Axonal Damage in Multiple Sclerosis

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CHAPTER 2

Phagocytosis of Neuronal Debris by Microglia is Associated with Neuronal Damage in Multiple Sclerosis

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Abstract
Neuroaxonal degeneration is a pathological hallmark of multiple sclerosis (MS) contributing to irreversible neurological disability. Pathological mechanisms leading to axonal damage include autoimmunity to neuronal antigens. In actively demyelinating lesions, myelin is phagocytosed by microglia and blood-borne macrophages, while the fate of degenerating or damaged axons is unclear. Phagocytosis is essential for clearing neuronal debris to allow repair and regeneration. However, phagocytosis may lead to antigen presentation and autoimmunity, as has been described for neuroaxonal antigens. Despite this notion, it is unknown whether phagocytosis of neuronal antigens occurs in MS. Here, we show using novel, well-characterised antibodies to axonal antigens, that axonal damage is associated with human leukocyte antigen (HLA)-DR expressing microglia/macrophages engulfing axonal bulbs, indicative of axonal damage. Neuronal proteins were frequently observed inside HLA-DR$^+$ cells in areas of axonal damage. *In vitro*, phagocytosis of neurofilament light (NF-L) present in white and grey matter, was observed in human microglia. The number of NF-L or myelin basic protein positive cells was quantified using the mouse macrophage cell line J774.2. Intracellular co-localisation of NF-L with the lysosomal membrane protein lysosomal-associated membrane protein 1 was observed using confocal microscopy confirming that NF-L is taken up and degraded by the cell. *In vivo*, NF-L and proteolipid protein was observed in cerebrospinal fluid cells from MS patients, suggesting neuronal debris is drained by this route following axonal damage.
In summary, neuroaxonal debris is engulfed, phagocytosed and degraded by HLA-DR$^+$ cells. Although uptake is essential for clearing neuronal debris, phagocytic cells could also play a role in augmenting autoimmunity to neuronal antigens.

Introduction
Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease of the central nervous system (CNS) in which the immune system plays a major role (1-3). The presence of T cells and antibodies directed to myelin and myelin-associated antigens (4, 5) has led to the widely accepted view that MS is an autoimmune disease targeting myelin and oligodendrocytes. Antibodies from patients with MS bind to myelin, facilitating phagocytosis of myelin *in vitro* (6, 7) indicating a role *in vivo*. In MS lesions, immunoglobulin on myelin sheaths may induce complement-mediated lysis, but also promote opsonisation of myelin and oligodendrocytes, resulting in macrophage-mediated phagocytosis (8, 9). In addition to myelin damage, there is a strong neurodegenerative component, which contributes to the irreversible neurological deficits (10). Axonal degeneration is already present early in disease (11). Despite a strong correlation between inflammation and axonal damage in MS, the mechanism of axonal damage is not clear. Proposed mechanisms include chronic demyelination, leading to increased energy demands by axons (12), mitochondrial dysfunction (13), and macrophage-derived reactive oxygen and nitrogen species (14).
Neurons and axons are also vulnerable to autoimmune-mediated damage, as is observed in other neurodegenerative diseases (15, 16). In MS, antibodies and T cells are also directed to neuroaxonal antigens (17-21), and axon-reactive B cells are present in the cerebrospinal fluid (CSF) (22). In mice, extensive axonal damage occurs following immunisation with the axonal protein neurofilament light (NF-L) (23, 24). In this model, axonal end bulbs as well as myelin sheaths devoid of axons are observed. Intact myelin sheaths containing damaged axons are also observed in

MS, and in myelin-induced experimental autoimmune encephalomyelitis (EAE) (14), indicating direct damage to axons. A pathogenic role of autoimmunity to neurons is further supported by the observation that antibodies to the axo-glial proteins neurofascin and contactin-2/TAG-1, as found in patients with MS, augment clinical EAE, axonal injury and grey matter damage in rats (17, 20).
Despite strong evidence for autoimmunity to neuronal antigens, it is unclear how these responses arise, but they could be due to infections or ischemia in the CNS. In MS and stroke in humans, and after EAE and neurological damage in experimental animals, CNS antigens released during damage drain to the local cervical lymph nodes (CLN) (25-27). How CNS antigens are transported to the CLN is unknown. Although such antigens could be subject to direct drainage into the CSF or blood, they could also be transported to the CLN after uptake by phagocytic cells. Depending on the appropriate signals, presentation of neuronal antigens in the CLN could invoke a pathogenic autoimmune response, leading to subsequent episodes of autoimmunity. More likely however, these responses can be protective because animals fail to develop recurrent clinical episodes of EAE after fibre tract injury in the CNS (28). After fibre tract injury, local antigen presentation by immature antigen-presenting cells, up regulation of CD95L and T cell apoptosis have been suggested to contribute to immunosuppression and thus reduction in EAE (28). This is in line with a report contradicting the idea that brain trauma may cause or exacerbate MS (29). Whatever the outcome, uptake, degradation and presentation of neuronal antigens remains a crucial step for activation of autoreactive T cells and autoantibody production.
Since commercially available monoclonal antibodies (mAbs) to neurofilaments were found to cross-react with cellular structures, new mAbs to NF-L were generated. Selecting the most specific mAb to NF-L, we show that neuronal proteins, such as NF-L were engulfed by human leukocyte antigen (HLA)-DR^+ cells in the CNS of patients with MS. Higher numbers of antigen-containing HLA-DR^+ cells were detected in areas of axonal damage. NF-L and proteolipid protein (PLP) was also observed in CSF cells from patients with MS, suggesting neuronal debris is drained by this route after axonal damage. *In vitro*, neuronal antigens were observed in human microglia and in a mouse macrophage cell line the presence of NF-L co-localises with lysosomal membrane proteins. The data show that in addition to myelin antigens, neuronal antigens are also taken up by potential antigen-presenting cells during MS.

Materials and methods

Autopsy tissues

All human tissues were obtained in collaboration with the Netherlands Brain Bank and consent from donors and in compliance with local legal and ethical regulations. Paraffin blocks of post-mortem brain material from 15 patients with clinically definite MS (20 blocks), were selected, three patients with cerebrovascular accident and five non-neurological controls (Table 1). MS lesions were identified by MRI-guided sampling of the CNS as described previously (30, 31). The blocks contained 12 active, 9 chronic active and 9 inactive lesions and 7 areas of remyelination, and 8 grey matter lesions in addition to regions of normal appearing white matter.
CSF was collected at post-mortem from five patients with MS and five non-neurological control subjects and centrifuged at 302.4 *g* (Hettich Rotanta/RP, UK) for 7 min. The cell pellet was treated with 0.15 M NH_4Cl and 1 mM $KHCO_3$ in 10% bovine serum albumin (BSA) (Gibco Invitrogen, NL) for 5 min to lyse the erythrocytes. Cells present in the CSF were washed in Dulbecco's modified essential media/nutrient mixture

F10 (DMEM/F10) (1:1) (Gibco Invitrogen, NL) and collected using the Cytospin 3 centrifuge (Shandon, Bohemia, USA) for 15 min at 22.4 *g* after which the cells were fixed in 4% formalin.

