Axonal Damage in Multiple Sclerosis

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2014

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

CHAPTER 6

Neurofilament Light as an Immune Target for Pathogenic Antibodies

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Submitted

Abstract
Antibodies directed to neuronal antigens are associated with several neurological diseases including paraneoplastic neurological disorders, epilepsy, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Antibodies directed to neurofilament light (NF-L), a neuron-specific intermediate filament located in axons, are widely considered to be surrogate biomarkers of axonal injury in ALS and MS. It is however at present unknown whether and how autoantibodies against NF-L are pathogenic and contribute to neurodegeneration. Here we show for the first time that a monoclonal antibody (mAb) directed to mouse NF-L induced axonopathy in rat spinal cord co-cultures and promoted neurodegeneration in a mouse model of optic neuritis related to MS. *In vivo* stereotactic administration of mAb to NF-L into the cerebral cortex of C57BL/6 mice revealed internalisation of the antibodies by $SMI32^+$ cells. To examine if such pathogenic antibodies are present in MS, we purified the immunoglobulin (Ig) G fraction from sera of people with MS with high levels of antibodies to NF-L. The pathogenicity of the purified IgG was revealed by their ability to augment clinical neurological experimental autoimmune encephalomyelitis (EAE) in ABH Biozzi mice. Taken together, our data reveals for the first time that mAb to NF-L contribute to experimentally-induced neurological disease, as do purified IgG from sera of people with MS with high levels of NF-L antibodies. Therefore, antibodies to NF-L may contribute to neurodegeneration in MS and other immune-mediated neurodegenerative diseases where NF-L antibodies are present.

Introduction
Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS that has a strong neurodegenerative component (1). Current therapies that target immune pathways are effective in people with MS with active inflammatory lesions, but are ineffective in people with MS with non-relapsing, progressive disease, who are generally considered to have irreversible axonal damage and neuronal loss. However, inflammation is observed in some people with primary and secondary progressive MS irrespective of the disease duration (2), indicating that immune responses may play a role in people with progressive disease.
Although the aetiology of MS is still unclear, T cells and immunoglobulins (Igs) directed to myelin and axonal proteins are detected in people with MS (3-7), although some may also be present in healthy controls (7-9). Support for a pathogenic role of the immune response in MS comes from successful immunotherapy as a treatment strategy for relapsing-remitting MS (10) and from similarities with experimental autoimmune encephalomyelitis (EAE) models of MS. In EAE, autoimmune responses to myelin proteins and peptides (11-13) as well as spinal cord homogenates (14) induce chronic-relapsing neurological disease and demyelination particularly in the presence of demyelinating antibodies (15, 16). Likewise, autoimmunity to neuronal proteins also leads to neurological disease (8, 17, 18). As such, immunisation of mice with the neuron specific cytoskeleton protein neurofilament light (NF-L) induces spasticity and limb paralysis, symptoms that are also present in people with MS. Furthermore, immunoglobulins (Igs) are detected inside axons of mice which develop neurological disease, but it is unknown what the effect of antibody internalisation in these axons is (8). The presence of oligoclonal intrathecal antibody production in people with MS has led to a search for the specificity of these antibodies and the assumption that the antibodies are involved in the disease pathogenesis. Some of these antibodies are directed to NF-L and are increased in the cerebrospinal

fluid (CSF) (19) and serum (20) in people with progressive MS. However, it has not been demonstrated yet whether antibodies to NF-L are pathogenic. Recently, it has been shown that IgG and IgM localise with neurofilaments in MS lesions (21). Furthermore, antibodies to the neuronal antigens connectin-2 and neurofascin are present in MS serum and shown to be pathogenic in experimental animal models (3, 5). Purified human IgG (hIgG) from serum of people with MS induces demyelination and axonal damage in a rat myelinating co-culture system (22). Although autoimmunity to NF-L provokes experimental disease, it is presently not known whether antibodies to NF-L contribute to MS.

The aim of this study was to determine the pathogenic effect of antibodies directed to NF-L both *in vivo* and *in vitro*. We demonstrated that IgG purified from sera of people with MS with high levels of antibodies to NF-L exacerbated active EAE in mice. Furthermore, for the first time we showed that monoclonal antibody (mAb) to NF-L induced axonopathy in rat spinal cord co-cultures and in EAE mice with optic neuritis. Taken together, our results corroborate the pathogenicity of antibodies to neuronal antigens and show that antibodies to NF-L exacerbate clinical symptoms in experimental disease. These findings indicate that these antibodies might contribute to neurodegeneration in MS.

Materials and methods

Subjects

People with relapsing-remitting MS (RRMS) were recruited at The Royal London Hospital, London, UK and La Fe Polytechnic and University Hospital in Valencia, Spain. People with secondary progressive MS (SPMS) were recruited from the National Hospital for Neurology and Neurosurgery, London, and the Royal Free Hospital, London, UK (Table 1). Patients were diagnosed according to the McDonald criteria (23).

Sera were obtained with informed consent from the donors and the procedures were approved by the local ethical committees. Ethical approval was obtained from the North London REC 2 (10/H0724/36), Hospital Universitario La Fe Committee, University College London Hospitals Committee and National Research Ethics Committee (REC:06/Q0512/16, EudraCT: 2005-005588-27). All studies were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Total IgG preparations were purified from serum samples showing high reactivity to NF-L protein in an enzyme-linked immunosorbent assay (ELISA, Table 1). Pooled samples were diluted 1:10 in phosphate buffered saline (PBS) and purified using a 1 mL HiTrap protein-G HP column (GE Healthcare) following manufacturers recommendations. Samples were concentrated using Amicon® Ultra centrifugal filters (UFC501096, Millipore, United Kingdom) and the buffer was exchanged for PBS. Concentration was measured using a NanoDrop ND-1000 Spectrophotometer. Reactivity of hIgG to NF-L was tested in mouse spinal cord homogenates and recombinant mouse NF-L by western blotting (Fig. 1).

