and P. Nacken, Delta Crystallon BV).
In summary, neuronal antigens phagocytosed and presented by antigen presenting cells have the potential to attract and activate the immune system. In this way they might contribute to pathogenic immune responses and neuronal damage in people with MS.

What are the mechanism(s) by which NF-L-specific T cells cause axonal damage?

The presence of T cells in MS lesions and myelin-reactive T cells in the circulation of people with MS as well as T cell targeted therapies implicate T lymphocytes in the pathogenesis of MS. Furthermore, $CD8^+$ T cells outnumber $CD4^+$ T cells in MS lesions (19). Both myelin-reactive T cells and T cells reactive to neuronal antigens, e.g. NF-L, are present in people with MS and healthy controls (13). However, these results do not confirm a pathogenic role of T cells in MS. Most support for a pathogenic role of T cells in MS is generated by using animal models. For example,

adoptive transfer of myelin-specific T cells from myelin-immunised mice causes disease in naive recipients (20). However, little is known about neuronal-specific T cells. Previous studies showed that immunisation with recombinant NF-L induces spasticity and paralysis accompanied by grey matter pathology in the dorsal column of the spinal cord of Biozzi ABH (antibody high) mice (21, 22). In **chapter 4** we further characterised the T cell response in these mice and demonstrated an association of T cells with axonal damage in spinal cord lesions. In addition, transfer of NF-L-specific T cells into naive recipients induced clinical disease after 25 days. Although clinical disease was mild (partial loss of tone tail) and present in 29% of mice it reveals that NF-L-specific T cells have the potential to be pathogenic in this model.
The mechanisms by which T cells might contribute to axonal damage are discussed below.

Mechanisms of axonal damage by T cells
Effector mechanisms of activated $CD4^+$ and $CD8^+$ T lymphocytes include secretion of perforin and granzyme B. Although $CD4^+$ T cells are initially thought not to have cytotoxic abilities, studies indicate that a subset of $CD4^+$ T cells is able to express cytotoxic molecules e.g. perforin and granzyme B (23, 24). In biological fluids of people with MS, $perforin^+$ $CD4^+$ and $CD8^+$ T cells are identified (25, 26). Several studies show neurotoxicity following exposure of perforin (27-29), or destabilisation of microtubules (30). In perforin-deficient mice infected with Theiler's murine encephalomyelitis virus (TMEV), axons are preserved despite demyelination (31, 32) indicating that perforin contributes to axonal damage in this model. We showed that perforin expression was increased in NF-L immunised Biozzi ABH mice **(chapter 4).** After pore formation of the cell membrane caused by perforin, granzyme B enters the cell (33). Induction of neuronal death by granzyme B is reported *in vitro* (34-36) and *in vivo* (37). Granzyme B can also induce apoptosis in a perforin-independent manner through endocytosis (35). A role of granzyme B in CNS damage in MS is supported by an increased granzyme B mRNA expression in active and chronic active MS lesions compared to normal appearing white matter (35) as well as increased granzyme B protein expression in the CSF of people with relapsing-remitting MS (RRMS) who experience a relapse (38). In line with these studies are preliminary results that show the presence of granzyme B^+ $CD4^+$ T cells in an active MS brain lesion (Fig. 1). Whether these granzyme B^+ granules are released is currently unknown. Furthermore, in **chapter 4** we show the presence of granzyme B^+ cells in spinal cord lesions in NF-L immunised Biozzi ABH mice. These results indicate that besides perforin, secretion of granzyme B by NF-L-reactive T cells may contribute to axonal damage.

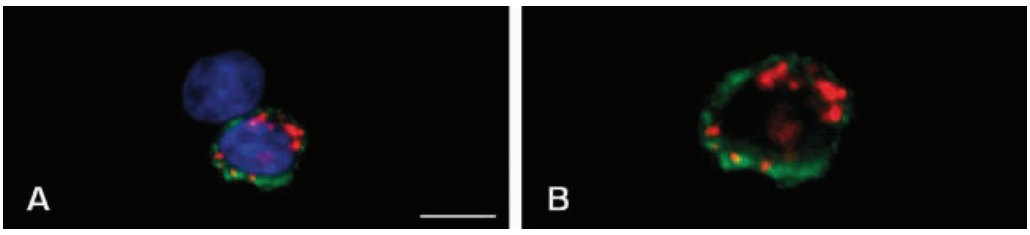

Figure 1. Granzyme B^+ $CD4^+$ T cell in the brain parenchyma in a MS lesion. **A***) Granzyme B expression (red) in CD4 T cells (green) in an active MS lesion.* **B***) Enlargement of (A). Nuclei are stained blue. Scale bar=5µm. In collaboration with prof. M. Kipp (RWTH, Aachen, Germany).*

In highly inflammatory MS lesions, neurons are shown to express MHC class I (39), suggesting that neurons might also be targets for $CD8^+$ T cells. Sauer and colleagues show direct axonal injury by $CD8^+$ T cells through a MHC class I and granzyme B-dependent mechanism (36). Expression of MHC class I is up regulated in the presence of interferon-gamma (36, 40), a pro-inflammatory molecule which is produced by NF-L-reactive T cells (13). This indicates that neurons might also be targets for $CD8^+$ T cells through the interaction with MHC class I molecules.
Other cell-cell contact dependent mechanisms by which T cells might contribute to axonal damage are through interaction of Fas- Fas ligand (FasL), tumor necrosis factor-related apoptosis inducing ligand (TRAIL), CD11a and CD40 (41-43). Giuliani and colleagues show decreased neuronal loss by $CD4^+$ and $CD8^+$ T cells following pre-treatment of T cells with antibodies to FasL, lymphocyte function-associated antigen-1 and CD40 (42). Furthermore, in MS lesions apoptotic spinal motor neurons are surrounded by $TRAIL^+$ $CD3^+$ T cells (43). To confirm a role for a TRAIL-dependent mechanism of neuronal loss, Vogt and colleagues compare neuronal loss in mice following adoptive transfer of MOG^{35-55}-specific T cells from wild-type mice or TRAIL-deficient mice. Significantly more neuronal loss is observed in mice following adoptive transfer of MOG^{35-55}-specific T cells, while the number of $CD3^+$ T cells in the lesions is comparable (43).
To conclude, several mechanisms causing neuronal and axonal damage by T cells are described. Whether these mechanism are also part of NF-L-reactive T cells in the CNS of people with MS needs further study.