Table 1. Clinical details of patients

Patient	Gender	Age	MS type or control classification	Disease duration (y)	Cause of Death
1	F	48	MS SP	8	Euthanasia
2	F	53	MS SP	27	Euthanasia
3	M	43	MS unknown	Unknown	Pneumonia
4	F	75	MS unknown	42	Pneumonia
5	F	48	MS PP/SP	25	Euthanasia
6	F	50	MS unknown	Unknown	Unknown
7	F	44	MS PP	8	Decompensation
8	F	77	MS unknown	Unknown	Respiratory insufficiency with aspirated pneumonia
9	M	63	MS PP	24	Cardiac arrest
10	M	56	MS SP	27	Pneumonia
11	F	69	MS PP	53	Respiratory and heart failure
12	M	66	MS unknown	Unknown	Sepsis, hypothermia, possible dehydration and pneumonia
13	F	70	MS PP (slow)	40	Urinary tract infection
14	M	47	MS PP	7	Urosepsis with organ failure
15	M	41	MS unknown	Unknown	Urosepsis and pneumonia
16	F	67	Stroke	n/a	Aortic dissection
17	F	72	Stroke	n/a	Bronchopneumonia
18	F	60	Stroke	n/a	Disseminated fungal infection
19	F	89	Non-neurological control	n/a	Cardiac arrhythmia
20	F	45	Non-neurological control	n/a	Cardiac arrhythmia
21	F	99	Non-neurological control	n/a	Pneumonia
22	F	63	Non-neurological control	n/a	Pneumonia and pulmonary embolism
23	F	58	Non-neurological control	n/a	Myocardial infarct

SP, secondary progressive; PP, primary progressive; n/a, not applicable.

In vitro phagocytosis assay

Human microglia were obtained from post-mortem control tissue using a rapid post-mortem isolation procedure as described previously (32) with omission of DNase. Isolated microglia or mouse macrophages (J774.2 cell line, Sigma-Aldrich, NL) were seeded in 4-well chamber slides (Nunc Thermo Scientific, UK). Human microglia were cultured in DMEM/F10 supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific, UK), 100 U/mL penicillin, 100 mg/mL streptomycin and 1 mM L-Glutamine (all from Gibco Invitrogen, Breda, NL) and mouse macrophages were cultured in DMEM supplemented as described for the human microglia. All cell types were cultures at 37°C in 5% CO_2 for 1 d prior to use in the phagocytosis assays.

As a source of neuroaxonal antigens control human grey matter and white matter was homogenised in DMEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 1 mM L-glutamine. Culture medium of the microglia was replaced by grey matter homogenate (1.8 mg/mL) for 1 h, after which the cells were washed thoroughly to remove excess grey matter. Microglia were fixed in 4% formalin after removal of grey matter (time point 1 h). For the macrophage cell line, cells were incubated with grey or white matter (1.8 mg/mL) also for 1 h followed by extensive washing. Subsequently, cells were fixed for the time point 1 h or incubated for another 5 h (time point 6 h) or 23 h (time point 24 h).

Production of mAbs to NF-L
Primary antibodies used for immunochemistry are detailed in Table 2. To produce specific mAbs to NF-L that did not cross-react with human tissues other than NF-L, splenocytes from NF-L-immunised Biozzi ABH mice (24) were fused with SP2/0-ag14 myeloma cells and cultured in the presence of 5% hybridoma cloning factor (Tebubio, NL). Positive clones were identified using enzyme-linked immunosorbent assay for recombinant mouse NF-L. The resultant immunoglobulins in the supernatant were purified using Protein A-coupled sepharose columns (Amersham Biosciences, NL). Three clones obtained recognised recombinant mouse NF-L as a band of 61 kDa on a western blot of human CNS white matter. Cross-reactivity with leukocyte antigens was assessed by staining human tonsil using immunohistochemistry as described below.

Enzyme digestion of NF-L protein
To verify that NF-L mAbs recognised degraded protein and breakdown products as would be present after phagocytosis, recombinant mouse NF-L (33) was treated with 50 μg/mL cathepsin B (12.1 U/mg) and 50 μg/mL cathepsin D (4.6 U/mg; Sigma-Aldrich Chemie, NL) at 37°C. Samples were collected at 0, 10, 20, 40, 60, 120, 180, 240 and 300 min, inactivated with 1 M Tris-HCl pH 9.0 and stored at -20°C. The samples were then diluted in NuPage sample buffer and reducing agent. Gel electrophoresis using 2 μg protein collected at each time point was performed for 1 h at 200 V using a 4-12% Bis-Tris gel with MOPS as running buffer (Invitrogen, NL). Proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) using an Ancos semi-dry electroblotter. The membranes were then incubated with NF-L antibodies (0.5 μg/mL) followed by horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (1:1,000; DakoCytomation, Denmark) for 1 h at room temperature (RT, 20°C) in TBS with 5% milk powder and 0.1% Tween. Bound antibodies were visualised on a Chemidoc XRS (Bio-Rad Laboratories, NL) using ECL reagent (Amersham Biosciences). Signal intensities were analysed using NIH Image J software (http://rsb.info.nih.gov-/ij/index.html).

Quantification of neuronal and myelin proteins in human grey and white matter
Protein was isolated using RIPA buffer (50 mM Tris HCl pH 8; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulphate) containing complete Mini Protease inhibitor cocktail tablets (Roche, Germany) in accordance with the manufacturers instructions. Supernatant was collected, and protein concentration determined by Pierce BC Protein Assay kit (Thermo Fischer, USA). The same amounts of protein (10 μg) were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, Germany) for 1 h at 10 V. The nitrocellulose membranes were blocked with 5% fat-free milk solution in phosphate-buffered saline (PBS) for 1 h. Membranes were then incubated with primary antibodies to NF-L and myelin basic protein (MBP, Table 2) diluted in 5% milk in PBS-Tween (PBST) overnight at 4°C. After washing, appropriate secondary antibodies conjugated to HRP were added to the membranes and incubated for 2 h at RT. Visualisation was performed with commercial enhanced chemiluminescence reagent kit (ABC kit; Amersham Bioscience, UK) and subsequently exposed to X-ray film (Kodak, Germany). Signal intensities were analysed using NIH Image J software.

Table 2. Antibodies used for immunohistochemistry and immunofluorescence

Antigen	Species and isotype	Clone	Dilution	Source
APP	mIgG1	LN27	1:4,000	Zymed
HLA-DR	mIgG2b	LN3	1:250	Gift from Dr Hilgers, VUMC, NL
MBP	mIgG1	26	1:4,000	Gift from Prof Groome, Oxford UK
MBP	mIgG2b	22	1:50	Millipore
NF-L	mIgG2b	4F8.1	5 μg/ml	In-house (this study)
NF-L	mIgG1	5B4.2	5 μg/ml	In-house (this study)
NF-L	mIgG1	10H9	IHC: 5 μg/mL IF: 8.4 μg/mL WB: 0.84 μg/mL	In-house (this study)
NF-L	mIgG1	DA2	1:100	Zymed
Non-phosphorylated NF-H	mIgG1	SMI32	1:500	Sternberger Monoclonals
PLP	mIgG2a	Plpc1	1:250	Serotec, UK
LAMP1	rbIgG		1:500	Abcam, UK
Isotype control	mIgG1	MOPC-31C	1:100	BD, NL
Isotype control	mIgG1		IF: 8.4 μg/mL	BD, NL
Isotype control	mIgG2a	C1.18.4	1:100	BD, NL
Isotype control	mIgG2b	MPC-11	1:100	BD, NL
Isotype control	rbIgG		1.4 μg/mL	Southern Biotech

APP, amyloid precursor protein; NF-H, neurofilament heavy; LAMP1, lysosomal-associated membrane protein 1; m, mouse; rb, rabbit; IHC, immunohistochemistry; IF, immunofluorescence; WB, western blot.