Mice

Biozzi ABH (H-2^{dq1}) female and male mice between 8-10 weeks old were obtained from Harlan Ltd (UK) or bred at Queen Mary University of London (UK). For optical coherence tomography and retinal ganglion cell (RGC) analysis, 10 weeks old female and male transgenic C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch.Cg-Tg(Thy1-CFP)23Jrs/J mice expressing T cell receptors specific for myelin oligodendrocyte

glycoprotein (MOG) and RGC were used (24). Stereotactic studies were performed in 10 week old C57BL/6 male mice obtained from Charles River (Germany). Animals were housed in a temperature-controlled room (25°C) and given access to food and water *ad libitum* (25). All procedures were performed following institutional ethical review in accordance to the United Kingdom Animals (Scientific Procedures) Act (1986) and European Union Directive 2010/63/EU and the German Review Board for the Care of Animal Subjects of the district government (North Rhine-Westphalia, Germany) under the recommendations of the Federation of European Laboratory Animal Science Associations. Further details relating to animal husbandry and operational experimental design relating to the ARRIVE guidelines reporting have been described previously (25).

Rats

Time-mated Sprague-Dawley rats (26, 27) were bought from Charles River (Germany) and housed in a temperature-controlled room and given access to food and water *ad libitum*. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experiments of the VU University Medical Center, Amsterdam, The Netherlands (PA 13-01).

Table 1. Clinical data of multiple sclerosis patients. Sera of these patients were pooled and IgG were purified

Patient	Age (years)	Gender	Disease course	EDSS	Disease duration (years)	Level of antibodies to NF-L (optical density)	
						mean	SD
1	58	Female	SPMS	6	8	1.79	0.52
2	42	Female	SPMS	6	16	1.42	0.09
3	41	Female	SPMS	4	4	1.49	0.01
4	43	Female	SPMS	4.5	4	1.37	0.05
5	54	Male	SPMS	6.5	8	1.57	0.04
6	51	Female	SPMS	6	11	1.53	0.03
7	52	Male	SPMS	6.5	2	1.41	0.03
8	44	Female	RRMS	5.5	12	1.84	0.14
9	24	Male	RRMS	6	6	1.83	0.01
10	30	Male	RRMS	2	3	1.85	0.12
11	40	Male	RRMS	6	17	2.51	0.44
12	26	Male	RRMS	2	3	2.02	0.09
13	36	Female	RRMS	5.5	5	1.98	0.14
14	47	Male	RRMS	3	3	2.54	0.12
15	44	Female	RRMS	2	2	1.97	0.01
16	25	Male	RRMS	1	1	1.86	0.09

EDSS, expanded disability status scale; SD, standard deviation.

Induction of EAE

Biozzi ABH mice were injected subcutaneously with 200 µg MOG^{35-55} peptide emulsified with incomplete Freund's adjuvant (Difco Laboratories, UK) supplemented with 48 µg *Mycobacterium tuberculosis* and 6 µg *M. butyricum* (Difco Laboratories) on day 0 and 7 as described previously (11, 25). Mice were also intraperitoneally injected with 200 ng of pertussis toxin (PTX) from *Bordetella pertussis* (Sigma, UK) 24 h and 8 d after immunisation. From day 10 mice (n=4) were injected

intraperitoneally every other day until day 26 with 0.5 mg hIgG or 0.5 mg NF-L 10H9. The other mice were not injected with hIgG. Mice were monitored daily and scored according to a neurological scale with 0=normal, 1=limp tail, 2=impaired righting reflex, 3=hindlimb paresis and 4=hindlimb paralysis (8, 28).

Western blotting

Western blotting was used to test the reactivity of hIgG to the NF-L protein after the purification procedure and to show the specificity of the mAb to NF-L clone 10H9 (NF-L 10H9)(4) to spinal cord homogenate or brain homogenate. Serum from NF-L immunised ABH mice (28) was taken as positive control. Briefly, 0.49 mg/ mL recombinant mouse NF-L (rmNF-L) or 10 µg mouse spinal cord or brain homogenate was separated using Mini-Protean TGX Precast Gel (Bio-Rad, Germany) and transferred onto Whatmann nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% fat-free milk solution and 0.5% bovine serum albumin for 1 h. Membranes were incubated with purified hIgG (1:1,000) or NF-L 10H9 (10 µg/mL) overnight. After washing, goat anti-human IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz, USA) or goat anti-mouse IgG-horseradish peroxidase (Dako, Denmark) was added to the membranes and incubated for 1 h at room temperature. Visualisation was performed with the chromogen 3,3'-diaminobenzidine (Dako) and enhanced chemiluminescence (Amersham, UK).

Induction of optic neuritis

MOG-specific T cell receptor transgenic mice with fluorescent RGC were used to study whether mAb NF-L 10H9 or hIgG induced loss of RGC as described previously with anti-myelin antibodies (24). Optic neuritis was induced by injection with 150 ng PTX (Sigma) on day 0 and day 2 (24). Subsequently, mice were randomly divided into two groups. One group (Group A) was injected with 0.5 mg fluorescently labelled mAb NF-L 10H9-PE (phycoerythrin) or hIgG on days 11, 12 and 13 and euthanised on day 15. The other group (Group B) was injected with 0.5 mg unlabelled mAb NF-L 10H9 or hIgG on days 14, 16 and 18 and euthanised at day 20. As a negative control for NF-L 10H9 mice were injected with isotype control IgG1 (Sigma). Optical coherence tomography was used to measure changes in the retinal nerve fibre layer (RNFL) thickness (24) comparing measurements before PTX injection (day 0) and after injection of antibodies (days 15 and 20) (24). In addition, retinal flatmounts were prepared for fluorescent microscopy and RGC density was calculated as described previously (24).

Intracerebral injection of NF-L 10H9

For stereotactic studies (29), C57BL/6 male mice were fixed in a mouse adapter for stereotactic frames (World Precision Instruments). After drilling a small hole in the skull, 1.92 µg NF-L 10H9 labelled with DyLight 594 (Microscale Antibody Labeling Kit, Thermo Scientific Pierce Protein, UK) was injected into the respective regions using a 10 µl syringe equipped with a 33 gauge needle (Hamilton Company) with a volume of 6 nL/s by using a Ultra Micro Pump (UMP3, World Precision Instruments). Coordinates for injection were X (-2.2), Y (0.6) and Z (0.8 for cerebral cortex and 1.5 for corpus callosum) relative to the anterior bregma. The needle was navigated by a motor-driven stereotactic frame (World precision instruments) utilising StereoDrive software (Neurostar). The injection was performed within a standardised time frame of approx 5 min injection time and 10 min deposition rest

in the cortex before the needle continued into the corpus callosum. Subsequently, the needle was retracted after 5 min deposition rest in order to prevent the reflux of the injected substances. Injection of fluorescently-labelled mouse isotype control IgG1 at the same coordinates was used as a vehicle control. Mice were euthanised 2 h post-injection and perfused transcardially with 4% paraformaldehyde (pH 7.4). After overnight post-fixation in the same fixative at 4°C, brains were submerged in 30% sucrose solution at 4°C.

