

Puentes, F. et al. (2017) Neurofilament light as an immune target for pathogenic antibodies. *Immunology*, 152(4), pp. 580-588.
(doi:[10.1111/imm.12797](https://doi.org/10.1111/imm.12797))

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

This is the peer-reviewed version of the following article: Puentes, F. et al. (2017) Neurofilament light as an immune target for pathogenic antibodies. *Immunology*, 152(4), pp. 580-588, which has been published in final form at [10.1111/imm.12797](https://doi.org/10.1111/imm.12797). This article may be used for non-commercial purposes in accordance with [Wiley Terms and Conditions for Self-Archiving](#).

<http://eprints.gla.ac.uk/144622/>

Deposited on: 08 August 2017

DR. FABIOLA PUENTES (Orcid ID : 0000-0002-3697-4726)

Article type : Original Article

Neurofilament Light as an Immune Target for Pathogenic Antibodies

Short title: NF-L antibodies contribute to axonal damage

Fabiola Puentes^{1*}, Baukje J van der Star^{2*}, Stephanie D. Boomkamp³, Markus Kipp⁴, Louis Boon⁵, Isabel Bosca^{1,6}, Joel Raffel¹, Sharmilee Gnanapavan¹, Paul van der Valk², Jodie Stephenson¹, Susan C. Barnett⁷, David Baker¹ and Sandra Amor^{1,2}

¹Neuroimmunology Unit, Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, UK

²Pathology Department, Vrije University Medical Center, Amsterdam, The Netherlands

³Regenerative Medicine Institute, School of Medicine, National University of Ireland, Galway, Ireland..

⁴Institute of Neuroanatomy, Faculty of Medicine, RWTH Aachen University, Aachen, Germany

⁵Bioceros Holdings BV, Yalelaan 46, 3584 CM, Utrecht, The Netherlands

⁶MS Unit, Neurology Department, La Fe University Hospital, Valencia, Spain

⁷Institute of Infection, Immunity & Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/imm.12797

This article is protected by copyright. All rights reserved.

Correspondence to: Dr Fabiola Puentes, Neuroimmunology Unit, Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, 4 Newark Street, London E1 2AT, UK. Email: f.puentes@qmul.ac.uk

Key words: Antibodies, neurofilament light, axonal damage, autoimmunity, neurodegeneration

*F Puentes and BJ van der Star contributed equally to this work and share first authorship

Abbreviations:

ALS amyotrophic lateral sclerosis

CNS central nervous system

CSF cerebrospinal fluid

EAE experimental autoimmune encephalomyelitis

ELISA enzyme-linked immunosorbent assay

hIgG human immunoglobulin G

mAb monoclonal antibody

MOG myelin oligodendrocyte glycoprotein

MS multiple sclerosis

NF-L neurofilament light

This article is protected by copyright. All rights reserved.

rmNF-L recombinant mouse NF-L

RGC retinal ganglion cells

RNFL retinal nerve fiber layer

pwMS people with multiple sclerosis

Summary

Antibodies to neuronal antigens are associated with many neurological diseases including paraneoplastic neurological disorders, epilepsy, amyotrophic lateral sclerosis and multiple sclerosis. Immunisation with neuronal antigens such as neurofilament light NF-L, a neuronal intermediate filament in axons, has been shown to induce neurological disease and spasticity in mice. Also, while antibodies to NF-L are widely used as surrogate biomarkers of axonal injury in amyotrophic lateral sclerosis and multiple sclerosis, it remains to be elucidated if antibodies to NF-L contribute to neurodegeneration and neurological disease. To address this, we examined the pathogenic role of antibodies directed to NF-L *in vitro* using spinal cord co-cultures and *in vivo* in experimental autoimmune encephalomyelitis (EAE) and optic neuritis animal models of multiple sclerosis. Here we show that peripheral injections of antibodies to NF-L augmented clinical signs of neurological disease in acute EAE, increased retinal ganglion cell loss in experimental optic neuritis and induced neurological signs following intracerebral injection into control mice. The pathogenicity of antibodies to NF-L was also observed in spinal cord co-cultures where axonal loss was induced. Taken together, our results reveal that as well as acting as reliable biomarkers of neuronal damage, antibodies to NF-L exacerbate neurological disease, suggesting that

antibodies to NF-L generated during disease may also be pathogenic and play a role in the progression of neurodegeneration.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that has a strong neurodegenerative component[1]. While several studies show that current therapies targeting the adaptive immune pathways are effective in people with MS (pwMS) with active inflammatory lesions, such approaches fail to show efficacy in progressive disease associated with irreversible axonal damage and neuronal loss. However, both B and T cells and immunoglobulin deposits are present in the CNS in primary and secondary progressive MS irrespective of the disease duration[2], indicating that immune responses may continue to play a role in progressive disease.

Although the aetiology of MS is still unclear, T cells and immunoglobulins (Ig) directed to myelin and axonal proteins as well as myelin associated antigens such as the heat shock protein HSPB5 are present in peripheral blood and the cerebrospinal fluid (CSF) in pwMS[3-7], though some studies also show that these are also present in healthy controls[7-9]. A clear role for immune responses in MS is evidenced by the efficacy of immunotherapy as a treatment strategy[10]. Additional evidence comes from the similarities between experimental autoimmune encephalomyelitis (EAE) and 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 that is enhanced in the presence of demyelinating antibodies[15, 16].