Can antibodies to NF-L serve as biomarkers of disease and/or do they contribute to axonal damage in MS?

Around 90% of people with MS have oligoclonal bands (OCB) in the CSF, which are a result of local immunoglobulin (Ig) production. It is unknown whether these Igs contribute to disease, although a pathogenic role of intrathecal Igs is supported by an increased risk of conversion to MS in people with CIS positive for OCB (44, 45). Oligoclonal antibody production in the CSF is not specific for MS (46, 47) and there is discussion whether OCB determination should still be part of the diagnostic tools for MS (47, 48). On the contrary, Igs in the CSF might serve as useful biomarkers to predict disease progression.
In the CSF and serum of people with MS, several studies report the presence of antibodies to neuronal antigens including NF-L (2, 4, 6). A recent study by Sádaba and colleagues has shown the binding of IgM and IgG on axons in active, chronic active and inactive MS lesions (49). Furthermore, levels of antibodies to NF-L are increased in the CSF and serum of people with progressive disease (2, 6) and correlate with clinical disability (6) and cerebral atrophy (3), suggesting the potential of antibodies to NF-L as biomarkers. Not all studies show a correlation between antibodies to NF-L and disability **(chapter 5** and (46)**)**. These inconsistencies might be a result of differences in cohorts or assays used (50) as well as the medical history of the subjects (46). Also important to mention is that antibodies to NF-L are likely to bind NF-L proteins, which are also present in biological fluids (51, 52), and form complexes, which might interfere with the assay used for detection (50). Thus, it should be noted that measurements of NF-L antibodies might underestimate the actual numbers. In **chapter 5** we investigated the influence of treatment on serum antibody levels. We reported reduced levels of serum antibodies to NF-L in people with RRMS treated with natalizumab. These results are in line with a study

by Bartos and colleagues, who report that levels of intrathecal NF-L antibodies are specifically reduced following immunosuppressive and/or immunomodulatory treatment (46). Decreased NF-L protein levels in the CSF of people with RRMS following natalizumab treatment (53) as well mitoxantrone and rituximab treatment in people with progressive MS (54) are also reported. This might suggests that reduction of anti-NF-L antibodies is secondary to a decrease in axonal damage and therefore a decline in the release of neurofilament protein or that the treatment decreases the levels of NF-L antibodies which results in a positive effect on axonal damage. The latter argument is supported by a negative correlation with NF-L antibody levels and time since last relapse as shown in **chapter 5**.
Thus, antibodies to NF-L may serve as useful biomarkers for treatment responses and reflect axonal damage, but there are also indications that argue for a potential pathogenic role of NF-L-specific antibodies. The question whether antibodies to NF-L might contribute to axonal damage was the focus in **chapter 6**. In this chapter we demonstrated that a monoclonal antibody (mAb) to NF-L caused axonopathy in spinal cord co-cultures and a mouse model of optical neuritis. Recently, Elliott and colleagues have shown demyelination and axonal reduction using the same spinal cord co-culture system following MOG and neurofascin-specific antibodies, respectively (55). Whether mAb to NF-L also have an effect on myelination would be of interest for future studies, although in NF-L-immunised ABH mice mainly axons are affected (22). To localise NF-L antibodies, stereotactic intracerebral injection with labelled mAb to NF-L was performed in a mouse. This revealed internalisation of mAb to NF-L in neurons, although the isotype control was also detected inside neurons following stereotactic injection. Uptake of control Ig is shown before (56, 57). In the study by Greenlee and colleagues, anti-Yo antibodies targeting cytoplasmic proteins e.g. ribosomes in Purkinje cells, are taken up and accumulate intracellular. This accumulation results in neuronal death whereas the control Ig does not accumulate in the Purkinje cell and therefore does not result in neuronal death (56). Fc receptor-mediated and clathrin-mediated endocytosis are possible non-epitope specific mechanisms by which antibodies (to NF-L) may be internalised by neurons (58-60). A recent study has shown that antibodies to an intracellular antigen enter neurons by endocytosis and cause depletion of adenosine triphosphate (ATP) and an increase in caspase levels leading to apoptosis and cell death (61). Activation of Fc-gamma receptor 1 by IgG-immune complex is shown to cause an increase in intracellular calcium and enhances excitability in sensory neurons (62). On the contrary, uptake of Ig by neurons might also serve as a protective mechanism because their internalisation inhibits complement activation (59) and assists in protein clearance (58).
In summary, antibodies to NF-L might serve as biomarkers but that does not exclude their possible pathogenic effects in inflammatory neurodegenerative diseases.