Immunofluorescence
Cryosections (6 µm) of the complete mouse brains were collected from mice stereotactically injected with labelled mAb NF-L 10H9, fixed in acetone and stained with an antibody to non-phosphorylated NF-H (mouse IgG1, clone SMI32, Millipore) overnight. After washing, sections were incubated with secondary goat anti-mouse IgG1 Alexa Fluor 488 (Invitrogen) for 1 h at room temperature. Sections were viewed on a Leica DMI6000B microscope with Leica DFC365 FX camera or a Leica DM5000B microscope with a Leica DFC360 FX camera.

Axonal density in myelinating spinal cord cultures
Myelinating co-cultures were prepared as described in detail in (26, 27). Briefly spinal cords were dissected from E16 rat pups and enzymatically dissociated. Cells were plated on a confluent monolayer of neurosphere derived astrocytes (26). After a culture period of 23-24 days, co-cultures were treated with mAb NF-L 10H9. In brief, spinal cord co-cultures were incubated with 10 µg/mL mAb NF-L 10H9 for 16 h at 37°C in the presence of 5% CO_2 in duplicate as described previously (22). Incubation with isotype control IgG1 (Sigma) served as negative control. Cultures were stained for phosphorylated axons/neurites with anti-mouse SMI310 IgG1 (1:1,500; Abcam, UK). Cells were imaged on a Leica DMR fluorescence microscope using a Leica DFC4000 camera. Briefly, 20 images were analysed per condition using ImageJ software (NIH systems, version 1.41o). The number of pixels of $SMI310^+$ neurites were measured and divided by the total number of pixels per field of view. At least three independent myelinating co-cultures were used.

Statistics
Data were analysed using GraphPad Prism (5.01) and expressed as means ± standard error of the mean (SEM) unless otherwise specified. RNFL thinning and RGC density were analysed using Student's *t*-test with Welch's correction. Statistical analysis of clinical scores was assessed using the non-parametric Mann-Whitney U test. Axonal density of rat spinal cord co-cultures were quantified with ImageJ and analysed with GraphPad Prism (5.01) using the Student's *t*-test with Welch's correction. P values of less than 0.05 were considered statistically significant.

Results
Specificity of IgG from MS serum
The specificity of mAb to NF-L clone 10H9 was verified in mouse spinal cord, brain homogenate and recombinant NF-L protein using western blot analysis (Fig. 1A). IgG from pooled sera were selected from 16 people with MS (RRMS n=9; SPMS n=7) with high antibody levels reactive to NF-L using ELISA (Table 1) in comparison to NF-L antibody levels in 24 healthy controls (0.79 ± 0.27 optical density). The reactivity was examined to rmNF-L and mouse spinal cord homogenate (Fig. 1B). The hIgG from people with MS shows a clear band in lane 1 at the molecular weight

of the linear NF-L protein (~65 kDa), confirming the presence of antibodies to NF-L in these samples. In lane 2 more bands can be discriminated, likely indicating the presence of antibodies to other antigens besides NF-L present in mouse spinal cord homogenate.

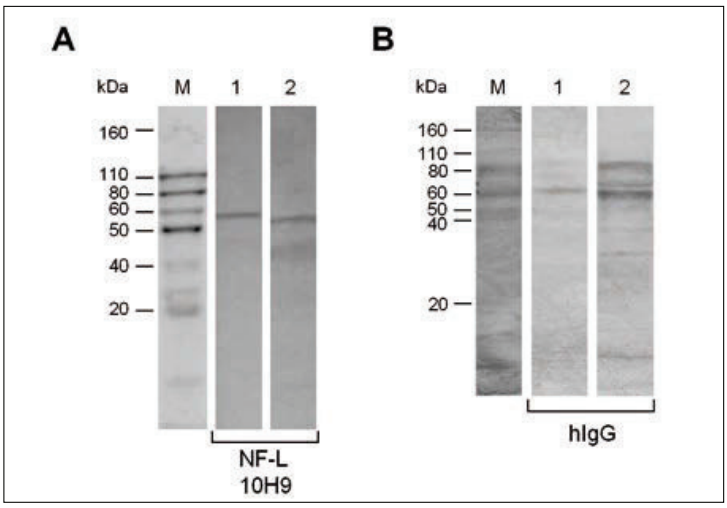

Figure. 1 Specificity of mAb to NF-L. ***A****) Western blot analysis shows specificity of mAb NF-L 10H9 to the NF-L protein (~68 kDa) in (1) spinal cord and (2) brain homogenates from ABH mice.* ***B****) Total IgG was purified from pooled sera of people with MS who had high levels to NF-L protein as confirmed by ELISA (Table 1) in comparison to NF-L antibody levels in healthy controls (0.79 ± 0.27 optical density). Reactivity of purified hIgG from people with MS to rmNF-L protein (1) and ABH spinal cord homogenate (2) was tested by western blotting and shows a clear band at ~65 kDa in lane 1, while besides a band at the molecular weight of NF-L, more bands are present in lane 2. M, protein standard.*

IgG from MS sera exacerbates MOG$^{35-55}$-induced EAE

To examine the pathogenic effect of the mAb NF-L 10H9 and IgG from MS sera, that contained high levels of antibodies to NF-L, *in vivo*, mice were immunised with MOG$^{35-55}$ (n=21) and injected intraperitoneally with NF-L 10H9 (n=6) or hIgG (n=4) every other day from day 10 post-immunisation (Fig. 2, Table 2). In MOG$^{35-55}$+ NF-L 10H9 mice clinical symptoms were statistically increased at day 12 compared to regular EAE mice (n=11; Mann-Whitney U test; $p<0.05$). However, throughout the rest of the disease course, clinical symptoms in MOG$^{35-55}$+ NF-L 10H9 mice were not statistically different in comparison to regular EAE mice. IgG from people with MS significantly exacerbated clinical neurological signs 12 to 14 days post-immunisation compared to regular EAE mice (Mann-Whitney U test; $p<0.05$). After this peak in clinical disease, no statistical differences were observed between MOG$^{35-55}$+ hIgG and regular EAE mice till the end of the experiment. Mice injected with IgG from people with MS showed a more rapid disease onset (11.8 ± 1.0 versus 14.1 ± 2.1; $p<0.05$; Table 2).