Immunohistochemistry

CNS sections from MS and control brain tissues were deparaffinised, rehydrated and endogenous peroxidase activity blocked with 0.3% H_2O_2 in methanol for 30 min. After washing in PBS, the sections were processed for heat-mediated antigen retrieval in 0.1 M citric acid pH 6.0. After washing with PBST, sections were incubated with 10% normal goat serum (DakoCytomation) and 5% normal human AB serum (NHS; Sanquin, Leiden, NL) in 0.1% BSA/PBS for 30 min. Primary antibodies (Table 2) were allowed to bind overnight at 4°C. After washing, sections were incubated with appropriate secondary antibodies HRP-conjugated goat anti-mouse IgG1, HRP-conjugated goat anti-mouse IgG2a and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG2b (all from Southern Biotechnology Associates, AL) in PBS containing 1% BSA and 1% NHS for 45 min at RT. Bound antibodies were visualised using Fast-Blue for AP-conjugated secondary antibodies and HRP-conjugated antibodies were visualised with 3-amino-9-ethylcarbazole. Isotype-matched antibodies replaced the primary antibodies as controls (Table 2).

Tissue sections for pathological evaluation and quantification were viewed on an Olympus microscope by two independent observers (R.H. and S.A.) and the numbers of HLA-DR$^+$ cells containing axonal antigens were tested for significance using the Student's paired *t*-test. To assess whether uptake of axonal antigens occurred more frequently in regions of axonal damage, the number of amyloid precursor

protein (APP) positive axonal ovoids per cm^2 was determined as a measure of axonal degeneration using the Mann-Whitney U test.

Immunocytochemistry
Human microglia, CSF cells from patients with MS and controls patients, and J774.2 mouse macrophages were permeabilised with 0.1% saponin (Sigma-Aldrich, NL) in PBS and endogenous peroxidase was blocked as described above. Cells were incubated with primary mAbs to NF-L, MBP, PLP, or HLA-DR (Table 2) in 0.1% saponin/1% BSA/PBS overnight at 4°C. Washing steps were performed with 0.1% saponin/PBS. Primary antibodies were detected using HRP-conjugated polyclonal rabbit anti-mouse IgG (DakoCytomation, Denmark) for 30 min at RT, followed by visualisation with 3,3'-diaminobenzidine for 10 min. Isotype mouse IgG1 or PBS replaced primary antibodies as controls, respectively. To visualise the uptake of neutral lipids, permeabilised cells were incubated for 10 min with 0.3% Oil-red-O, followed by extensive washing with 0.1% saponin/PBS. Nuclei were counterstained with haematoxylin. The percentage of $HLA\text{-}DR^+$ cells or cells containing neutral lipids, NF-L, MBP or PLP was performed using 100x objective counting 700 – 1,000 of cells in each sample.

Confocal microscopy
For co-localisation studies of NF-L within the lysosomal compartment in mouse macrophages (J774.2), immunocytochemistry was performed as described previously with some minor modifications (34). In brief, fixed macrophages were washed in PBST for 1 h, followed by permeabilisation of the membrane by incubation with 0.01% Triton X-100 in PBS for 1 h. Cells were subsequently washed for 1 h in PBST, blocked in 1% BSA/PBST and incubated with primary antibodies (Table 2) overnight at 4°C. After washing, slides were incubated with a combination of fluorescent anti-mouse secondary antibodies (Alexa Fluor 488 donkey IgG; Invitrogen, Germany; 1:500) and fluorescent anti-rabbit secondary antibodies (Alexa Fluor 594 goat IgG; Invitrogen, Germany; 1:500) both diluted in 1% BSA/PBST. Slides were then incubated with Hoechst 33342 (Invitrogen, Germany; 1:1,000) diluted in PBS to visualise cell nuclei. To exclude unspecific binding of the fluorescent secondary antibodies to primary antibodies, appropriate negative controls were performed by first incubating sections with the primary antibodies of murine origin and subsequently incubating these sections with fluorescent anti-rabbit secondary antibody. Unspecific secondary antibody binding was excluded by performing negative controls by incubating sections with each of the fluorescent secondary antibodies alone. Stained and processed slides were documented with the microscope working station Zeiss LSM 7 Duo.

Results

Phagocytosis and degradation of NF-L by human microglia and mouse macrophages
NF-L is a member of the intermediate filament protein family, a group of homologous cytoskeletal proteins expressed in different cell types. Since commercially available mAb to NF-L cross-react with cellular structures, new mAbs to NF-L antibodies were generated. Of the three mAbs examined, only clone 10H9 showed no cross-reactivity with human tonsil (Supp. Table 1) and was able to detect breakdown products after proteolytic degradation of recombinant NF-L (Supp. Fig. 1). Therefore, this mAb was selected for *in vitro* studies of microglia-mediated phagocytosis of grey matter tissue samples and immunochemistry.

To determine whether NF-L is taken up by human microglia, grey matter from a control subject was added to cultured microglia. The majority of cells were positive for HLA-DR (Fig. 1A). After 1 h digestion of grey matter, human microglia showed extensive Oil-red-O staining (Fig. 1B), indicating marked breakdown of myelin. Additionally, microglia were positive for MBP (Fig. 1C, arrows) and NF-L (Fig. 1D, arrows) after 1 h digestion, confirming phagocytosis of both myelin and neuronal antigens by human microglia. Cells with omitted primary antibodies or with isotype controls were negative (data not shown). Using the mouse macrophage cell line J774.2, the numbers of cells containing myelin or neuronal antigens were quantified at 1, 6 and 24 h after incubation with either human grey or white matter. It was observed that many more cells were positive for MBP than NF-L at all time points regardless of the source (white matter or grey matter; Fig. 1E). This was despite semi-quantification of grey and white matter showing 7.5 more NF-L than the MBP in grey matter and 3 times more NF-L than MBP in white matter (Supp. Fig. 2).