Mechanisms of axonal damage by antibodies
Antibodies directed to targets in the CNS contribute to pathology in several autoimmune disorders. In people with neuromyelitis optica, antibodies to aquaporin-4, a protein expressed on astrocytes, lead to demyelination possibly through complement-dependent cytotoxicity (63-68). In GBS antibodies to specific lipids, gangliosides, present on the surface of neurons, including anti-GM1 and anti-GQ1b cause neurotoxicity through complement-mediated calcium influx resulting in paralysis (12). Furthermore, the neuronal protein neurofascin is also demonstrated as a target for autoimmunity in MS (69, 70). Neurofascin has two

isoforms (155 kDa or 186 kDa) and is located at the nodes of Ranvier and functions in cell adhesion (69). Co-transfer of anti-neurofascin specific monoclonal antibodies and MOG-specific T cells results in complement-dependent, although reversible, axonal damage in rats. The authors show that anti-neurofascin antibodies interfere with electrophysiological properties of hippocampal neurons *in vitro* (70).
In the above mentioned examples the target antigen is located extracellular, but antibody-mediated axonal damage to intracellular located antigens is also reported. For example, in stiff person syndrome, passive transfer of antibodies to amphiphysin, an intracellular protein involved in vesicle release, is pathogenic in rats (71, 72). Geis and colleagues also demonstrate epitope-specific uptake of anti-amphiphysin antibodies by neurons and disturb synaptic activity (71). Whether antibodies to NF-L have an impact on neuronal communication and electrophysiology could be relevant for future studies by using e.g. patch clamping (Box 2). Other mechanisms of antibody-mediated axonal damage include disruption of axonal transport (73, 74) and alterations in the function of the target antigen (75). Antibodies to enolase, an enzyme involved in glycolysis, inhibit enolase function resulting in ATP depletion and apoptosis of retinal cells (75, 76). Whether antibodies to NF-L are able to disrupt axonal transport would be interesting for future research (Box 2). A previous study showed accumulation of mitochondria in axons located in the spinal cord following immunisation with NF-L in ABH mice (22). Accumulation of mitochondria is demonstrated early in the course of Wallerian degeneration (77) suggesting that internalised antibodies to NF-L might cause an increase in mitochondria interfering with neuronal function. It could be of interest to investigate mitochondrial function in spinal cord co-cultures following incubation with NF-L antibodies or investigate co-localisation of intracellular antibodies to NF-L and mitochondria accumulation in NF-L-immunised mice. The presence of antibodies to NF-L in the CNS might interfere with axon regeneration similar as has been reported for GM1-specific antibodies, one of the targets of autoimmunity in GBS patients (78, 79). On the contrary, antibody internalisation might also lead to degradation in lysosomes, which decreases the chance of a pathogenic effect of antibodies (59).
To conclude, besides the use of antibodies to NF-L as a biomarker, accumulating data supports a role for these antibodies in axonal damage.

Targeting autoimmunity to neuronal antigens; is this relevant for disease progression in MS?
The association of active white matter MS lesions with inflammatory cells suggests that in people with MS, the balance between protection and autoimmunity is lost. Nevertheless, grey matter pathology in the CNS of people with MS is accompanied by less inflammation. This suggests that pathological mechanisms independent of inflammation might be possible as well or that the grey matter is more vulnerable to pro-inflammatory cytokines **(chapter 3)**.
Treatment strategies prohibiting entry of T cells in the CNS show that MS is more complex than originally thought, namely reactivation of CNS-specific T cells and in particular myelin-reactive T cells. The complex interplay between both the innate and adaptive immune system suggests that targeting of whole cell populations has major implications for the immune response. This is in line with an increased risk of neurotropic viruses attacking the CNS, such as increased risk of progressive multifocal leukoencephalopathy following treatment with natalizumab (80).
Increased knowledge regarding B cells and antibodies has led to targeting B cells in people with MS. Rituximab, a monoclonal antibody to CD20, reduces relapse rate

and newly forming lesions in people with RRMS (81, 82). Rituximab depletes $CD20^+$ B cells which also leads to reduced levels of T lymphocytes in the CNS, although the treatment does not have an effect on OCB or CSF-IgG (83). Nevertheless, removal of humoral factors including antibodies using plasma exchange is beneficial for some people with MS, suggesting that perhaps in a (small) group of people with MS, pathogenic antibodies contribute to the disease. Unfortunately, plasma exchange provides only a temporary relief and can also worsen disability in some cases (84, 85). Depletion of B cells and antibodies might impede repair as well, since B cells and antibodies also aid in the removal of debris (86, 87) and promote repair (87, 88). A positive effect of antibodies is supported by favourable results in people with MS following administration of naturally occurring antibodies (intravenous immunoglobulin, IVIg) (89), although controversial (90).

In people with progressive MS with little inflammatory activity, anti-inflammatory agents do not seem to be effective (91) or even make the disease worse (92). Although progressive MS gains more and more attention, it is unclear why disability continues while inflammation in the CNS is less than in the CNS of people with RRMS (93). One hypothesis suggests a secondary response to inflammation present earlier in disease, for example in people with SPMS following RRMS (94).

A study by Ehling and colleagues and Silber and colleagues shows increased levels of antibodies to NF-L in progressive MS compared to people with RRMS (2, 6). This observation might be explained by the possibility that these antibodies are a remainder of an autoimmune response to NF-L in the active phase of the disease and B cell clones in the CNS produce these antibodies locally. This is in line with studies identifying B cell maturation and expansion in the CSF of people with MS (95, 96). Repair of the blood-brain barrier might contribute to this so-called compartmentalisation of antibodies (94). Another possibility includes the selection of subjects. Perhaps the selected people with progressive MS in these two studies show more inflammation than other people with progressive MS, although antibodies to other antigens, e.g. the above mentioned gangliosides, have also been shown to be increased in progressive MS (97).

To conclude, currently we know little about ongoing progression of neurological deficits in people with MS. Therapies targeting the inflammatory response are effective in diminishing neuroinflammation, but have no effect on continuation of axonal damage. Possibly a more specific targeting of the pathological mechanisms, such as antibodies to NF-L, in combination with neuroprotective agents and promoting repair mechanisms (94, 98) may be more effective. In addition, early treatment is a must to decrease clinical disability in later stages (98), which also urges the need for reliable biomarkers. Also the identification and targeting of pathogenic components in people with MS will be a challenge for future research.