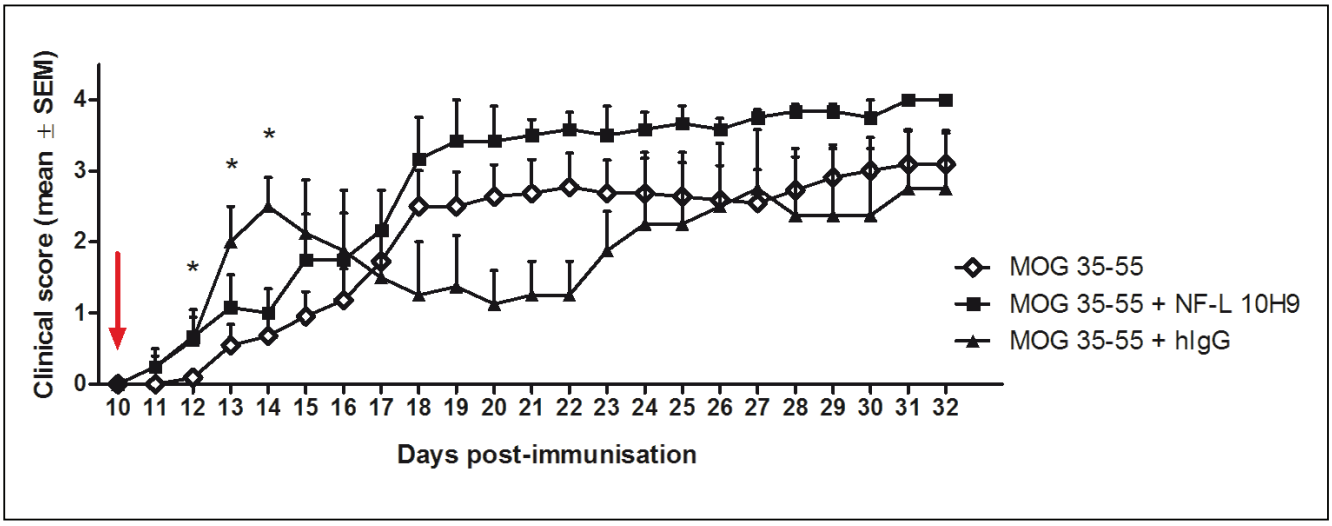

Figure. 2 Exacerbation of MOG$^{35-55}$-induced EAE by hIgG. *EAE was induced with MOG$^{35-55}$ peptide. ABH mice were repeatedly injected intraperitoneally with NF-L 10H9 (n=6) or hIgG (n=4) from day 10 post-immunisation (red arrow). Clinical scores were significantly increased in EAE mice injected with NF-L 10H9 or hIgG compared to regular EAE mice (n=11) at day 12 and days 12 till 14, respectively (Mann-Whitney U test; p<0.05). Also the day of onset was significantly earlier in mice treated with hIgG compared to non-treated EAE mice (Table 2; Mann-Whitney U test; p<0.05). See figure on the previous page.*

Table 2. Exacerbation of MOG$^{35-55}$-induced EAE in ABH by hIgG

Treatment	No. EAE	Mean group score[a]	Mean EAE score[b]	Mean day of onset[c]
MOG$^{35-55}$	9/11	3.3 ± 1.6	4.0 ± 0.0	14.1 ± 2.1
MOG$^{35-55}$ + NF-L 10H9	6/6	4.0 ± 0.0	4.0 ± 0.0	12.3 ± 1.0
MOG$^{35-55}$ + hIgG	4/4	3.6 ± 0.8	3.6 ± 0.8	11.8 ± 1.0*

[a] *mean ± SEM of maximum clinical score of EAE from all mice in the group.*
[b] *mean ± SEM of maximum clinical score from mice exhibiting EAE within a group.*
[c] *mean ± standard deviation of day of onset of clinical disease. * p<0.05, Mann-Whitney U test.*

RGC density is significantly affected by mAb to NF-L

Since the majority of people with MS develop ocular deficits, we investigated whether hIgG from sera of people with MS with high serum levels of antibodies to NF-L and the mAb NF-L 10H9 were pathogenic to RGC that can occur as a downstream consequence of damage to the optic nerve during optic neuritis (30). Delivery of NF-L 10H9 after the onset of optic neuritis did not significantly augment the development of RGC loss 15 days (group A) post-induction (Fig. 3A). However, 20 days post-induction of optic neuritis (group B) mice injected with NF-L 10H9 showed a significant reduction in the mean RGC density compared to mice injected with the isotype IgG1 control (p<0.05). On the contrary, intraperitoneal injection of hIgG with high levels of antibodies to NF-L after mice developed optic neuritis did not show a reduction in RGC density in both groups (Fig. 3A). In addition to measuring RGC loss to examine damage to the eye is thinning of the RNFL. Both NF-L 10H9 and hIgG did not seem to have a significant effect on the thickness (thickening or thinning) of the RNFL in mice with optic neuritis (Fig. 3B).