In the CSF of pwMS, the presence of intrathecal oligoclonal antibody production has led to a search for the specificity of these antibodies based on the concept that they are

This article is protected by copyright. All rights reserved.

involved in the pathogenesis of MS. Some of these antibodies are directed to the neuronal cytoskeleton protein neurofilament light (NF-L) and are increased in the CSF[17] and serum[18] in progressive MS. Likewise, CNS resident B cells have shown characteristics of an antigen driven response, including specificity to NF-L[19]. However, the question arises whether antibodies to CNS antigens such as NF-L are pathogenic. Previously, we have shown that mice immunized with NF-L develop neurological disease characterized by spasticity and limb paralysis associated with axonal degeneration and inflammation in the dorsal column and the grey matter[8, 20, 21]. Furthermore, in these mice immunoglobulin is observed inside axons of mice immunized with NF-L which develop neurological disease[8]. This finding is in line with the observations that neuronal intracellular antigens are targets of specific humoral immune responses[22-26]. However, in the NF-L induced spasticity it was unclear whether the antibodies directed to NF-L are themselves responsible for the pathogenicity in this model. In humans, it has recently been shown that IgG and IgM localize with neurofilaments in MS lesions[27]. Nonetheless, the contribution of antibodies to NF-L in MS remains unclear.

Here, we examined the pathogenic effect of antibodies directed to NF-L *in vitro* using spinal cord co-cultures and *in vivo* in mice. We show that a monoclonal antibody (mAb) to NF-L induced axonopathy in rat dissociated spinal cord co-cultures, promoted neurodegeneration in a mouse model of optic neuritis, exacerbated active EAE in mice and induced neurological signs after intracerebral injection in naive mice. Taken together, our results indicate a pathogenic role of antibodies to neuronal antigens and show that antibodies to NF-L exacerbate neuronal damage in experimental disease. These findings indicate that NF-L antibodies might also contribute to neurodegeneration in MS.

Materials and methods

Animals

Mice: For EAE studies 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 expressing cyan fluorescent protein (CFP) were used (about 25% of the offspring)[28]. Animals were housed in a temperature-controlled room (25°C) and given access to food and water *ad libitum*[29]. 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[29].

Rats: Time-mated Sprague-Dawley rats[30, 31] purchased from Charles River (Germany) were 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 Medical Centre, Amsterdam, The Netherlands (PA 13-01).

Human 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 (Supplementary Table 1). Sera were obtained with informed consent from the donors. All 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).

Antibodies

The mouse monoclonal antibody to NF-L used in the current study, NF-L 10H9, has been described in a previous study[4]. The affinity purified anti-human NF-L rabbit polyclonal antibody was obtained from (Abnova, Cambridge, UK). Total human IgG preparations were purified from serum of either pwMS that showed high reactivity to NF-L protein in an enzyme-linked immunosorbent assay (ELISA, Supplementary Table 1) or healthy controls. Pooled IgG fractions were diluted 1:10 in phosphate buffered saline (PBS) and purified using a 1 ml HiTrap protein-G HP column (GE Healthcare). IgG bound to the column was eluted with 0.1 M citric acid (pH 3.0) and neutralized with 1 M Tris-HCl (pH 9.1). Samples were concentrated using Amicon® Ultra centrifugal filters (UFC501096, Millipore, UK) and the buffer was exchanged for PBS. The IgG concentration was measured using a NanoDrop ND-1000 Spectrophotometer.

Western blotting

Western blotting was carried out to show the reactivity of NF-L 10H9⁴ and purified hlgG to spinal cord, brain homogenates and recombinant NF-L. Serum from NF-L immunized ABH mice³² was used as a positive control. Briefly, 0.49 mg/ml recombinant mouse NF-L (rmNF-L), or 10 µg mouse spinal cord or brain homogenate were 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 NF-L 10H9 (10 µg/ml) or hlgG (50 µg/ml) overnight. After washing, goat anti-mouse IgG-horseradish peroxidase (Dako, Denmark) was added to the membranes and incubated for 1 h at room temperature. Visualization was performed with the chromogen 3,3'-diaminobenzidine (Dako) and enhanced chemiluminescence (Amersham, UK).

Induction of EAE and administration of NF-L antibodies

ABH mice were injected subcutaneously with 200 µg MOG³⁵⁻⁵⁵ 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, 29]. Mice were also injected intraperitoneally with 200 ng of *Bordetella pertussis* toxin (Sigma, UK) immediately after the immunisation and at 24 h and 8 d after immunisation. Mice were intraperitoneally injected with 0.5 mg NF-L 10H9 or 0.5 mg hlgG from day 10 every other day until day 26. 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, 32].

This article is protected by copyright. All rights reserved.

Induction of optic neuritis

Transgenic mice were used to study whether NF-L 10H9 induced loss of RGC as described previously with anti-myelin antibodies[28]. Optic neuritis was induced in MOG-specific T cell receptor transgenic mice with fluorescent RGC by injection with 150 ng *B. pertussis* toxin (Sigma) on day 0 and day 2[28]. Subsequently, mice were randomly divided into two groups. One group (Group A) was injected with 0.5 mg fluorescently labeled NF-L 10H9-PE (phycoerythrin) on days 11, 12 and 13 and euthanized on day 15. The other group (Group B) was injected with 0.5 mg mAb unlabeled NF-L 10H9 on days 14, 16 and 18 and euthanized at day 20. As a negative control mice were injected with isotype control IgG1 (Sigma). Optical coherence tomography was used to measure changes in the retinal nerve fiber layer (RNFL) thickness comparing measurements before *B. pertussis* toxin injection (day 0) and after injection of antibodies to NF-L (days 15 and 20)[28]. Retinal flat-mounts were prepared for fluorescent microscopy and RGC density was calculated as described previously[28].