Conclusions and future perspectives

We hypothesise that autoimmunity to NF-L contributes to axonal damage in MS (Fig. 2). Our research has implicated that in MS, autoimmunity to NF-L indeed might contribute to axonal damage. This conclusion is supported by our main findings as summarised in Box 1. We show that in the CNS of people with MS $HLA\text{-}DR^+$ cells phagocytose neuronal antigens. This could lead to antigen presentation by professional antigen presenting cells that could serve as a key event in development of autoimmunity to NF-L. Furthermore, using an experimental mouse model for inflammatory-mediated neurodegeneration, we show that T cells secreted cytotoxic molecules that harm neurons and that immunodominant epitopes in the NF-L

sequence also induced experimental disease in mice. Antibodies to NF-L may be added to the growing panel of biomarkers to reflect treatment responses in MS, but are also shown to be pathogenic *in vitro* and *in vivo*. Therefore, antibodies to NF-L should not be ruled out as part of the disease mechanism in (a subgroup of) people with MS.

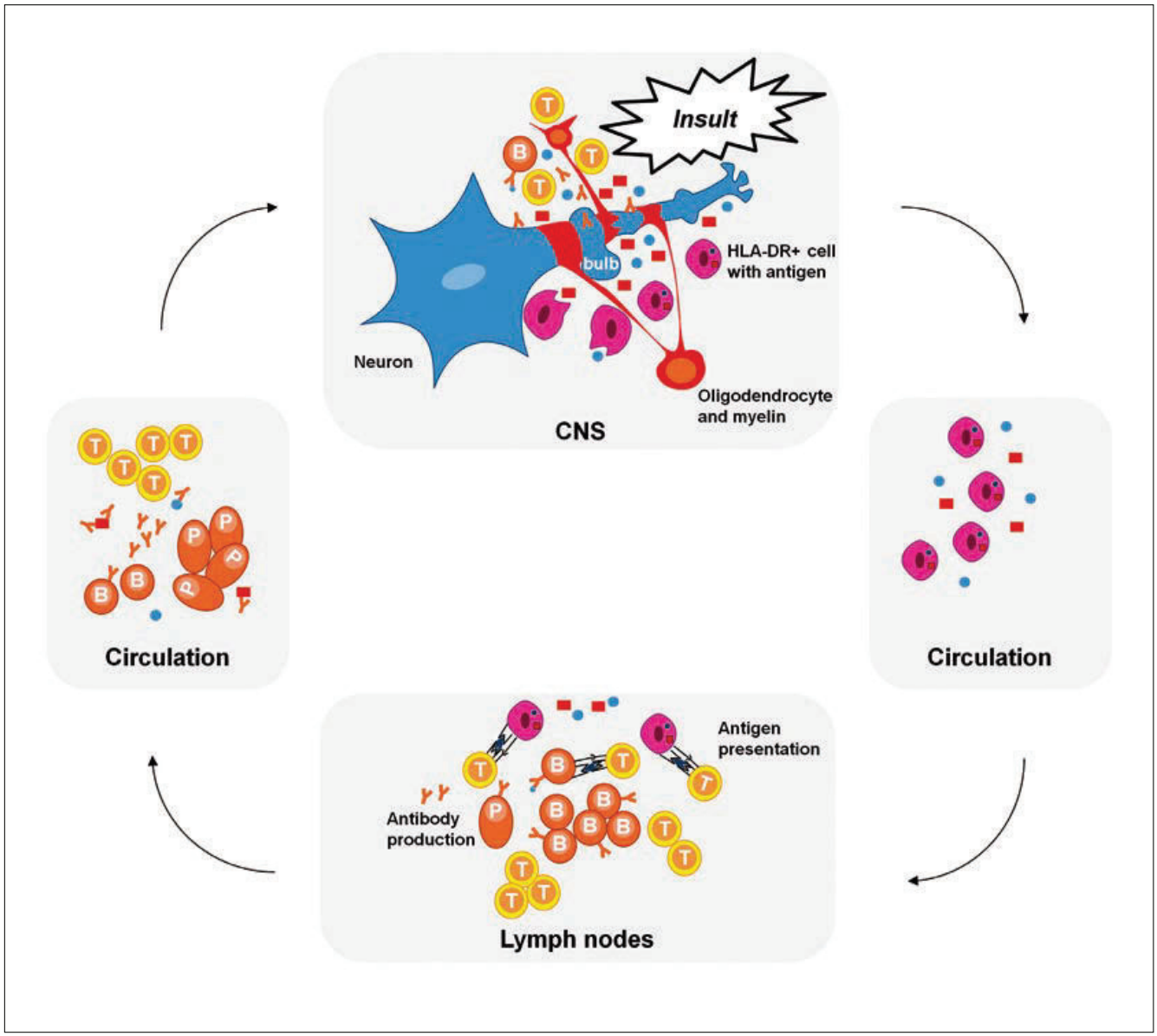

Figure 2. Hypothesis of autoimmunity to NF-L in inflammatory neurodegenerative diseases. *Axonal and myelin damage in the CNS caused by an unknown insult (or multiple insults). Neuronal debris (blue, rounds) and myelin debris (red, squares) are cleared by HLA-DR cells which enter the circulation and reach the lymph nodes. Antigen presentation and (re)activation of T cells takes place followed by B cell activation and maturation in plasma cells and antibody production. Activated cells and antibodies enter the circulation and reach the CNS where T cells might contribute to neuronal damage through secretion of inflammatory molecules and antibodies that interfere with (intra)cellular processes leading e.g. to the formation of axonal bulbs. B, B lymphocyte; T, T lymphocyte; P, plasma cell.*

Box 1 Conclusions of this thesis

- HLA-DR$^+$ cells phagocytose neuronal antigens including NF-L in MS lesions and there is a higher frequency of HLA-DR$^+$ cells containing NF-L in the CSF of people with MS **(Chapter 2)**.
- Functional differences between HLA-DR$^+$ cells in the grey and white matter might be responsible for the extent of axonal damage observed in the grey matter part of leukocortical MS lesions, despite the lower degree of inflammation compared with the white matter **(Chapter 3)**.
- Immunodominant epitopes in the NF-L sequence can induce experimental disease as well **(Chapter 4)**.
- Antibodies to NF-L are increased in the serum of people with MS compared to healthy controls and are decreased following natalizumab treatment **(Chapter 5)**.
- Monoclonal antibodies to NF-L cause axonopathy *in vitro* and *in vivo* **(Chapter 6)**.