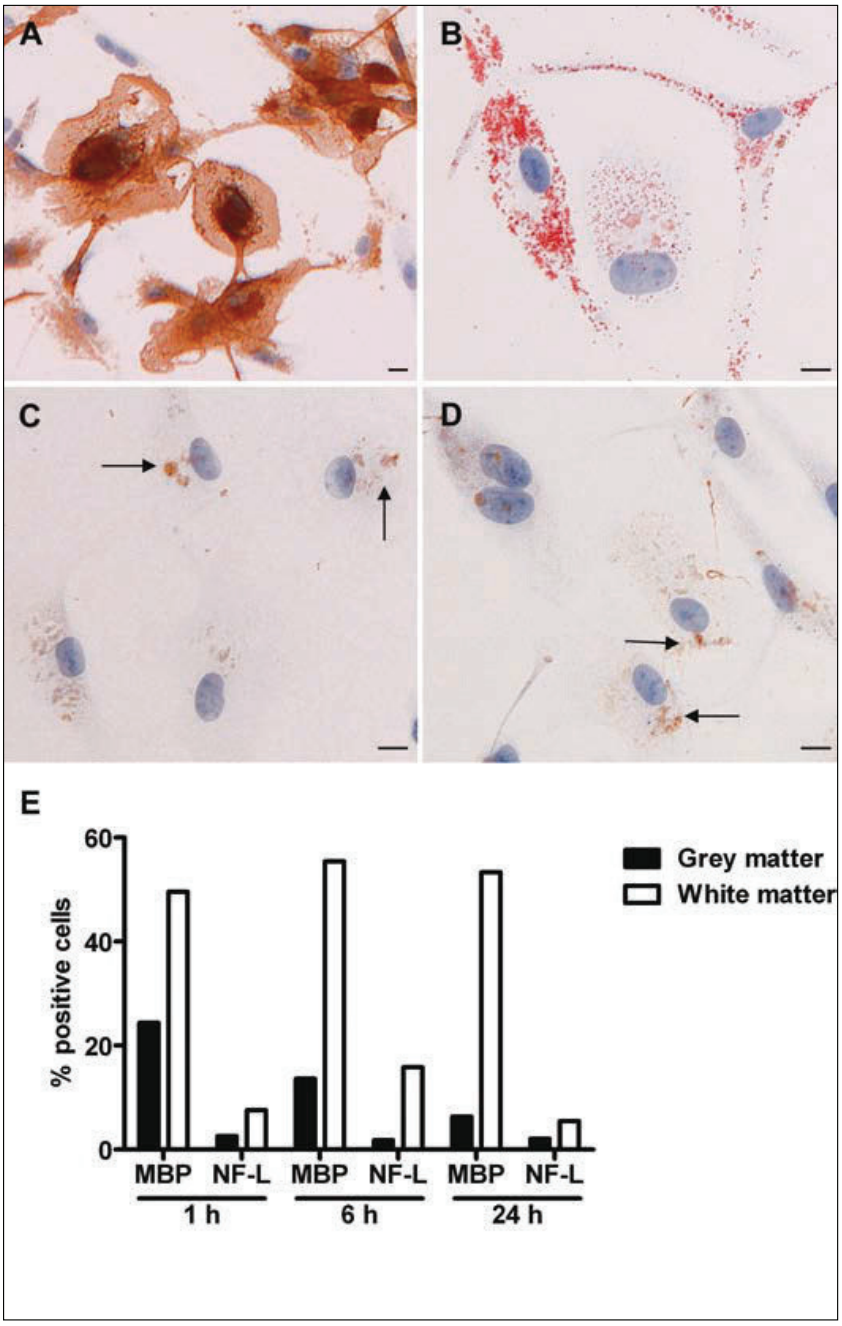

Figure 1. NF-L and MBP are phagocytosed by human microglia. *Human microglia were incubated with human grey matter, and immunocytochemistry used to detect MBP and NF-L after 1 h incubation.* ***A****) Microglia express HLA-DR and contain (****B****) Oil-red-O neutral lipids (red) and particles of MBP (****C****) and NF-L (****D****) (brown, arrows).* ***E****) Percentage of J774.2 mouse macrophages containing MBP and NF-L positive particles after incubation with human grey matter or white matter for 1 h. Cells were washed to remove excess debris and sampled immediately (1 h) or incubated for a further 5 h (time point 6 h) or a further 23 h (time point 24 h). Scale bars=7.5 µm.*

To confirm that NF-L was located intracellular and enters a proteolytic pathway in the phagocytosis assay *in vitro*, confocal microscopy was performed. Here we show that in the mouse macrophage cell line J774.2 (Fig. 2A, nucleus) positive structures of the late endosomal/lysosomal membrane protein LAMP1 (Fig. 2B) co-localised with ingested NF-L (Fig. 2C and 2D, merge).

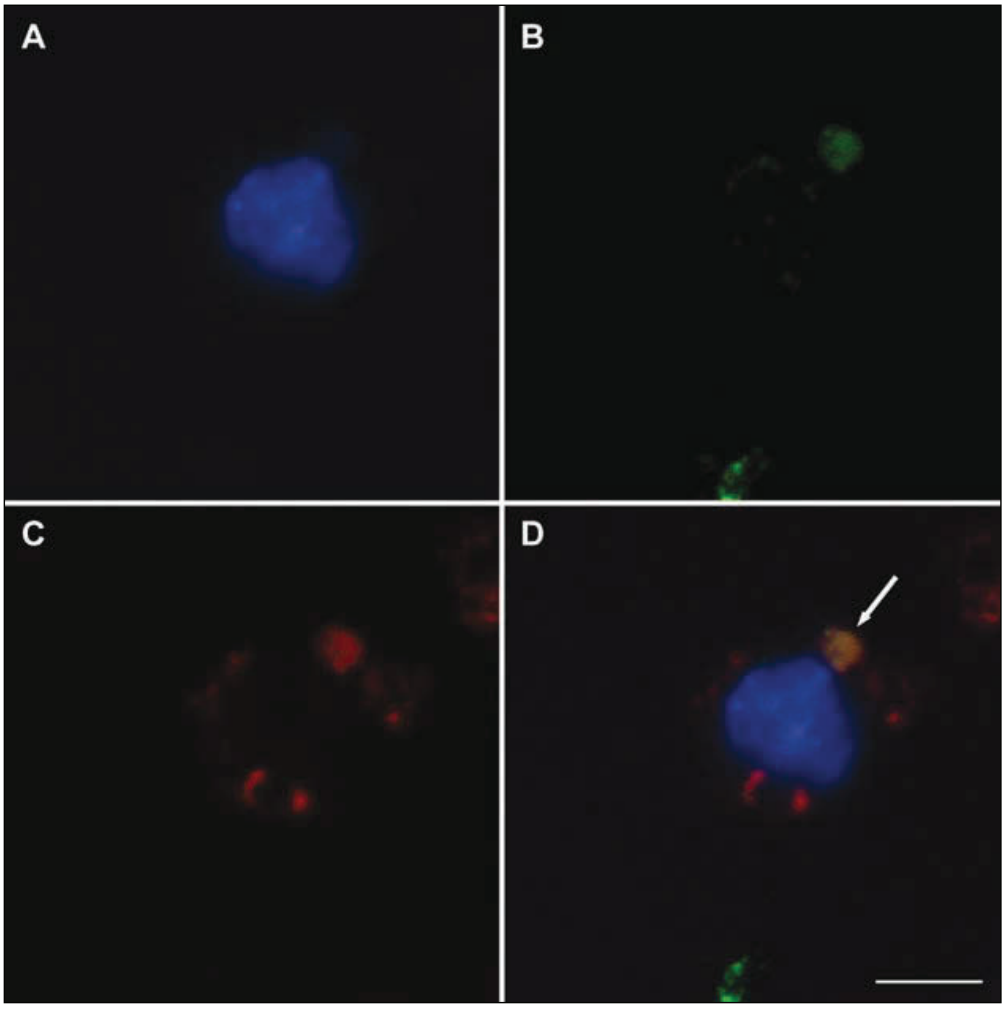

Figure 2. Neurofilament enters a proteolytic pathway after phagocytosis in vitro. *J774.2 mouse macrophages were incubated with human grey matter for 1 h and confocal microscopy used to detect NF-L particles after phagocytosis.* ***A****) Single nucleus.* ***B****) The late endosomal/lysosomal membrane protein LAMP1 (green) co-localises with bulbs of neurofilament light (****C****, red). The merge is shown in (****D****) (arrow). Scale bars=7.5 µm.*

In MS, damaged axons are engulfed by HLA-DR⁺ cells

To examine whether axonal antigens are engulfed by cells in MS lesions, the presence of APP, non-phosphorylated NF-H, NF-L, or the myelin proteins PLP and MBP inside HLA-DR⁺ cells was examined. As expected, HLA-DR⁺ cells containing MBP were closely associated with active lesions, the border of chronic active lesions and in the perivascular space (Fig. 3A, inset). In the hypercellular rim, APP⁺ axonal bulbs indicative of axonal damage were engulfed by HLA-DR⁺ cells (Fig. 3B, inset). Similar to APP, NF-L⁺ bulbs were also detected in a chronic MS lesion (Fig. 3C) and also associated with HLA-DR⁺ cells (Fig. 3C, insets). This was also the case for NF-L⁺ ovoids in an active MS lesion (Fig. 3D, inset). The edge of the demyelinated area is shown in Fig. 3E (MBP, red). Engulfment of neuronal and myelin proteins by HLA-DR⁺ cells was also present in the normal appearing white matter (non-phosphorylated NF-H Fig. 3F, inset; MBP Fig. 3G).