Intracerebral administration of NF-L antibodies

Intracerebral injection of NF-L 10H9, mAb MOG Z12[33] and hIgG was performed in female ABH mice anesthetized by inhalation with isoflurane. Briefly, 30 µg of NF-L 10H9, mAb MOG Z12 and 180 µg hIgG from pwMS or healthy controls was administered intracerebrally to the forebrain of mice[34].

PC12 cell viability

Undifferentiated PC12 cells were cultured in DMEM (Invitrogen, UK) supplemented with 10% FCS (Invitrogen) and 10% horse serum (Sigma, UK), 100 µg/ml streptomycin, 100 U/ml penicillin (Invitrogen) and incubated at 37°C with 5% CO₂ humidified atmosphere. The cells were differentiated by plating at a density of 1×10^5 in 96 well plates (Nunc, Thermofisher, UK) in DMEM medium with 0.1% horse serum supplemented with NGF (50 ng/ml). After 48 h, the differentiated PC12 cells were treated for 24 h, 48 h or 72 h with 20 µg/ml of either rabbit IgG directed to human NF-L (Abbexa), rabbit IgG as control (Sigma) or medium only. The cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For analysis the treated cells were incubated with MTT (0.5 mg/ml) during the final 4 h of incubation, the supernatants were discarded and 200 µL dimethyl sulfoxide was added to solubilise the formazan crystals. The absorbance was determined at 590 nm using an ELISA reader (ELISA, Synergy HT microplate reader). The percentage of cell viability was calculated as the absorbance of experimental group/ absorbance of control (media only).

Axonal density in myelinating spinal cord cultures

Myelinating co-cultures were prepared as described previously[30, 31]. Briefly, spinal cords were dissected from E15 rat pups and enzymatically dissociated. Cells were plated on a confluent monolayer of neurosphere derived astrocytes[30] and allowed to develop to form a co-culture containing axons, glia and the various cell types that make up the spinal cord for their detailed study. After 23-24 days, the co-cultures were treated with the monoclonal antibody to NF-L. Spinal cord co-cultures were incubated with 10 µg/ml NF-L 10H9 or the isotype control (mouse IgG1, Sigma) for 0.5 h to 48 h

Accepted Article

at 37°C in the presence of 5% CO₂ in duplicate as described previously[35]. Cultures were stained for phosphorylated axons/neurites with unlabeled anti-mouse SMI310 IgG1 and goat-anti mouse- Alexa Fluor-594 F(ab')₂ fragment was used as secondary antibody (1:1500; Abcam, UK). Cells were imaged on a Leica DMR fluorescence microscope using a Leica DFC4000 camera. Briefly, 20 images were analyzed per condition using ImageJ software (NIH systems, version 1.41o). For each time point, three independent myelinating co-cultures were used and per condition the images were analyzed (10 per coverslip, 2 coverslips per condition). The number of pixels of SMI310⁺ neurites were measured and divided by the total number of pixels per field of view, resulting in the percentage of axonal density. Subsequently, for each time point, the percentage of axonal density from NF-L 10H9 treated co-cultures was calculated in relation to the isotype control IgG1 treated co-cultures, resulting in the percentage of axonal loss.

Statistics

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

Results

Specificity of mAb to NF-L

The specificity of NF-L clone 10H9 was verified in western blot analysis using mouse spinal cord, brain homogenate and recombinant NF-L protein (Fig. 1). Similarly, hlgG purified from selected pwMS with high levels of NF-L antibodies, showed reactivity mainly to a protein at the molecular weight of the linear NF-L protein (68 kDa) (Supplementary Figure 1).

Antibodies to NF-L exacerbates MOG³⁵⁻⁵⁵ induced EAE

To examine the *in vivo* pathogenic effect of NF-L 10H9 antibody, mice were immunized with MOG³⁵⁻⁵⁵ (n=11) and injected i.p. with NF-L 10H9 (n=6) every other day from 10 d post-immunisation. The cumulative EAE score in mice immunized with MOG³⁵⁻⁵⁵ and injected with NF-L 10H9 was significantly increased compared to control EAE mice (p=0.01; Table 2). Likewise, injection of serum IgG from pwMS results in an early onset of clinical signs of disease compared to untreated mice (11.8 ± 1.0 days versus 14.1 ± 2.1 days; *p<0.05) (Supplementary Figure 2).

RGC density is significantly affected by antibodies to NF-L

Since the majority of people with MS develop ocular deficits, we investigated whether the NF-L 10H9 antibodies were pathogenic to RGCs. Damage to the RGC can occur as a downstream consequence of damage to the optic nerve during optic neuritis[36]. Delivery of fluorescently labeled NF-L 10H9-PE after the onset of optic neuritis caused a significant reduction in the mean RGC density at 20 days post-induction (group B),

compared to mice injected with the isotype IgG1 control ($p < 0.05$). This effect was not apparent at 15 days (Fig. 2A, group A). NF-L 10H9 had no significant effect on the thickness of the RNFL in mice with optic neuritis (Fig. 2B).

Intracerebral injection of antibodies to NF-L causes transient neurological signs

To further investigate the pathogenicity of antibodies to NF-L, NF-L 10H9 was injected intracerebrally into naive ABH mice. Significant neurological symptoms were apparent after recovery from the anaesthetic (Supplementary Figure 3A and B). This effect was also observed following injection of hIgG from pwMS (Supplementary Figure 3C). The neurological signs included stiffness of hind limbs, tail stiffness, paresis of the hind limbs and subsequent movement abnormalities. In contrast, mice injected with mouse serum, mAb MOG Z12 or saline solution did not develop signs of disease.