Further research regarding autoimmunity to NF-L in MS should focus on the pathogenicity *in vitro* and *in vivo* of NF-L-specific antibodies purified from the serum and CSF of people with MS and other inflammatory neurodegenerative disorders. For example, the myelinating spinal cord co-cultures could be used as an *in vitro* model as we show in chapter 6 and by Elliott and colleagues (55). This model could be used to examine the impact of NF-L-specific antibodies on axonal development and regeneration as well as to investigate whether antibodies to NF-L interfere with NF-L assembly and function in axons.

A limitation of our studies is the unknown binding affinity of NF-L-specific antibodies to the three neurofilament subunits as well as whether steric hindrance of the other neurofilament subunits influence the binding capacity of neurofilament antibodies. These questions should be addressed in the future.

To conclude, the results in this thesis add to the growing knowledge about the pathological impact of neuronal-reactive antibodies and the mechanisms that may operate in progressive neurological deficits in MS.

Box 2 Outstanding questions

- Do NF-L-containing HLA-DR$^+$ cells in MS tissue express co-stimulatory molecules? And can NF-L containing HLA-DR$^+$ cells activate T cells or do they induce T cell tolerance?
- Which NF-L peptide epitopes are recognised by NF-L-reactive T cells from people with MS?
- Is exacerbation of EAE by purified hIgG with high antibody levels to NF-L from the sera of people with MS due to pathogenic NF-L antibodies? Is there still exacerbation of EAE when antibodies to NF-L are absorbed out from the hIgG?
- Do people with MS with high antibodies to neuronal proteins including NF-L also have high levels of antibodies to other proteins such as myelin proteins?
- Can antibodies to NF-L interfere with remyelination and/or neuronal communication?
- What is the mechanism by which antibodies enter neurons? Are antibodies still taken up by Fc receptor-deficient neurons or if F(ab')$_2$ fragments are used?
- Once internalised, do antibodies to NF-L interfere with neurofilament assembly and axonal transport?

References

1. Bjartmar, C., Kidd, G., Mork, S., Rudick, R., and Trapp, B.D. 2000. Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. *Ann Neurol* 48:893-901.
2. Ehling, R., Lutterotti, A., Wanschitz, J., Khalil, M., et al. 2004. Increased frequencies of serum antibodies to neurofilament light in patients with primary chronic progressive multiple sclerosis. *Mult Scler* 10:601-606.
3. Eikelenboom, M.J., Petzold, A., Lazeron, R.H., Silber, E., et al. 2003. Multiple sclerosis: Neurofilament light chain antibodies are correlated to cerebral atrophy. *Neurology* 60:219-223.
4. Fialova, L., Bartos, A., Svarcova, J., Zimova, D., et al. 2013. Serum and cerebrospinal fluid light neurofilaments and antibodies against them in clinically isolated syndrome and multiple sclerosis. *J Neuroimmunol* 262:113-120.
5. Newcombe, J., Gahan, S., and Cuzner, M.L. 1985. Serum antibodies against central nervous system proteins in human demyelinating disease. *Clin Exp Immunol* 59:383-390.
6. Silber, E., Semra, Y.K., Gregson, N A., and Sharief, M.K. 2002. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* 58:1372-1381.
7. Fujinami, R.S., and Oldstone, M.B. 1989. Molecular mimicry as a mechanism for virus-induced autoimmunity. *Immunol Res* 8:3-15.
8. Carrizosa, A.M., Nicholson, L.B., Farzan, M., Southwood, S., et al. 1998. Expansion by self antigen is necessary for the induction of experimental autoimmune encephalomyelitis by T cells primed with a cross-reactive environmental antigen. *J Immunol* 161:3307-3314.
9. Greene, M.T., Ercolini, A.M., DeGutes, M., and Miller, S.D. 2008. Differential induction of experimental autoimmune encephalomyelitis by myelin basic protein molecular mimics in mice humanized for HLA-DR2 and an MBP(85-99)-specific T cell receptor. *J Autoimmun* 31:399-407.
10. Mokhtarian, F., Zhang, Z., Shi, Y., Gonzales, E., and Sobel, R.A. 1999. Molecular mimicry between a viral peptide and a myelin oligodendrocyte glycoprotein peptide induces autoimmune demyelinating disease in mice. *J Neuroimmunol* 95:43-54.
11. Brocke, S., Gaur, A., Piercy, C., Gautam, A., et al. 1993. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature* 365:642-644.
12. Willison, H.J. 2005. The immunobiology of Guillain-Barre syndromes. *J Peripher Nerv Syst* 10:94-112.
13. Huizinga, R., Hintzen, R.Q., Assink, K., van Meurs, M., and Amor, S. 2009. T-cell responses to neurofilament light protein are part of the normal immune repertoire. *Int Immunol* 21:433-441.
14. Pfleger, C.C., Koch-Henriksen, N., Stenager, E., Flachs, E.M., and Johansen, C. 2009. Head injury is not a risk factor for multiple sclerosis: a prospective cohort study. *Mult Scler* 15:294-298.
15. Mutlu, L., Brandt, C., Kwidzinski, E., Sawitzki, B., et al. 2007. Tolerogenic effect of fiber tract injury: reduced EAE severity following entorhinal cortex lesion. *Exp Brain Res* 178:542-553.
16. Schutzer, S.E., Angel, T.E., Liu, T., Schepmoes, A.A., et al. 2013. Gray matter is targeted in first-attack multiple sclerosis. *PLoS One* 8:e66117.
17. Van Zwam, M., Huizinga, R., Melief, M.J., Wierenga-Wolf, A.F., et al. 2008. Brain antigens in functionally distinct antigen-presenting cell populations in cervical