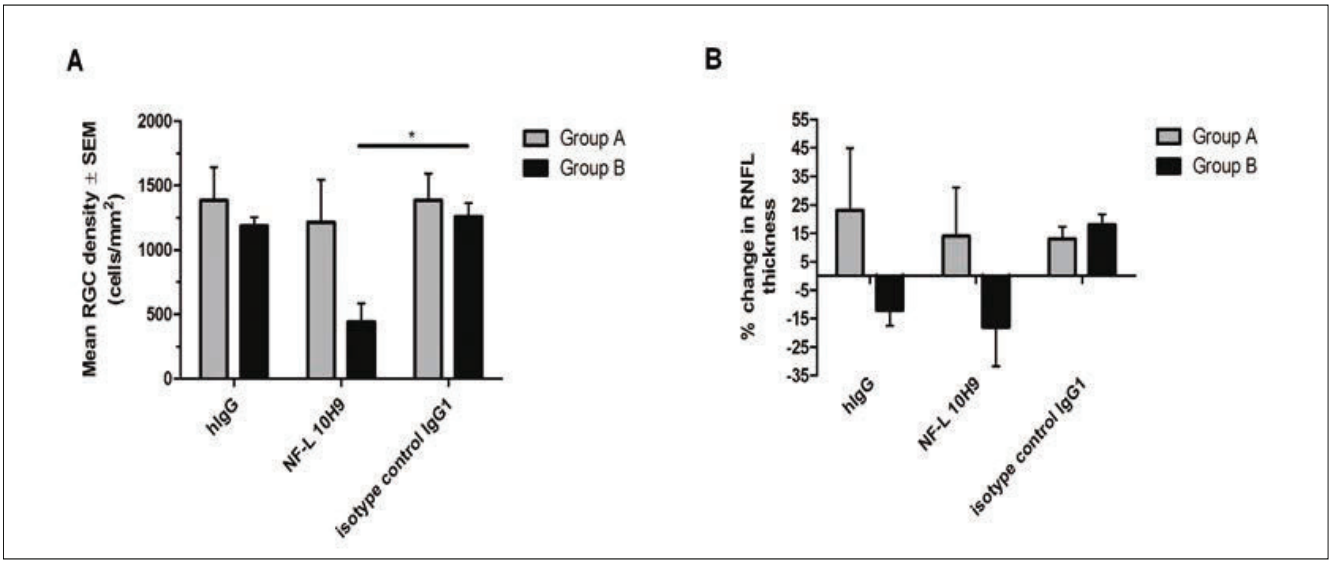

***Figure. 3 RGC density is significantly affected by NF-L 10H9. A**) RGC density (n=3 mice per group except isotype control which were 2 mice) and (**B**) RNFL thickness (n=2 mice per group) 15 days (group A, grey bars) or 20 days (group B, black bars) after treatment with hIgG, (un)labelled mAb NF-L 10H9 or isotype control IgG1. **A**) Only mice in group B treated with NF-L 10H9 showed a statistically significant reduction in RGC density compared with mice treated with isotype control IgG1 (Student's t-test with Welch's correction, $p<0.05$). **B**) No statistical differences in percentage change in RNFL thickness were observed in hIgG or NF-L 10H9 treated mice 15 or 20 days post-induction. See figure on the previous page.*

Stereotactic injection of mAb to NF-L is taken up by neurons but is not specific

Since neurofilaments are intracellular proteins, experiments were performed to investigate whether antibodies were taken up by neurons following stereotactic intracerebral injection. The mAb NF-L 10H9 was fluorescently labelled with DyLight 594 using the Microscale Antibody Labeling Kit. Subsequently, 1 adult mouse was stereotactically injected with the labelled mAb in the cortex and corpus callosum after which the brain was collected 2 h post-injection. As a control, another mouse was injected with labelled isotype control IgG1 at the same locations in the cortex and corpus callosum. Cortical neurons, between the medial longitudinal fissure and the injection channel, were examined for expression of the labelled mAb NF-L 10H9-DyLight 594 (Fig. 4C). To verify that these cells were neurons, double immunofluorescence was performed with SMI32 (Fig. 4C and D). Nevertheless, antibody uptake was not specific for NF-L 10H9, since injection of the labelled isotype IgG1 also showed uptake by cells (data not shown).