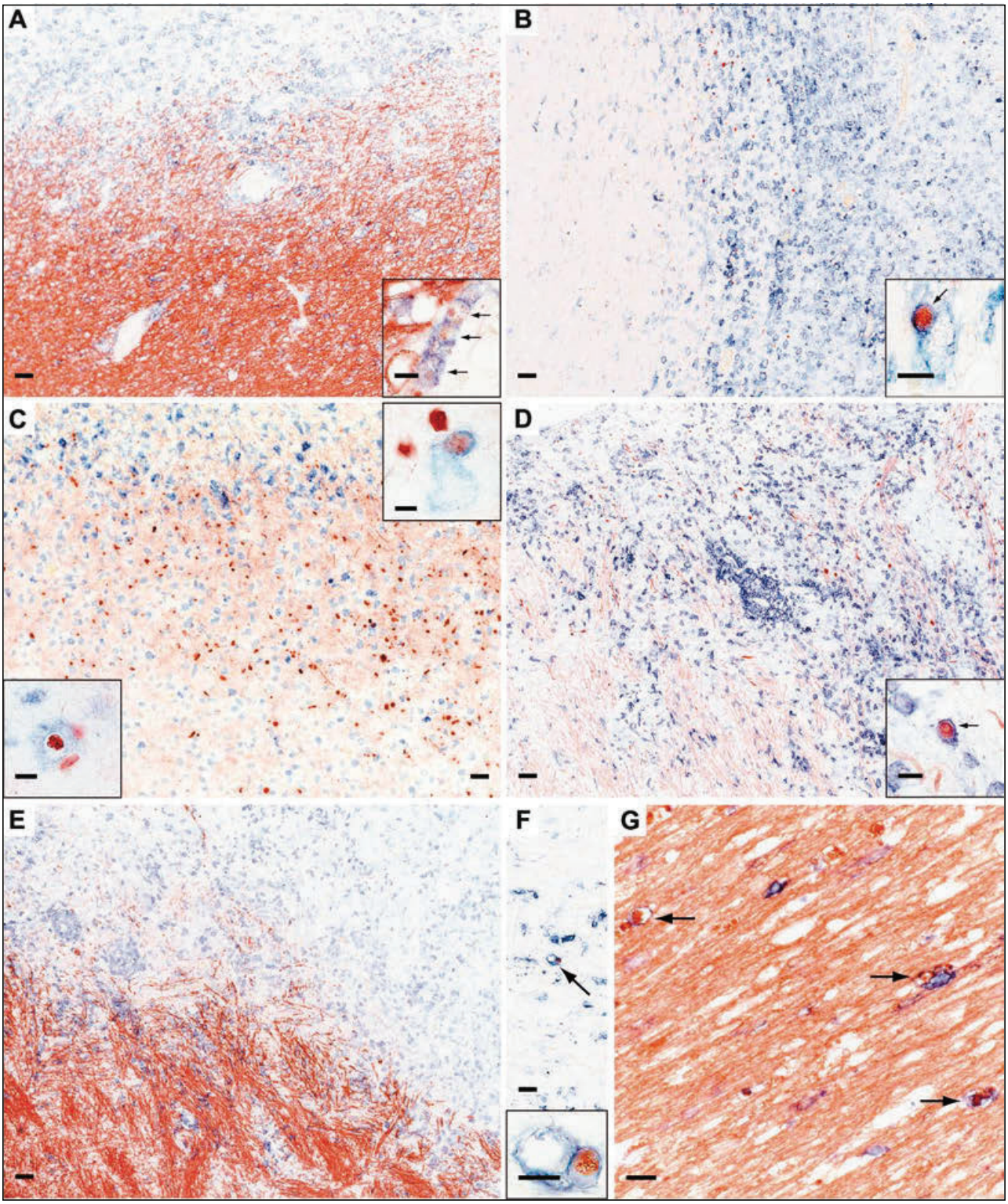

Figure 3. Axons are engulfed by HLA-DR⁺ cells in MS tissue. A*) MBP (red, arrows) inside HLA-DR cells (blue) in the perivascular space of blood vessels at the edge of a chronic active MS lesion.* ***B****) Axonal damage indicated by APP ovoids (red) in the hypercellular rim containing HLA-DR cells (blue); some ovoids are totally engulfed by HLA-DR cells (inset, arrow).* ***C****) Numerous ovoids positive for NF-L (red) in chronic active MS lesion (insets) and active MS lesion (****D****) are engulfed by HLA-DR cells (inset, arrow). The lack of space between the axon and the HLA-DR cell suggests that the myelin sheath has already degenerated, resulting in direct interaction between the axon and the HLA-DR cell. The border of myelination is shown in (****E****) (MBP, red).* ***F****) HLA-DR cells also engulf non-phosphorylated NF-H axons in normal appearing white matter (inset) where HLA-DR cells also engulf MBP (red,* ***G****, arrows). Scale bars represent 50 µm in (A)-(E), 20 µm in (F) and (G) and 10 µm in the insets.*

Axonal engulfment by HLA-DR⁺ cells correlates to the extent of axonal damage
We next assessed the frequency of HLA-DR⁺ cells containing neuronal or myelin antigens in MS lesions. Figure 4A shows that the number of HLA-DR⁺ cells containing MBP or PLP is significantly higher than those containing APP, NF-L or non-phosphorylated NF-H ($p<0.05$, Student's paired *t*-test). Figure 4 is a graphical representation of the quantification shown in Supplementary Table 2 in which details of the lesions are given. Axonal bulbs engulfed by HLA-DR⁺ cells were observed in all lesion types and also observed in normal appearing white matter (Fig. 3D, E). In contrast to myelin antigens, axonal antigens were not observed within HLA-DR⁺ cells in the perivascular spaces or in the meninges (data not shown). In CNS tissues containing a large number of damaged axons (>100 APP⁺ ovoids per cm^2), a larger number of axons engulfed by HLA-DR⁺ cells were observed compared to sections containing only few (<100) APP⁺ ovoids/cm^2 (Fig. 4B, $p<0.01$ Mann-Whitney U test).