lymph nodes in MS and distinct EAE models. *Submitted for publication.*
18. Fissolo, N., Haag, S., de Graaf, K.L., Drews, O., et al. 2009. Naturally presented peptides on major histocompatibility complex I and II molecules eluted from central nervous system of multiple sclerosis patients. *Mol Cell Proteomics* 8:2090-2101.
19. Babbe, H., Roers, A., Waisman, A., Lassmann, H., et al. 2000. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 192:393-404.
20. Miller, S.D., Karpus, W.J., and Davidson, T.S. 2010. Experimental autoimmune encephalomyelitis in the mouse. *Curr Protoc Immunol* Chapter 15:Unit 15 11.
21. Huizinga, R., Gerritsen, W., Heijmans, N., and Amor, S. 2008. Axonal loss and gray matter pathology as a direct result of autoimmunity to neurofilaments. *Neurobiol Dis* 32:461-470.
22. Huizinga, R., Heijmans, N., Schubert, P., Gschmeissner, S., et al. 2007. Immunization with neurofilament light protein induces spastic paresis and axonal degeneration in Biozzi ABH mice. *J Neuropathol Exp Neurol* 66:295-304.
23. Hombach, A., Kohler, H., Rappl, G., and Abken, H. 2006. Human CD4+ T cells lyse target cells via granzyme/perforin upon circumvention of MHC class II restriction by an antibody-like immunoreceptor. *J Immunol* 177:5668-5675.
24. Williams, N.S., and Engelhard, V.H. 1996. Identification of a population of CD4+ CTL that utilizes a perforin- rather than a Fas ligand-dependent cytotoxic mechanism. *J Immunol* 156:153-159.
25. Giovanni, F., Domenico, P., Alessandro, M., Raffaele, I., et al. 2011. Circulating CD8+CD56-perforin+ T cells are increased in multiple sclerosis patients. *J Neuroimmunol* 240-241:137-141.
26. Tejera-Alhambra, M., Alonso, B., Teijeiro, R., Ramos-Medina, R., et al. 2012. Perforin Expression by CD4+ Regulatory T Cells Increases at Multiple Sclerosis Relapse: Sex Differences. *Int J Mol Sci* 13:6698-6710.
27. Deb, C., Lafrance-Corey, R.G., Schmalstieg, W.F., Sauer, B.M., et al. 2010. CD8+ T cells cause disability and axon loss in a mouse model of multiple sclerosis. *PLoS One* 5:e12478.
28. Deb, C., Lafrance-Corey, R.G., Zoecklein, L., Papke, L., et al. 2009. Demyelinated axons and motor function are protected by genetic deletion of perforin in a mouse model of multiple sclerosis. *J Neuropathol Exp Neurol* 68:1037-1048.
29. Kroner, A., Ip, C.W., Thalhammer, J., Nave, K.A., and Martini, R. 2010. Ectopic T-cell specificity and absence of perforin and granzyme B alleviate neural damage in oligodendrocyte mutant mice. *Am J Pathol* 176:549-555.
30. Miller, N.M., Shriver, L.P., Bodiga, V.L., Ray, A., et al. 2013. Lymphocytes with cytotoxic activity induce rapid microtubule axonal destabilization independently and before signs of neuronal death. *ASN Neuro* 5:e00105.
31. Howe, C.L., Adelson, J.D., and Rodriguez, M. 2007. Absence of perforin expression confers axonal protection despite demyelination. *Neurobiol Dis* 25:354-359.
32. Murray, P.D., McGavern, D.B., Lin, X., Njenga, M.K., et al. 1998. Perforin-dependent neurologic injury in a viral model of multiple sclerosis. *J Neurosci* 18:7306-7314.
33. Trapani, J.A., and Smyth, M.J. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2:735-747.
34. Haile, Y., Pasychniyk, D., Turner, D., Bleackley, R.C., and Giuliani, F. 2011. CD4+CD25+CD127dimFoxp3+ T cells are cytotoxic for human neurons. *J Leukoc Biol* 89:927-934.
35. Haile, Y., Simmen, K.C., Pasichnyk, D., Touret, N., et al. 2011. Granule-derived

granzyme B mediates the vulnerability of human neurons to T cell-induced neurotoxicity. *J Immunol* 187:4861-4872.