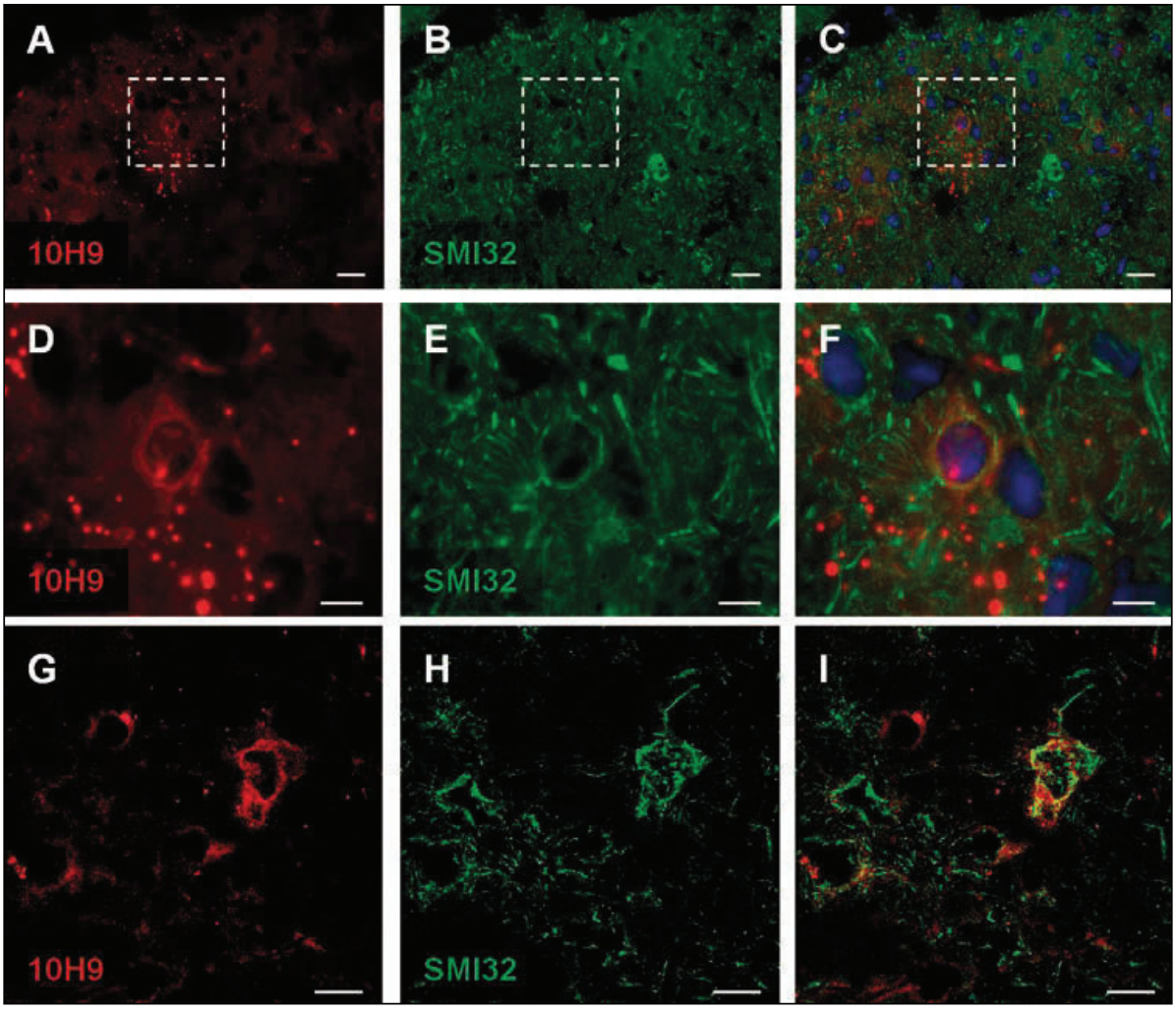

Figure. 4 Internalisation of mAb to NF-L by neurons. *mAb NF-L 10H9 was fluorescently labelled with DyLight-594. A) Neuron in the cortex next to the medial longitudinal fissure of a stereotactically injected mouse (n=1) positive for mAb NF-L 10H9-DyLight 594 (red, square). Sections were stained for SMI32 (B, green; scale bar=10 µm).* ***C****) Merge of A and B. (****D-F****) Higher magnification pictures of the dotted squares in (A-C) (scale bars=10 µm). Not all injected and labelled mAb has been taken up. Also confocal imaging shows co-localisation with NF-L 10H9-DyLight 594 (red,* ***G****) and a SMI32 neuron (green,* ***H****). The merge is shown in I (scale bar=8 µm). See figure on the previous page.*

Pathogenic effect of mAb to NF-L in spinal cord cultures

Since injection of mAb to NF-L *in vivo* led to loss of function, we determined whether the mAb NF-L 10H9 was pathogenic to neurons *in vitro*. Spinal cord co-cultures from rat were treated with mAb to NF-L (Fig. 5A) and the density of phosphorylated axons and neurites used as a measure of axonal damage (22, 27, 31). The mAb NF-L 10H9 induced axonal loss in this culture system relative to the isotype control IgG1 (n=3-4; $p<0.05$). After 1 h incubation with NF-L 10H9, axonal loss was significant ($p<0.001$; Fig. 5A). Figure 5 B and C show representative immunofluorescent images of cultures incubated with isotype control IgG1 (Fig. 5B) and NF-L 10H9 (Fig. 5C) revealing significant loss of the axonal network.

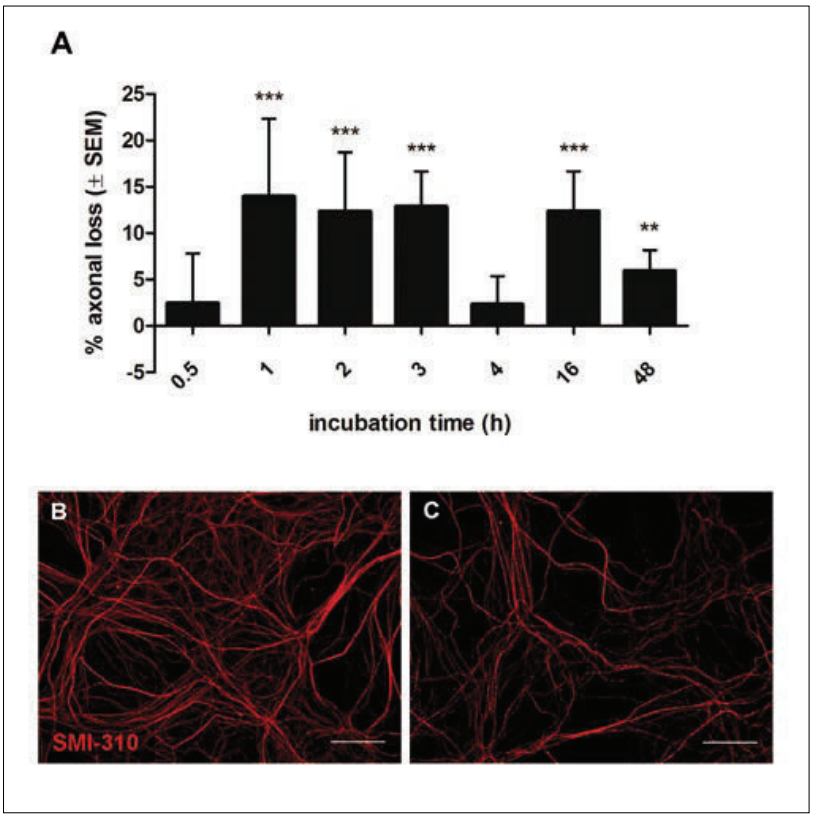

Figure. 5 mAb to NF-L reduces axonal density in rat spinal cord co-cultures. ***A****) Co-cultures were treated with NF-L 10H9 for 0.5; 1; 2; 3; 4; 16 or 48 h with 10 µg/mL mAb. Subsequently, cultures were stained for SMI310 and axonal density was determined using ImageJ and represented as percentage axonal loss relative to the isotype control IgG1. Axonal loss was significant after 1, 2, 3, 16, or 48 h of treatment with NF-L 10H9 (n=4 for 0.5 and 48 h, n=3 for 1-16 h, *** $p<0.0001$, ** $p<0.001$, * $p<0.05$, Student's t-test with Welch's correction).* ***B****) Representative immunofluorescent images of cultures treated with 10 µg/mL isotype control IgG1 or mAb NF-L 10H9 (****C****) for 16 h and stained with SMI310 (scale bars=35 µm).*