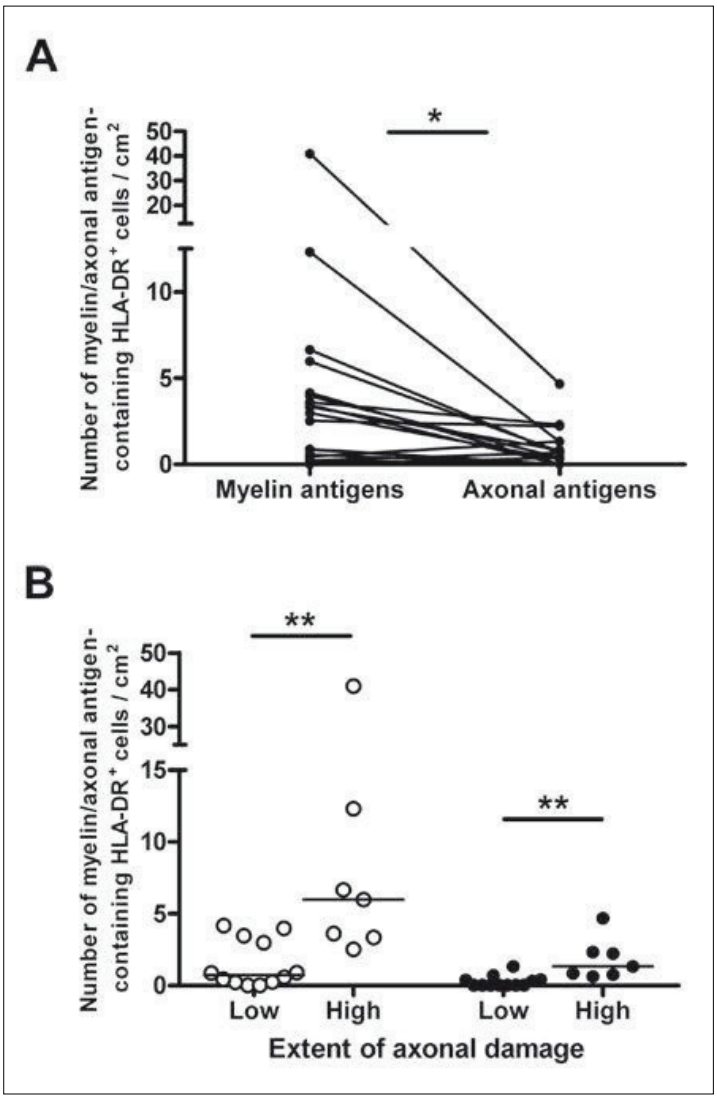

Figure 4. Phagocytosis of axonal antigens by HLA-DR⁺ cells is less frequent than myelin antigens and directly related to axonal damage. *(**A**) HLA-DR cells containing myelin (PLP and MBP) or axonal antigens (APP, NF-L, non-phosphorylated NF-H) in tissue sections containing MS lesions (* $p<0.05$, paired t-test). (**B**) Numbers of HLA-DR cells containing myelin antigens (open circles) or neuroaxonal antigens (filled circles) in areas of extensive axonal degeneration (>100 APP ovoids/cm^2) compared to areas with low levels of axonal degeneration (<100 APP ovoids/cm^2; ** $p<0.01$, Mann-Whitney U test).*

NF-L and PLP are phagocytosed by cells present in the CSF
To examine the possible fate of cells containing axonal and myelin debris, the presence of HLA-DR⁺ cells (Fig. 5A) collected from CSF samples from five patients with MS and five non-neurological control subjects at autopsy were examined. The number of Oil-red-O⁺ cells (Fig. 5B) and cells containing PLP (Fig. 5C) or NF-L

positive particles (Fig. 5D) were evaluated. As shown in Fig. 5E, CSF obtained from patients with MS contained a significantly higher number of HLA-DR$^+$, and Oil-red-O$^+$ cells ($p<0.01$, Mann-Whitney U test) and for NF-L$^+$ and PLP$^+$ cells ($p<0.05$, Mann-Whitney U test) compared with controls.

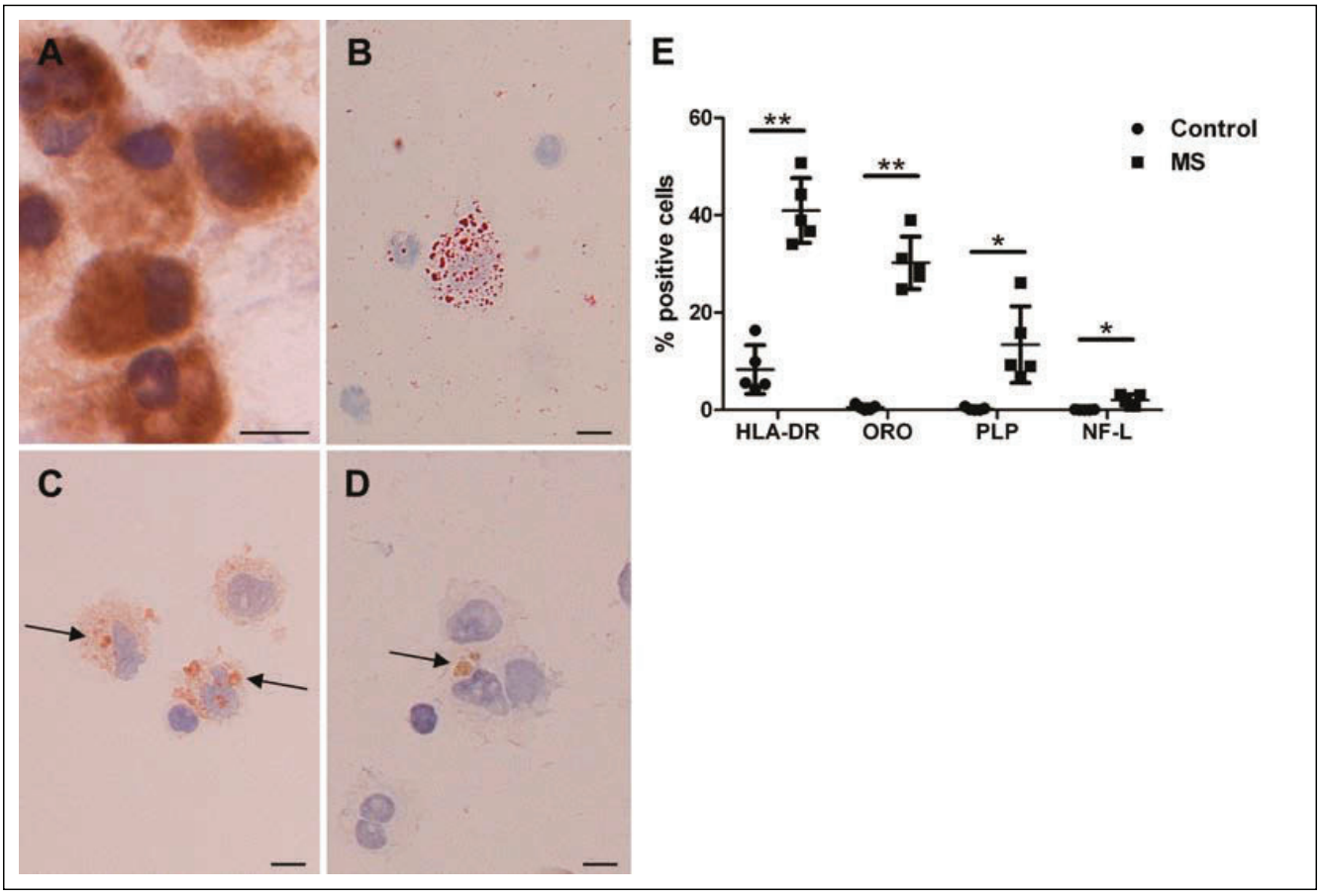

Figure 5. CSF cells phagocytosing neuronal and myelin proteins in MS. *CSF cells were collected at autopsy from patients with MS (n=5) and non-neurological controls (n=5) and examined for the expression of (**A**) HLA-DR molecules, (**B**) Oil-red-O deposits of neutral lipids, (**C**) PLP particles (arrows), and (**D**) NF-L particles (arrow). (**E**) The number of cells positive for HLA-DR expression or PLP and NF-L particles in CSF samples were compared with controls (** p<0.01; * p<0.05, Mann-Whitney U test). Scale bars=7.5 μm.*