Antibodies to NF-L decrease neuronal viability

The pathogenic effect of antibodies to NF-L was examined using the PC12 cell line. No effect of the control rabbit Ig was observed over the study. In contrast a neurotoxic effect of NF-L antibody was observed within 24 h that decreased the cell viability compared to control rabbit IgG ($p < 0.01$) and media only ($p < 0.001$) (Fig. 3). This was mirrored at 48 and 72h. By contrast, the control IgG antibody had no significant effect on cell viability.

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

To further examine the *in vivo* effect of NF-L antibodies the antibody 10H9 was applied to rat spinal cord co-cultures and the density of phosphorylated axons and neurites used as a measure of axonal damage as described previously[31, 35, 37]. The monoclonal NF-L 10H9 induced axonal loss in the culture system relative to the isotype control IgG1 (Fig. 4A); $n = 3-4$; $p < 0.05$). After 1 h incubation with NF-L 10H9, axonal loss was significantly increased compared to isotype control treated cultures ($p < 0.001$; Fig. 4A). Representative immunofluorescent images of cultures incubated with isotype control IgG1 (Fig. 4B) and NF-L 10H9 (Fig. 4C) reveal significant loss of the axonal network.

Discussion

Antibodies to NF-L have been previously evaluated as surrogate biomarkers of neuronal damage. We show, for the first time, that antibodies to neurofilaments contribute to neuronal damage. Our data indicate that antibodies to NF-L might be involved in disease progression in neurodegenerative diseases such as MS and other neurodegenerative diseases in which antibodies to NF-L have been reported[17, 18, 38]. Thus, therapeutic approaches targeting humoral immunity to neurons and axons could be beneficial in a variety of neurological disorders. There is some evidence of the efficacy of this approach already. In MS therapeutic strategies targeting B cells reduce disease severity[39, 40] although such efficacy might not be due to a direct effect of reducing autoantibodies. In general, subgroups of pwMS respond well to plasma exchange[41-43], indicating that pathogenic antibodies contribute to disease.

Although pwMS experience various symptoms, visual problems commonly precede other neurological symptoms. Optical coherence tomography can be used to assess

This article is protected by copyright. All rights reserved.

Accepted Article

damage to the retina. Here, we used a new optic neuritis model[28] to investigate whether mAb to NF-L have a pathogenic effect on RNFL thickness and RGC density consequent to damage of axons within the optic nerve. Similar to effects noted with injection of mAb to MOG[28], injection of NF-L 10H9 into animals with optic neuritis significantly reduced RGC density 20 days post-injection. The significance of antibodies to NF-L in pwMS who develop optic neuritis has not been accounted before and deserves further investigation.

Here we also show early onset of disease and exacerbation of MOG³⁵⁻⁵⁵-induced EAE, following injection of IgG purified from sera of multiple pwMS with high levels of NF-L antibodies (Supplementary Figure 1), and a significant increase in the cumulative score induced following injection with the mAb NF-L. This suggests that antibodies to neuronal antigens, such as those to NF-L, may elicit an additional reaction causing neuronal damage. The differential effect observed on the EAE course after injection of the mAb NF-L or hIgG could be due to differences in specificity and therefore pathogenicity of these antibodies in pwMS compared to the mouse monoclonal. This is in line with previous studies by Pedotti et al[16] showing that IgG purified from plasma from a RRMS patient exacerbated PLP-induced EAE in mice. Absorption and affinity studies are required to identify the contribution of the NF-L antibodies as well as other antibodies present in the purified human IgGs. Nevertheless, we have previously shown that autoimmune T cell responses to neuronal antigens augment experimental autoimmune disease[32] while other reports show that antibodies to neurofascin augments neuronal damage[3] underscoring the potential importance of autoimmunity to neurons in humans.

To overcome the influence of the blood-brain barrier in the penetration of antibodies into the CNS, mAb were injected intracerebrally in mice. These antibodies induced the development of MS-like symptoms, including ataxia, spasticity and paralysis. Some studies have shown that antibodies to an intracellular antigen have the ability to enter

This article is protected by copyright. All rights reserved.

neurons causing depletion of adenosine triphosphate and increase of caspase levels that could lead to apoptosis and cell death[44]. This mechanism together with an elevation in intracellular Ca^{2+} mediated by anti-alpha-enolase antibodies has also been described in autoimmune retinopathy[45]. In addition, activation of FcγRI by IgG-immune complex has been shown to cause an increase in intracellular calcium and enhance excitability in sensory neurons[46]. Patch-clamping might be used to investigate whether mAb to NF-L impact on neuronal signaling. Our data also show that both NF-L specific and isotype antibodies can be internalized by neurons (data not shown). Once internalized, antibodies to NF-L might disturb axonal transport processes by binding to their target. Here we show that an anti-human NF-L antibody decreased remarkably the viability of differentiated neurons as measured in an MTT assay that reflects the amount of mitochondrial activity. We also show that mAb to NF-L 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[47, 48].

Further research needs to be performed to determine the mechanism by which antibodies to NF-L cause cytotoxicity. It is also possible that antibody-antigen complexes of neurofilament proteins and antibodies crosslink with Fc-receptors on immune cells which can trigger phagocytosis, antibody-dependent cell-mediated cytotoxicity and cytokine release[49] thereby contributing to progression in neuroinflammatory conditions.

Our previous studies show that antibodies to NF-L are diminished in response to disease modifying therapies in MS[50] and are also correlated with fast progression of ALS[38], which further suggest a pathogenic role of these antibodies. In this study, we expand some of our previous observations of the presence of Ig deposits within axons in lesions of mice exhibiting clinical disease[8]. Our results show that antibodies to intracellular antigens contribute to axonal damage and are not just surrogate markers

This article is protected by copyright. All rights reserved.

of disease. This may open novel potential therapeutic targets for antibodies interfering with these responses.