Discussion

The focus on antibodies to be usable as biomarkers of neuronal damage has been extensively reported. However, besides the possible predictive value of these antibodies, we show that antibodies to neurofilaments contribute to neuronal damage. Our data indicate that antibodies to NF-L might be involved in axonal damage in neurodegenerative diseases such as MS and other neurodegenerative diseases in which antibodies to NF-L have been reported (19, 20, 32). Thus, antibodies to neurofilaments are not specific for MS and they are clearly present in several neurodegenerative disorders indicating that therapeutic approaches targeting

humoral immunity to neurons and axons could be beneficial in many neurological disorders. In rheumatoid arthritis and MS therapeutic strategies targeting B cells reduce disease severity (33, 34) although such efficacy might not be due to a direct effect of reducing autoantibodies. However, subgroups of people with MS respond well to plasma exchanges (35-37), indicating that pathogenic antibodies contribute to disease. Therefore, new insights into axonal damage might lead to improved therapies to suppress (ongoing) neurodegeneration in MS, especially since neurodegeneration occurs early in disease (38, 39). Our results suggest that autoimmunity to neuronal antigens might be an important pathogenic mechanism inducing early neuronal damage in people with MS as well as contributing to chronic disease. The ineffectiveness of intravenous Ig and other therapies targeting B cells in progressive disease could represent the inability of these therapies to reduce (intracellular) autoantibodies to NF-L within the CNS, at this stage of disease. In support of our studies Pedotti and colleagues (16) show that IgG purified from plasma from one single MS case exacerbate PLP-induced EAE. In our study, we induced chronic EAE with MOG^{35-55} and used purified IgG from sera of 16 people with MS (9 RRMS and 7 SPMS) who were selected for high levels of NF-L antibodies compared to healthy controls. Although Pedotti and colleagues show localisation on axon bundles in the CNS grey matter of treated mice, they do not discuss whether the IgG cause any axonal damage. Nevertheless, autoimmune responses to neuronal antigens augment experimental autoimmune disease (28) and neuronal damage (3) underscoring the importance of such responses in humans.

Although people with MS experience various symptoms, visual problems commonly precede other neurological symptoms. Optical coherence tomography can be used to assess damage to the retina. Here, we used a new optic neuritis model (24) to investigate whether mAb to NF-L and hIgG have a pathogenic effect on RGC density and RNFL thickness consequent to damage of axons within the optic nerve. Similar to effects noted with injection of mAb to MOG (24), injection of NF-L 10H9 into mice with optic neuritis significantly reduced RGC density. However, contrary to the exacerbation of MOG^{35-55}-induced EAE, no loss in RGC density was observed by hIgG from people with MS with high serum levels of antibodies to NF-L. Possibly the hIgG could not penetrate the blood-brain-barrier or that effector functions of human Igs were not sufficiently compatible with mouse effector mechanisms.

Previously our group showed Igs bound to axons in NF-L immunised ABH mice (8). To examine the localisation of antibodies to NF-L in the CNS, mice were stereotacticcally injected with the fluorescently labelled mAb NF-L 10H9 or isotype control IgG1. Injection in the cortex resulted in the detection of the labelled NF-L 10H9 in $SMI32^{+}$ neurons. This result is in line with the observation that neurons generally are able to take up antibodies targeting intracellular antigens (40-44). However, the labelled isotype control IgG1 could also be observed intracellular, possibly indicating uptake through receptor-dependent, clathrin-mediated endocytosis or Fc-receptor-mediated mechanisms as reported previously (43, 45). Using mAb depleted of the Fc fragment might result in a different effect. A recent study shows that antibodies to an intracellular antigen enter neurons by endocytosis and cause depletion of adenosine triphosphate and an increase in caspase levels leading to apoptosis and cell death (46). This mechanism together with an elevation in intracellular calcium ions mediated by anti-alpha-enolase antibodies has also been described in autoimmune retinopathy (47). As well, activation of Fc-gamma receptor 1 by IgG-immune complex has been shown to cause an increase in intracellular calcium and enhance excitability in sensory neurons (48). Patch-clamping might be used to

investigate whether mAb to NF-L impact on neuronal signalling. Once internalised antibodies to NF-L might disturb axonal transport processes by binding to their target. We show that NF-L 10H9 reduced axonal density in rat spinal cord co-cultures which might be the consequence of failure of proper axonal transportation. The ability of antibodies directed to intracellular neuronal targets to disrupt axonal transport has been shown before (49, 50).

The hIgG isolated from sera of people with MS were selected for high levels of antibodies to NF-L, but to identify the contribution of antibodies to NF-L and other neuronal antigens requires further studies. In addition, further research needs to be performed to determine the mechanism by which antibodies to NF-L cause cytotoxicity. It is possible that antibody-antigen complexes of neurofilament proteins and antibodies can crosslink with Fc-receptors on immune cells which can trigger phagocytosis, antibody-dependent cell-mediated cytotoxicity and cytokine release (51) thereby contributing to progression in neuroinflammatory conditions. In addition, mAb to NF-L, as well as hIgG to NF-L, might interfere with neurofilament assembly.

In summary, our results show that antibodies to intracellular antigens contribute to axonal damage and are not just surrogate markers of disease.

Acknowledgements

We would like to thank Wouter Gerritsen for his exceptional technical skills. We would also like to thank Kamilla Nurtaeva and Adib Zendedel for excellent technical assistance with stereotactic injections.

Funding

This work was supported by Stichting MS Research, The Netherlands (grant number 07–627), the DANA foundation and by the MS Society of the United Kingdom (grant number NSCG-1F7R).

Conflict of interest

None declared.

References

1. Trapp, B.D., and Nave, K.A. 2008. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* 31:247-269.
2. Frischer, J.M., Bramow, S., Dal-Bianco, A., Lucchinetti, C.F., et al. 2009. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 132:1175-1189.
3. Derfuss, T., Parikh, K., Velhin, S., Braun, M., et al. 2009. Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc Natl Acad Sci U S A* 106:8302-8307.
4. Huizinga, R., van der Star, B.J., Kipp, M., Jong, R., et al. 2012. Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. *Glia* 60:422-431.
5. 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.
6. Thompson, E.J., Kaufmann, P., Shortman, R.C., Rudge, P., and McDonald, W.I. 1979. Oligoclonal immunoglobulins and plasma cells in spinal fluid of patients with multiple sclerosis. *Br Med J* 1:16-17.
7. Van Noort, J.M., Van Sechel, A.C., Bajramovic, J.J., El Ouagmiri, M., et al. 1995. The