Discussion

The presence of neuroaxonal debris as a result of neuronal and axonal damage in MS could contribute to ongoing neurodegeneration in MS. Several studies have shown that neuronal antigens are targets for autoimmune responses (20, 23, 24) and thus uptake and presentation of neuronal antigens may augment pathogenic autoimmune responses. Here, we demonstrate the presence of HLA-DR$^+$ cells in close association with axonal bulbs in MS lesions, particularly in areas of extensive axonal damage in MS lesions, however axon-engulfing HLA-DR$^+$ cells were not as frequent as myelin-containing HLA-DR$^+$ cells. *In vitro* primary human microglia and the mouse macrophage J774.2 cell line take up and degrade myelin and neuronal proteins present in human brain tissue. Despite the observation that both white and grey matter contain higher concentrations of NF-L compared to MBP, the *in vitro* studies do not reflect this and instead show that more cells take up myelin compared to neuronal antigen. Whether this is due to more rapid degradation of

neuronal antigens or higher uptake and slower degradation of myelin is unknown. Neuronal and myelin antigen-containing cells were also detected in the CSF of MS patients in significant higher numbers compared to cells in the CSF of control subjects. This suggests that after damage to myelin, axons and neurons during MS antigens may drain via the CSF to local lymph nodes in which myelin and neuronal antigen positive cells have been reported (27). Lastly, although myelin is present in the meninges in MS, as a reflection of demyelination (35), we did not detect neuronal antigens in the meningeal spaces despite extensive axonal damage (data not shown). These observations, together with our finding that NF-L is phagocytosed and degraded *in vitro* indicate that neuronal antigens could be presented to T cells in MS. Whether this leads to pathogenic autoimmunity or long-term tolerogenic effects depends on several factors. Although MHC class II is key for presentation of antigens and T cell receptor engagement, co-stimulatory molecules are responsible for the second signal that induce T cell activation and proliferation. These include CD80/CD86 interacting with CD28, and additional co-stimulatory molecules including members of the tumour necrosis family. The positive signals induced by these molecules are regulated by other co-stimulatory molecules, including cytotoxic T lymphocyte-associated antigen (CTLA)-4, programmed death-1, and B and T lymphocyte attenuator, which regulate the immune responses. While expression of co-stimulatory molecules has been reported in MS (36), current studies are underway to elucidate whether such expression in MS is also observed in cells engulfing neuronal antigens or indeed myelin.

Axonal damage is widely considered to be the underlying cause of the chronic irreversible neurological disability observed in patients with MS (10). Acute axonal damage reflected by disturbed axonal transport occurs early in MS and is most pronounced during active demyelination (11, 37). Axons may degenerate as a consequence of increased energy demands subsequent to myelin loss. Alternatively, it may result from nonspecific immune mediators. In addition, neuronal and axonal antigens may be the target of the immune response (18, 20, 21, 23, 24, 38). Similar to the idea that immune responses to myelin and myelin-associated antigens contribute to myelin destruction in MS, autoimmune responses to neurons could therefore contribute to axonal degeneration and/or grey matter pathology (17, 23). A crucial step for immune activation is processing and presentation of antigens by antigen-presenting cells. Our data support earlier studies showing that phagocytosis and degradation of apoptotic neurons by microglia was accomplished within 2 h (39). Rapid removal of degenerated myelin sheaths and axonal debris is required for axon regeneration and remyelination (40). In addition, persistence of tissue debris is more likely to lead to autoimmunity. This is apparent from mice deficient in milk fat globule-EGF factor 8 (MFG-E8), essential for apoptosis of cells. MFG-E8 deficient mice that fail to clear apoptotic cells develop lupus-like autoimmunity (41). Severe damage in the CNS and subsequent inefficient removal of antigens may result in the development of autoimmune responses in humans. In MS, neuronal antigens are readily detected in the CSF (42), in CLN (27) and, as we show here within cells in the CSF. That such release of neuronal antigens may be pathogenic has been shown in adoptive transfer of antibodies form mice with spinal cord injury that on intraspinal injection into healthy mice cause hind limb paralysis (43). Naturally occurring antibodies that distinguish damaged tissue as non-self are required for the rapid and efficient removal of damaged axons and myelin after nerve crush (44). Due to the immune privileged state of the CNS, there may be a failure of this antibody-mediated repair mechanism because naturally occurring antibodies may

be unable to cross the blood-brain barrier. Thus, on damage, CNS antigens may not be cleared efficiently, ultimately resulting in a humoral response that is directed to self-antigens, rather than altered-self antigens, aggravating neuronal damage in the CNS.

With regard to T cell responses, much of the evidence points to tolerogenic effects towards brain antigens. Phagocytosis of degenerating axons is observed in the entorhinal lesion model in mice (25) in which CD95L up regulation is associated with leukocyte apoptosis in areas of axonal damage. Autoreactive T cells secrete neurotrophins, regulate microglia activation and augment regeneration in the CNS (45, 46), arguing against the idea that release of neuronal antigens in brain injury could augment or initiate MS (29). Moreover, in mice experimental brain injury significantly reduces the clinical signs of EAE, considered an animal model of MS (28). Not only does brain trauma enhance T cell apoptosis but the presence of NF-L in CLN cells expressing MHC class II is associated with the expression of the pro-apoptotic gene BAD, increased T cell apoptosis and reduction of clinical signs of EAE. In MS, T cell responses to NF-L have been detected in the peripheral blood (19) but it remains unclear whether uptake of neuronal antigens in the CNS is sufficient for reactivation of the T cells and subsequent maturation into pathogenic effector cells. In MS humoral responses to neuronal antigens are also observed. Axon-reactive B cells are present in MS CNS, and antibodies to NF-L are present in the CSF of patients with MS (21, 47, 48). Antibodies to neuronal antigens isolated from patients with MS induce damage in animals (17, 20). How these antibodies arise is as yet unknown.

The detection and degradation of myelin proteins in phagocytosing macrophages in MS lesions has been used to estimate lesion age. Major myelin proteins such as MBP remain detectable in phagocytic vesicles inside macrophages for a longer period than the minor myelin protein, myelin oligodendrocyte glycoprotein (49) or the myelin-associated protein alpha B-crystallin (50). Because alpha B-crystallin is rapidly degraded within 6 h in macrophages *in vitro*, it has been suggested that the presence of alpha B-crystallin is the earliest marker of very recent myelin uptake. Here we show that phagocytosis and degradation of NF-L *in vitro* may also be a very rapid event because despite higher concentrations of NF-L in white and grey matter very few cells are positive for this antigen. This probably also explains the low frequency of HLA-DR$^+$ cells containing neuronal antigens in the MS lesions and the CSF samples. Nevertheless, the presence of neuronal antigens in macrophages/microglia although a rare event in established MS, can also be useful to assess the time lapse after axonal damage and better identify the factors directly involved in axonal damage.

In conclusion, this study provides evidence for phagocytic clearance of damaged axons in MS lesions. The processing of axonal antigens by HLA-DR positive cells may be important in the development of anti-neuronal responses.