Acknowledgments

We thank Wouter Gerritsen for his exceptional technical skills and Kamilla Nurtaeva and Adib Zendedel for excellent technical assistance with stereotactic injections. PC12 cells were kindly provided by Dr. Alexandra Chittka. We also thank Katie Lidster for technical assistance on optic neuritis studies. 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).

Disclosures

The authors declare that there are no conflicts of interest.

Contribution of authors

FP, BJS, SA, contributed to the conception and design of the study. FP, BJS, SB, MK, LB, JS, SCB, performed the experiments. IB, JR, SG, provided the samples from MS patients. FP, BJS, SB, MK, LB, IB, JS, JR, SG, PV, SCB, DB and SA contributed to the interpretation of the results, writing and revision of the manuscript.

References

1. Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci* 2008;**31**:247-69.
2. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, Laursen H, Sorensen PS, Lassmann H. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 2009;**132**:1175-89.
3. Derfuss T, Parikh K, Velhin S, Braun M, Mathey E, Krumbholz M, Kumpfel T, Moldenhauer A, Rader C, Sonderegger P, Pollmann W, Tiefenthaller C, Bauer J, Lassmann H, Wekerle H, Karagogeos D, Hohlfeld R, Linington C, Meinl E. 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* 2009;**106**:8302-7.
4. Huizinga R, van der Star BJ, Kipp M, Jong R, Gerritsen W, Clarner T, Puentes F, Dijkstra CD, van der Valk P, Amor S. Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. *Glia* 2012;**60**:422-31.
5. Mathey EK, Derfuss T, Storch MK, Williams KR, Hales K, Woolley DR, Al-Hayani A, Davies SN, Rasband MN, Olsson T, Moldenhauer A, Velhin S, Hohlfeld R, Meinl E, Linington C. Neurofascin as a novel target for autoantibody-mediated axonal injury. *J Exp Med* 2007;**204**:2363-72.
6. Thompson EJ, Kaufmann P, Shortman RC, Rudge P, McDonald WI. Oligoclonal immunoglobulins and plasma cells in spinal fluid of patients with multiple sclerosis. *Br Med J* 1979;**1**:16-7.
7. Van Noort JM, Van Sechel AC, Bajramovic JJ, El Ouagmiri M, Polman CH, Lassmann H, Ravid R. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. *Nature* 1995;**375**:798-801.
8. Huizinga R, Heijmans N, Schubert P, Gschmeissner S, t Hart BA, Herrmann H, Amor S. Immunization with neurofilament light protein induces spastic paresis and axonal degeneration in Biozzi ABH mice. *J Neuropathol Exp Neurol* 2007;**66**:295-304.
9. Van Noort JM, Bsibsi M, Gerritsen WH, van der Valk P, Bajramovic JJ, Steinman L, Amor S. Alphas-crystallin is a target for adaptive immune responses and a trigger of innate responses in preactive multiple sclerosis lesions. *J Neuropathol Exp Neurol* 2010;**69**:694-703.
10. Compston A, Coles A. Multiple sclerosis. *Lancet* 2008;**372**:1502-17.

11. Amor S, Baker D, Groome N, Turk JL. 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* 1993;**150**:5666-72.
12. Amor S, Groome N, Linington C, Morris MM, Dornmair K, Gardinier MV, Matthieu JM, Baker D. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol* 1994;**153**:4349-56.
13. Amor S, O'Neill JK, Morris MM, Smith RM, Wraith DC, Groome N, Travers PJ, Baker D. 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* 1996;**156**:3000-8.
14. Baker D, O'Neill JK, Gschmeissner SE, Wilcox CE, Butter C, Turk JL. Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J Neuroimmunol* 1990;**28**:261-70.
15. Morris-Downes MM, Smith PA, Rundle JL, Piddlesden SJ, Baker D, Pham-Dinh D, Heijmans N, Amor S. Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. *J Neuroimmunol* 2002;**125**:114-24.
16. Pedotti R, Musio S, Scabeni S, Farina C, Poliani PL, Colombo E, Costanza M, Berzi A, Castellucci F, Ciusani E, Confalonieri P, Hemmer B, Mantegazza R, Antozzi C. Exacerbation of experimental autoimmune encephalomyelitis by passive transfer of IgG antibodies from a multiple sclerosis patient responsive to immunoadsorption. *J Neuroimmunol* 2013;**262**:19-26.
17. Silber E, Semra YK, Gregson NA, Sharief MK. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* 2002;**58**:1372-81.
18. Ehling R, Lutterotti A, Wanschitz J, Khalil M, Gneiss C, Deisenhammer F, Reindl M, Berger T. Increased frequencies of serum antibodies to neurofilament light in patients with primary chronic progressive multiple sclerosis. *Mult Scler* 2004;**10**:601-6.
19. Willis SN, Stathopoulos P, Chastre A, Compton SD, Hafler DA, O'Connor KC. Investigating the Antigen Specificity of Multiple Sclerosis Central Nervous System-Derived Immunoglobulins. *Front Immunol* 2015;**6**:600.