36. Sauer, B.M., Schmalstieg, W.F., and Howe, C.L. 2013. Axons are injured by antigen-specific CD8(+) T cells through a MHC class I- and granzyme B-dependent mechanism. *Neurobiol Dis* 59:194-205.
37. Chaitanya, G.V., Kolli, M., and Babu, P.P. 2009. Granzyme-b mediated cell death in the spinal cord-injured rat model. *Neuropathology* 29:270-279.
38. Malmestrom, C., Lycke, J., Haghighi, S., Andersen, O., et al. 2008. Relapses in multiple sclerosis are associated with increased CD8+ T-cell mediated cytotoxicity in CSF. *J Neuroimmunol* 196:159-165.
39. Hoftberger, R., Aboul-Enein, F., Brueck, W., Lucchinetti, C., et al. 2004. Expression of major histocompatibility complex class I molecules on the different cell types in multiple sclerosis lesions. *Brain Pathol* 14:43-50.
40. Neumann, H., Cavalie, A., Jenne, D.E., and Wekerle, H. 1995. Induction of MHC class I genes in neurons. *Science* 269:549-552.
41. Aktas, O., Smorodchenko, A., Brocke, S., Infante-Duarte, C., et al. 2005. Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL. *Neuron* 46:421-432.
42. Giuliani, F., Goodyer, C.G., Antel, J.P., and Yong, V.W. 2003. Vulnerability of human neurons to T cell-mediated cytotoxicity. *J Immunol* 171:368-379.
43. Vogt, J., Paul, F., Aktas, O., Muller-Wielsch, K., et al. 2009. Lower motor neuron loss in multiple sclerosis and experimental autoimmune encephalomyelitis. *Ann Neurol* 66:310-322.
44. Dobson, R., Ramagopalan, S., Davis, A., and Giovannoni, G. 2013. Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *J Neurol Neurosurg Psychiatry* 84:909-914.
45. Tintore, M., Rovira, A., Brieva, L., Grive, E., et al. 2001. Isolated demyelinating syndromes: comparison of CSF oligoclonal bands and different MR imaging criteria to predict conversion to CDMS. *Mult Scler* 7:359-363.
46. Bartos, A., Fialova, L., Soukupova, J., Kukal, J., et al. 2007. Antibodies against light neurofilaments in multiple sclerosis patients. *Acta Neurol Scand* 116:100-107.
47. Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., et al. 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 69:292-302.
48. Petzold, A. 2013. Intrathecal oligoclonal IgG synthesis in multiple sclerosis. *J Neuroimmunol* 262:1-10.
49. Sadaba, M.C., Tzartos, J., Paino, C., Garcia-Villanueva, M., et al. 2012. Axonal and oligodendrocyte-localized IgM and IgG deposits in MS lesions. *J Neuroimmunol* 247:86-94.
50. Petzold, A. 2005. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. *J Neurol Sci* 233:183-198.
51. Kuhle, J., Malmestrom, C., Axelsson, M., Plattner, K., et al. 2013. Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis. *Acta Neurol Scand* 128:e33-36.
52. Kuhle, J., Plattner, K., Bestwick, J.P., Lindberg, R.L., et al. 2013. A comparative study of CSF neurofilament light and heavy chain protein in MS. *Mult Scler* 19:1597-1603.
53. Gunnarsson, M., Malmestrom, C., Axelsson, M., Sundstrom, P., et al. 2011. Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. *Ann*

Neurol 69:83-89.
54. Axelsson, M., Malmestrom, C., Gunnarsson, M., Zetterberg, H., et al. 2014. Immunosuppressive therapy reduces axonal damage in progressive multiple sclerosis. *Mult Scler* 20:43-50.
55. Elliott, C., Lindner, M., Arthur, A., Brennan, K., et al. 2012. Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis. *Brain* 135:1819-1833.
56. Greenlee, J.E., Clawson, S.A., Hill, K.E., Wood, B.L., et al. 2010. Purkinje cell death after uptake of anti-Yo antibodies in cerebellar slice cultures. *J Neuropathol Exp Neurol* 69:997-1007.
57. Hill, K.E., Clawson, S.A., Rose, J.W., Carlson, N.G., and Greenlee, J.E. 2009. Cerebellar Purkinje cells incorporate immunoglobulins and immunotoxins in vitro: implications for human neurological disease and immunotherapeutics. *J Neuroinflammation* 6:31.
58. Congdon, E.E., Gu, J., Sait, H.B., and Sigurdsson, E.M. 2013. Antibody uptake into neurons occurs primarily via clathrin-dependent Fcgamma receptor endocytosis and is a prerequisite for acute tau protein clearance. *J Biol Chem* 288:35452-35465.
59. Fewou, S.N., Rupp, A., Nickolay, L.E., Carrick, K., et al. 2012. Anti-ganglioside antibody internalization attenuates motor nerve terminal injury in a mouse model of acute motor axonal neuropathy. *J Clin Invest* 122:1037-1051.
60. Gu, J., Congdon, E.E., and Sigurdsson, E.M. 2013. Two novel Tau antibodies targeting the 396/404 region are primarily taken up by neurons and reduce Tau protein pathology. *J Biol Chem* 288:33081-33095.
61. Douglas, J., Gardner, L., and Levin, M. 2013. Antibodies to an Intracellular Antigen Penetrate Neuronal Cells and Cause Deleterious Effects. *J Clin Cell Immunol* 4.
62. Qu, L., Zhang, P., LaMotte, R.H., and Ma, C. 2011. Neuronal Fc-gamma receptor I mediated excitatory effects of IgG immune complex on rat dorsal root ganglion neurons. *Brain Behav Immun* 25:1399-1407.
63. Lennon, V.A., Kryzer, T.J., Pittock, S.J., Verkman, A.S., and Hinson, S.R. 2005. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med* 202:473-477.
64. Lennon, V.A., Wingerchuk, D.M., Kryzer, T.J., Pittock, S.J., et al. 2004. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet* 364:2106-2112.
65. Lucchinetti, C.F., Mandler, R.N., McGavern, D., Bruck, W., et al. 2002. A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. *Brain* 125:1450-1461.
66. Misu, T., Fujihara, K., Kakita, A., Konno, H., et al. 2007. Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain* 130:1224-1234.
67. Roemer, S.F., Parisi, J.E., Lennon, V.A., Benarroch, E.E., et al. 2007. Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. *Brain* 130:1194-1205.
68. Saadoun, S., and Papadopoulos, M.C. 2010. Aquaporin-4 in brain and spinal cord oedema. *Neuroscience* 168:1036-1046.
69. Lindner, M., Ng, J.K., Hochmeister, S., Meinl, E., and Linington, C. 2013. Neurofascin 186 specific autoantibodies induce axonal injury and exacerbate disease severity in experimental autoimmune encephalomyelitis. *Exp Neurol* 247:259-266.
70. Mathey, E.K., Derfuss, T., Storch, M.K., Williams, K.R., et al. 2007. Neurofascin as a novel target for autoantibody-mediated axonal injury. *J Exp Med* 204:2363-2372.