small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. *Nature* 375:798-801.
8. 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.
9. Van Noort, J.M., Bsibsi, M., Gerritsen, W.H., van der Valk, P., et al. 2010. Alphab-crystallin is a target for adaptive immune responses and a trigger of innate responses in preactive multiple sclerosis lesions. *J Neuropathol Exp Neurol* 69:694-703.
10. Compston, A., and Coles, A. 2008. Multiple sclerosis. *Lancet* 372:1502-1517.
11. Amor, S., Baker, D., Groome, N., and Turk, J.L. 1993. Identification of a major encephalitogenic epitope of proteolipid protein (residues 56-70) for the induction of experimental allergic encephalomyelitis in Biozzi AB/H and nonobese diabetic mice. *J Immunol* 150:5666-5672.
12. Amor, S., Groome, N., Linington, C., Morris, M.M., et al. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol* 153:4349-4356.
13. Amor, S., O'Neill, J.K., Morris, M.M., Smith, R.M., et al. 1996. Encephalitogenic epitopes of myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein for experimental allergic encephalomyelitis induction in Biozzi ABH (H-2Ag7) mice share an amino acid motif. *J Immunol* 156:3000-3008.
14. Baker, D., O'Neill, J.K., Gschmeissner, S.E., Wilcox, C.E., et al. 1990. Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J Neuroimmunol* 28:261-270.
15. Morris-Downes, M.M., Smith, P.A., Rundle, J.L., Piddlesden, S.J., et al. 2002. Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. *J Neuroimmunol* 125:114-124.
16. Pedotti, R., Musio, S., Scabeni, S., Farina, C., et al. 2013. Exacerbation of experimental autoimmune encephalomyelitis by passive transfer of IgG antibodies from a multiple sclerosis patient responsive to immunoadsorption. *J Neuroimmunol* 262:19-26.
17. 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.
18. Krishnamoorthy, G., Saxena, A., Mars, L.T., Domingues, H.S., et al. 2009. Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis. *Nat Med* 15:626-632.
19. 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.
20. 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.
21. 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.
22. 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.
23. Polman, C.H., Reingold, S.C., Edan, G., Filippi, M., et al. 2005. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 58:840-

846.
24. Lidster, K., Jackson, S.J., Ahmed, Z., Munro, P., et al. 2013. Neuroprotection in a novel mouse model of multiple sclerosis. *PLoS One* 8:e79188.
25. Al-Izki, S., Pryce, G., O'Neill, J.K., Butter, C., et al. 2012. Practical Guide to the Mouse Induction of Relapsing Progressive Experimental Autoimmune Encephalomyelitis in the Biozzi ABH. *Multiple Sclerosis and Related Disorders* 1:29-38.
26. Sorensen, A., Moffat, K., Thomson, C., and Barnett, S.C. 2008. Astrocytes, but not olfactory ensheathing cells or Schwann cells, promote myelination of CNS axons in vitro. *Glia* 56:750-763.
27. Boomkamp, S.D., Riehle, M.O., Wood, J., Olson, M.F., and Barnett, S.C. 2012. The development of a rat in vitro model of spinal cord injury demonstrating the additive effects of Rho and ROCK inhibitors on neurite outgrowth and myelination. *Glia* 60:441-456.
28. Puentes, F., van der Star, B.J., Victor, M., Kipp, M., et al. 2013. Characterization of immune response to neurofilament light in experimental autoimmune encephalomyelitis. *J Neuroinflammation* 10:118.
29. Clarner, T., Diederichs, F., Berger, K., Denecke, B., et al. 2012. Myelin debris regulates inflammatory responses in an experimental demyelination animal model and multiple sclerosis lesions. *Glia* 60:1468-1480.
30. Lidster, K., and Baker, D. 2012. Optical coherence tomography detection of neurodegeneration in multiple sclerosis. *CNS Neurol Disord Drug Targets* 11:518-527.
31. Thomson, C.E., Hunter, A.M., Griffiths, I.R., Edgar, J.M., and McCulloch, M.C. 2006. Murine spinal cord explants: a model for evaluating axonal growth and myelination in vitro. *J Neurosci Res* 84:1703-1715.
32. Puentes, F., Topping, J., Kuhle, J., van der Star, B.J., et al. 2013. Immune reactivity to neurofilament proteins in the clinical staging of amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry*.
33. Gredler, V., Mader, S., Schanda, K., Hegen, H., et al. 2013. Clinical and immunological follow-up of B-cell depleting therapy in CNS demyelinating diseases. *J Neurol Sci* 328:77-82.
34. 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.
35. Keegan, M., Konig, F., McClelland, R., Bruck, W., et al. 2005. Relation between humoral pathological changes in multiple sclerosis and response to therapeutic plasma exchange. *Lancet* 366:579-582.
36. Weinshenker, B.G., O'Brien, P.C., Petterson, T.M., Noseworthy, J.H., et al. 1999. A randomized trial of plasma exchange in acute central nervous system inflammatory demyelinating disease. *Ann Neurol* 46:878-886.
37. Kaynar, L., Altuntas, F., Aydogdu, I., Turgut, B., et al. 2008. Therapeutic plasma exchange in patients with neurologic diseases: retrospective multicenter study. *Transfus Apher Sci* 38:109-115.
38. Dziedzic, T., Metz, I., Dallenga, T., Konig, F.B., et al. 2010. Wallerian degeneration: a major component of early axonal pathology in multiple sclerosis. *Brain Pathol* 20:976-985.
39. 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.
40. Stein, T.D., Fedynyshyn, J.P., and Kalil, R.E. 2002. Circulating autoantibodies recognize and bind dying neurons following injury to the brain. *J Neuropathol Exp*

Neurol 61:1100-1108.
41. Tampellini, D., Magrane, J., Takahashi, R.H., Li, F., et al. 2007. Internalized antibodies to the Abeta domain of APP reduce neuronal Abeta and protect against synaptic alterations. *J Biol Chem* 282:18895-18906.
42. 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.
43. 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.
44. Michalak, Z., Lebrun, A., Di Miceli, M., Rousset, M.C., et al. 2012. IgG leakage may contribute to neuronal dysfunction in drug-refractory epilepsies with blood-brain barrier disruption. *J Neuropathol Exp Neurol* 71:826-838.
45. 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.
46. 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.
47. 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.
48. 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.
49. 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.
50. 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.
51. Nimmerjahn, F., and Ravetch, J.V. 2008. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 8:34-47.