20. Huizinga R, Gerritsen W, Heijmans N, Amor S. Axonal loss and gray matter pathology as a direct result of autoimmunity to neurofilaments. *Neurobiol Dis* 2008;**32**:461-70.
21. Krishnamoorthy G, Saxena A, Mars LT, Domingues HS, Mentele R, Ben-Nun A, Lassmann H, Dornmair K, Kurschus FC, Liblau RS, Wekerle H. Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis. *Nat Med* 2009;**15**:626-32.
22. Stein TD, Fedynyshyn JP, Kalil RE. Circulating autoantibodies recognize and bind dying neurons following injury to the brain. *J Neuropathol Exp Neurol* 2002;**61**:1100-8.
23. Tampellini D, Magrane J, Takahashi RH, Li F, Lin MT, Almeida CG, Gouras GK. Internalized antibodies to the Abeta domain of APP reduce neuronal Abeta and protect against synaptic alterations. *J Biol Chem* 2007;**282**:18895-906.
24. Geis C, Weishaupt A, Hallermann S, Grunewald B, Wessig C, Wulsch T, Reif A, Byts N, Beck M, Jablonka S, Boettger MK, Uceyler N, Fouquet W, Gerlach M, Meinck HM, Siren AL, Sigrist SJ, Toyka KV, Heckmann M, Sommer C. Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain* 2010;**133**:3166-80.
25. Fewou SN, Rupp A, Nickolay LE, Carrick K, Greenshields KN, Padiani J, Plomp JJ, Willison HJ. Anti-ganglioside antibody internalization attenuates motor nerve terminal injury in a mouse model of acute motor axonal neuropathy. *J Clin Invest* 2012;**122**:1037-51.
26. Michalak Z, Lebrun A, Di Miceli M, Rousset MC, Crespel A, Coubes P, Henshall DC, Lerner-Natoli M, Rigau V. IgG leakage may contribute to neuronal dysfunction in drug-refractory epilepsies with blood-brain barrier disruption. *J Neuropathol Exp Neurol* 2012;**71**:826-38.
27. Sadaba MC, Tzartos J, Paino C, Garcia-Villanueva M, Alvarez-Cermenio JC, Villar LM, Esiri MM. Axonal and oligodendrocyte-localized IgM and IgG deposits in MS lesions. *J Neuroimmunol* 2012;**247**:86-94.
28. Lidster K, Jackson SJ, Ahmed Z, Munro P, Coffey P, Giovannoni G, Baker MD, Baker D. Neuroprotection in a novel mouse model of multiple sclerosis. *PLoS One* 2013;**8**:e79188.
29. Al-Izki S, Pryce G, O'Neill JK, Butter C, Giovannoni G, Amor S, Baker D. Practical Guide to the Mouse Induction of Relapsing Progressive Experimental

Autoimmune Encephalomyelitis in the Biozzi ABH. *Multiple Sclerosis and Related Disorders* 2012;**1**:29-38.

30. Sorensen A, Moffat K, Thomson C, Barnett SC. Astrocytes, but not olfactory ensheathing cells or Schwann cells, promote myelination of CNS axons in vitro. *Glia* 2008;**56**:750-63.
31. Boomkamp SD, Riehle MO, Wood J, Olson MF, Barnett SC. 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* 2012;**60**:441-56.
32. Puentes F, van der Star BJ, Victor M, Kipp M, Beyer C, Peferoen-Baert R, Ummenthum K, Pryce G, Gerritsen W, Huizinga R, Reijerkerk A, van der Valk P, Baker D, Amor S. Characterization of immune response to neurofilament light in experimental autoimmune encephalomyelitis. *J Neuroinflammation* 2013;**10**:118.
33. Van der Goes A, Kortekaas M, Hoekstra K, Dijkstra CD, Amor S. The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages. *J Neuroimmunol* 1999;**101**:61-7.
34. Baker D, O'Neill JK, Davison AN, Turk JL. Control of immune-mediated disease of the central nervous system requires the use of a neuroactive agent: elucidation by the action of mitoxantrone. *Clin Exp Immunol* 1992;**90**:124-8.
35. Elliott C, Lindner M, Arthur A, Brennan K, Jarius S, Hussey J, Chan A, Stroet A, Olsson T, Willison H, Barnett SC, Meinl E, Linington C. Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis. *Brain* 2012;**135**:1819-33.
36. Lidster K, Baker D. Optical coherence tomography detection of neurodegeneration in multiple sclerosis. *CNS Neurol Disord Drug Targets* 2012;**11**:518-27.
37. Thomson CE, Hunter AM, Griffiths IR, Edgar JM, McCulloch MC. Murine spinal cord explants: a model for evaluating axonal growth and myelination in vitro. *J Neurosci Res* 2006;**84**:1703-15.
38. Puentes F, Topping J, Kuhle J, van der Star BJ, Douiri A, Giovannoni G, Baker D, Amor S, Malaspina A. Immune reactivity to neurofilament proteins in the clinical staging of amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry* 2013.