71. Geis, C., Weishaupt, A., Hallermann, S., Grunewald, B., et al. 2010. Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain* 133:3166-3180.
72. Sommer, C., Weishaupt, A., Brinkhoff, J., Biko, L., et al. 2005. Paraneoplastic stiff-person syndrome: passive transfer to rats by means of IgG antibodies to amphiphysin. *Lancet* 365:1406-1411.
73. Brady, S.T., Pfister, K.K., and Bloom, G.S. 1990. A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm. *Proc Natl Acad Sci U S A* 87:1061-1065.
74. Johnston, K.M., Brady, S.T., van der Kooy, D., and Connolly, J A. 1987. A unique tubulin antibody which disrupts particle movement in squid axoplasm. *Cell Motil Cytoskeleton* 7:110-115.
75. Magrys, A., Anekonda, T., Ren, G., and Adamus, G. 2007. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol* 27:181-192.
76. Adamus, G., Amundson, D., Seigel, G.M., and Machnicki, M. 1998. Anti-enolase-alpha autoantibodies in cancer-associated retinopathy: epitope mapping and cytotoxicity on retinal cells. *J Autoimmun* 11:671-677.
77. Webster, H.D. 1962. Transient, focal accumulation of axonal mitochondria during the early stages of wallerian degeneration. *J Cell Biol* 12:361-383.
78. Lehmann, H.C., Kohne, A., Meyer zu Horste, G., and Kieseier, B.C. 2007. Incidence of Guillain-Barre syndrome in Germany. *J Peripher Nerv Syst* 12:285.
79. Lopez, P.H., Zhang, G., Zhang, J., Lehmann, H.C., et al. 2010. Passive transfer of IgG anti-GM1 antibodies impairs peripheral nerve repair. *J Neurosci* 30:9533-9541.
80. Bloomgren, G., Richman, S., Hotermans, C., Subramanyam, M., et al. 2012. Risk of natalizumab-associated progressive multifocal leukoencephalopathy. *N Engl J Med* 366:1870-1880.
81. Bar-Or, A., Calabresi, P.A., Arnold, D., Markowitz, C., et al. 2008. Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. *Ann Neurol* 63:395-400.
82. Hauser, S.L., Waubant, E., Arnold, D.L., Vollmer, T., et al. 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 358:676-688.
83. Piccio, L., Naismith, R.T., Trinkaus, K., Klein, R.S., et al. 2010. Changes in B- and T-lymphocyte and chemokine levels with rituximab treatment in multiple sclerosis. *Arch Neurol* 67:707-714.
84. Matzke, B., Metze, C., Winkelmann, A., Grossmann, A., et al. 2011. Severe relapse of multiple sclerosis during plasma exchange treatment. *Mult Scler* 17:759-762.
85. Papeix, C., Depaz, R., Tourbah, A., Stankoff, B., and Lubetzki, C. 2011. Dramatic worsening following plasma exchange in severe post-natalizumab withdrawal multiple sclerosis relapse. *Mult Scler* 17:1520-1522.
86. Kuhlmann, T., Lingfeld, G., Bitsch, A., Schuchardt, J., and Bruck, W. 2002. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* 125:2202-2212.
87. Vargas, M.E., Watanabe, J., Singh, S.J., Robinson, W.H., and Barres, B.A. 2010. Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. *Proc Natl Acad Sci U S A* 107:11993-11998.
88. Rodriguez, M., Miller, D.J., and Lennon, V.A. 1996. Immunoglobulins reactive with myelin basic protein promote CNS remyelination. *Neurology* 46:538-545.
89. Fazekas, F., Deisenhammer, F., Strasser-Fuchs, S., Nahler, G., and Mamoli, B. 1997. Randomised placebo-controlled trial of monthly intravenous immunoglobulin

therapy in relapsing-remitting multiple sclerosis. Austrian Immunoglobulin in Multiple Sclerosis Study Group. *Lancet* 349:589-593.

90. Fazekas, F., Lublin, F.D., Li, D., Freedman, M.S., et al. 2008. Intravenous immunoglobulin in relapsing-remitting multiple sclerosis: a dose-finding trial. *Neurology* 71:265-271.
91. Wolinsky, J.S., Narayana, P.A., O'Connor, P., Coyle, P.K., et al. 2007. Glatiramer acetate in primary progressive multiple sclerosis: results of a multinational, multicenter, double-blind, placebo-controlled trial. *Ann Neurol* 61:14-24.
92. Coles, A.J., Cox, A., Le Page, E., Jones, J., et al. 2006. The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy. *J Neurol* 253:98-108.
93. Pender, M.P. 2004. The pathogenesis of primary progressive multiple sclerosis: antibody-mediated attack and no repair? *J Clin Neurosci* 11:689-692.
94. Antel, J., Antel, S., Caramanos, Z., Arnold, D.L., and Kuhlmann, T. 2012. Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity? *Acta Neuropathol* 123:627-638.
95. Colombo, M., Dono, M., Gazzola, P., Roncella, S., et al. 2000. Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. *J Immunol* 164:2782-2789.
96. Qin, Y., Duquette, P., Zhang, Y., Talbot, P., et al. 1998. Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J Clin Invest* 102:1045-1050.
97. Sadatipour, B.T., Greer, J.M., and Pender, M.P. 1998. Increased circulating antiganglioside antibodies in primary and secondary progressive multiple sclerosis. *Ann Neurol* 44:980-983.
98. Compston, A., and Coles, A. 2008. Multiple sclerosis. *Lancet* 372:1502-1517.