39. Gredler V, Mader S, Schanda K, Hegen H, Di Pauli F, Kuenz B, Deisenhammer F, Berger T, Reindl M, Lutterotti A. Clinical and immunological follow-up of B-cell depleting therapy in CNS demyelinating diseases. *J Neurol Sci* 2013;**328**:77-82.
40. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, Langer-Gould A, Smith CH. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 2008;**358**:676-88.
41. Keegan M, Konig F, McClelland R, Bruck W, Morales Y, Bitsch A, Panitch H, Lassmann H, Weinshenker B, Rodriguez M, Parisi J, Lucchinetti CF. Relation between humoral pathological changes in multiple sclerosis and response to therapeutic plasma exchange. *Lancet* 2005;**366**:579-82.
42. Weinshenker BG, O'Brien PC, Petterson TM, Noseworthy JH, Lucchinetti CF, Dodick DW, Pineda AA, Stevens LN, Rodriguez M. A randomized trial of plasma exchange in acute central nervous system inflammatory demyelinating disease. *Ann Neurol* 1999;**46**:878-86.
43. Kaynar L, Altuntas F, Aydogdu I, Turgut B, Kocyigit I, Hacıoglu SK, Ismailogullari S, Turgut N, Erkurt MA, Sari I, Oztekin M, Solmaz M, Eser B, Ersoy AO, Unal A, Cetin M. Therapeutic plasma exchange in patients with neurologic diseases: retrospective multicenter study. *Transfus Apher Sci* 2008;**38**:109-15.
44. Douglas J, Gardner L, Levin M. Antibodies to an Intracellular Antigen Penetrate Neuronal Cells and Cause Deleterious Effects. *J Clin Cell Immunol* 2013;**4**.
45. Magrys A, Anekonda T, Ren G, Adamus G. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol* 2007;**27**:181-92.
46. Qu L, Zhang P, LaMotte RH, Ma C. Neuronal Fc-gamma receptor I mediated excitatory effects of IgG immune complex on rat dorsal root ganglion neurons. *Brain Behav Immun* 2011;**25**:1399-407.
47. Johnston KM, Brady ST, van der Kooy D, Connolly JA. A unique tubulin antibody which disrupts particle movement in squid axoplasm. *Cell Motil Cytoskeleton* 1987;**7**:110-5.
48. Brady ST, Pfister KK, Bloom GS. A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm. *Proc Natl Acad Sci U S A* 1990;**87**:1061-5.

49. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. *Nat Rev Immunol* 2008;**8**:34-47.
50. Amor S, van der Star BJ, Bosca I, Raffel J, Gnanapavan S, Watchorn J, Kuhle J, Giovannoni G, Baker D, Malaspina A, Puentes F. Neurofilament light antibodies in serum reflect response to natalizumab treatment in multiple sclerosis. *Mult Scler* 2014;**20**:1355-62.

Figure legends

Figure 1. Specificity of the monoclonal antibody NF-L 10H9. (A) Western blot analysis illustrates the specificity of NF-L 10H9 to NF-L protein (68 kDa). Lane 1 shows the reactivity to spinal cord and Lane 2 the reactivity to mouse brain homogenate. (B) Indicates the reactivity to the recombinant NF-L protein. MW, protein standard.

Figure 2. RGC density is significantly affected by NF-L 10H9. (A) RGC density and (B) RNFL thickness at 15 days (group A, grey bars, n = 3 mice per group, n = 2 for isotype control) or at 20 days (group B, black bars, n = 2 mice per group) after treatment with (un)labeled NF-L 10H9 or isotype control IgG1. (A) Only mice in group B treated with unlabeled NF-L 10H9 showed a statistically significant reduction in RGC density compared with mice treated with unlabeled isotype control IgG1 (Student's t-test, p<0.05). (B) No significant differences in percentage change in RNFL thickness were observed in NF-L 10H9 treated mice 15 or 20 days post-induction.

Figure 3. Effect of an anti-human NF-L antibody on neuronal viability

Differentiated PC12 cells were treated for 24h, 48h and 72h with 20 µg/ml rabbit anti-human NF-L antibody or rabbit IgG as a control of the assay. Cells were incubated with

MTT (0.5 mg/ml) for the last 4 h of incubation, then supernatants were discarded and 200 μ L dimethyl sulfoxide was added to solubilise the formazan crystals. Absorbance was determined at 590 nm using an ELISA reader.

Figure 4. mAb to NF-L reduces axonal density in rat spinal cord co-cultures. (A)

Co-cultures were treated with NF-L 10H9 or isotype control for 0.5, 1, 2, 3, 16 or 48 h with 10 μ g/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). Representative immunofluorescent images of cultures treated with (B) 10 μ g/ml isotype control IgG1 or (C) NF-L 10H9 for 16 h and stained with SMI310 (scale bars=35 μ m).

Supplementary Figure 1. Specificity of IgG from MS serum. IgG from pooled sera were selected from 16 pwMS (RRMS n=9; SPMS n=7) with high antibody levels reactive to NF-L using ELISA (Supplementary Table 1) in comparison to NF-L antibody levels in 24 healthy controls (0.79 ± 0.27 optical density). Western blotting show the reactivity of purified hIgG to a protein at the molecular weight of the linear NF-L protein (68 kDa) in mouse spinal cord (lane 1) and brain homogenates (lane 2).

Supplementary Figure 2. Exacerbation of MOG³⁵⁻⁵⁵-induced EAE by hIgG. IgG from pwMS significantly exacerbated clinical neurological signs 12 to 14 days post-immunisation compared to regular EAE mice (Mann-Whitney U test; p<0.05). On day

15, the animals showed a remission phase that last approximately for a week and afterwards the severity of disease increase again. After the peak in clinical disease, no statistical differences were observed between MOG³⁵⁻⁵⁵+ hIgG and regular EAE mice. Mice injected with IgG from pwMS showed a more rapid disease onset (11.8 ± 1.0) days versus (14.1 ± 2.1 days; $p < 0.05$).

Supplementary Figure 3. Intracerebral injection of mAb to NF-L causes transient neurological signs. ABH mice were injected intracerebrally with NF-L 10H9 (n=6 mice), hIgG from pwMS (n=12 mice) or hIgG from healthy controls (n=5 mice). **A** and **B**) Still of a video of a mouse injected with NF-L 10H9 showed neurological signs including partial paralysis of hind limbs and tail stiffness. **C**) Video still of a mouse injected with hIgG from pwMS. Besides paralysis of the hind limb, mice injected with hIgG from pwMS showed extensive tail stiffness. **D**) Mouse injected with hIgG from healthy controls showed no signs of disease.

Table 2. Effect of NF-L specific antibodies in MOG³⁵⁻⁵⁵-induced EAE.

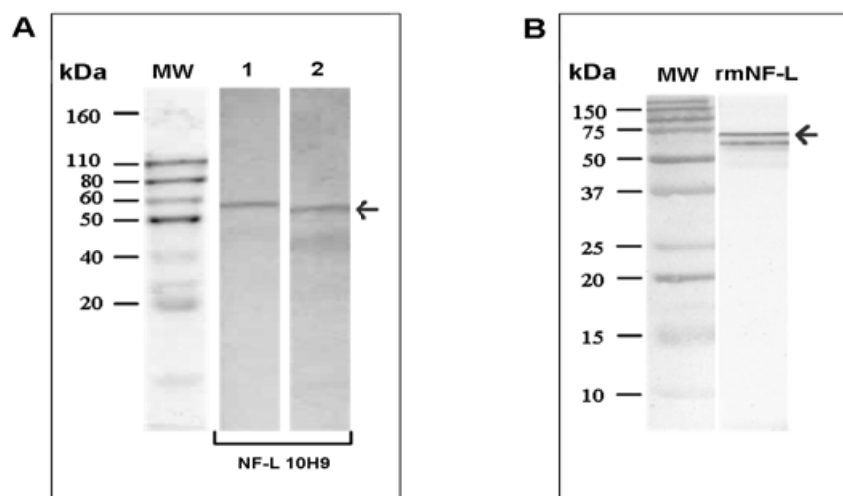
Treatment	No. EAE	Mean group score ^a	Mean EAE score ^b	Mean day of onset ^c	AUC ^d
MOG ³⁵⁻⁵⁵	9/11	3.3 ± 1.6	4.0 ± 0.0	14.1 ± 2.1	46.4 ± 1.1
MOG ³⁵⁻⁵⁵ + NF-L 10H9	6/6	4.0 ± 0.0	4.0 ± 0.0	12.3 ± 1.0	63.2 ± 1.4

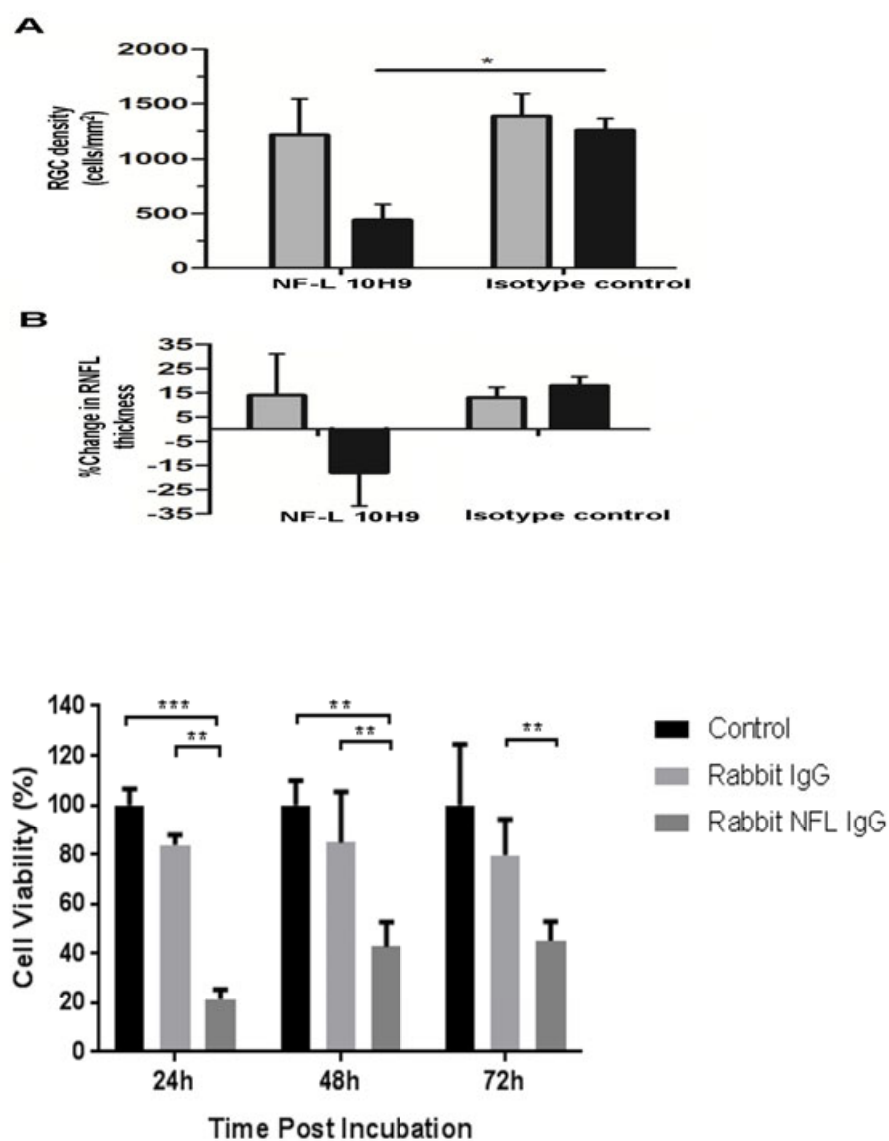
^a mean ± SEM of maximum clinical score of EAE from all mice in the group

^b mean ± SEM of maximum clinical score from mice exhibiting EAE within a group

^c mean ± SD of day of onset of clinical disease

^d AUC ± SD Cumulative scores are expressed as area under the curve (AUC). Cumulative clinical scores were calculated as the sum of all the daily scores over the course of EAE, **p=0.01.





A