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Supplementary Table 1. Binding characteristics of NF-L antibodies

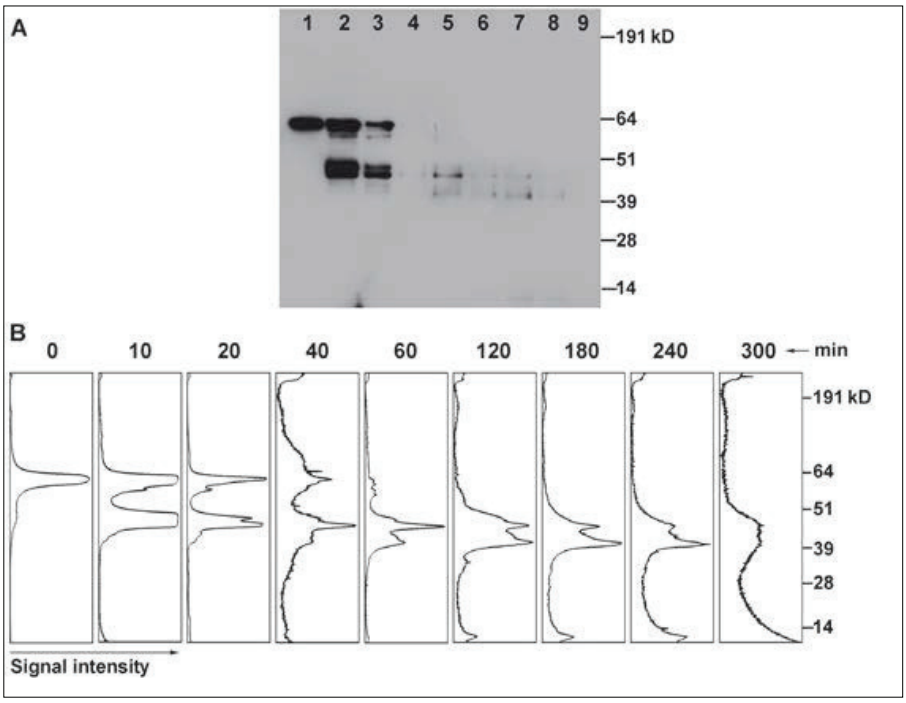
Antibody clone	Brain[a]	Tonsil[a]	Detection of NF-L breakdown products[b]
NF-L 4F8.1	+	++ (follicles)	+++
NF-L 5B4.2	+	+	n.d.
NF-L 10H9	+	-	++
NF-L DA2	+	++ (follicles)	+

[a]*immunohistochemistry;* [b]*Western blotting; n.d., not determined.*

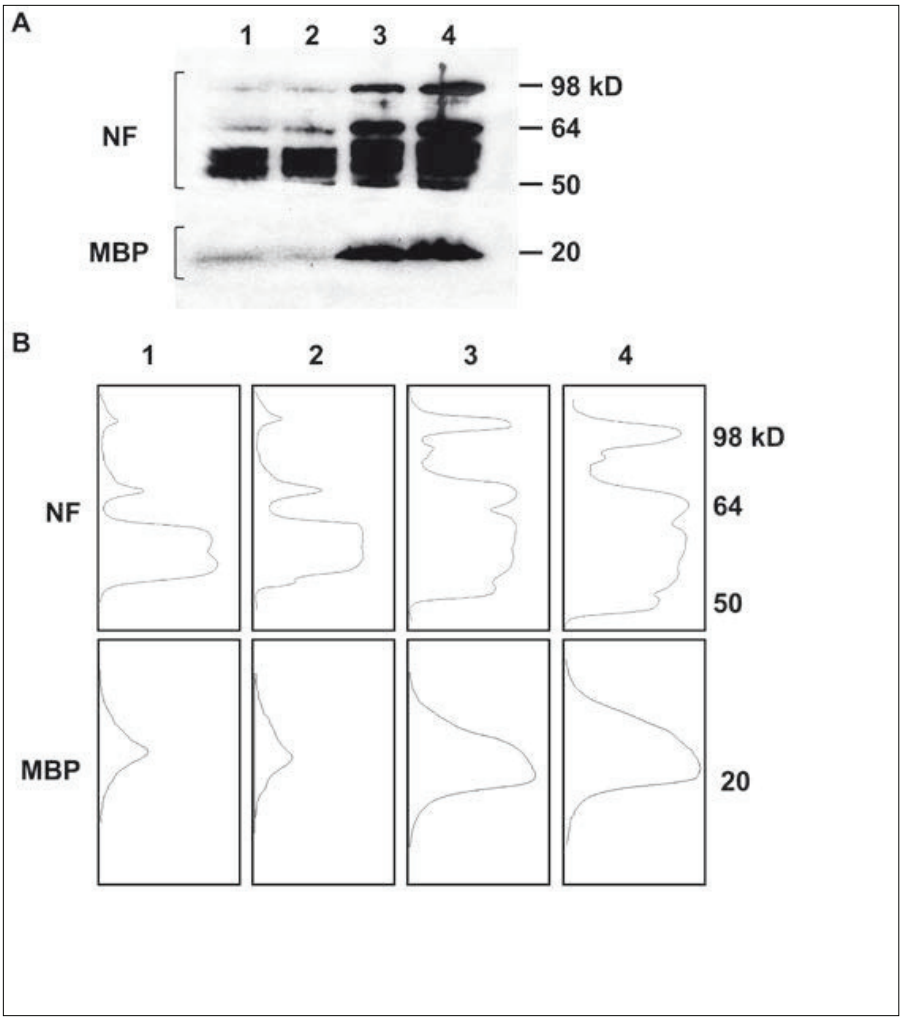
Supplementary Table 2. Number of HLA-DR$^+$ cells containing myelin or axonal proteins

						HLA-DR$^+$ cells /slide with		HLA-DR$^+$ cells /cm^2 with	
Patient and block	Area (mm^2)	No. HLA-DR$^+$ cells /slide	No. HLA-DR$^+$ cells /cm^2	No. APP$^+$ bulbs /slide	No. APP$^+$ bulbs /cm^2	Myelin Ag	Axonal Ag	Myelin Ag	Axonal Ag
1-1	519.07	78	15	53	10.2	3	0	0.58	0
1-2	267.48	4087	1528	>1100	>411.3	16	2	5.98	0.75
2-1	341.94	115	34	22	6.4	3	0	0.88	0
2-2	227.42	2355	1036	151	66.4	28	3	12.31	1.32
3-1	316.23	1315	416	>3000	>948.7	21	2	6.64	0.63
3-2	317.43	33434	10533	719	226.5	8	7	2.52	2.21
4	133.29	1970	1478	patches	n.d.	0	0	3.97	0.40
5	240.47	72	30	140	58.2	8	2	3.33	0.83
6	351.74	912	259	29	8.2	3	0	0.85	0
7	336.46	6808	2023	9	2.7	10	1	2.97	0.30
8	520.61	20478	3933	6	1.2	0	2	0	0.38
9	437.96	6817	1557	17	3.9	1	3	0.23	0.69
10-1	226.32	1351	597	15	6.6	1	3	0.44	1.33
10-2	232.14	33	14	17	7.3	8	0	3.45	0
11	265.29	23474	8848	2	0.8	11	0	4.15	0
12	278.66	194	70	135	48.4	114	13	40.91	4.66
13-1	252.05	5210	2067	11	4.4	10	1	3.97	0.40
13-2	417.04	424	102	9	2.2	1	0	0.24	0
14	388.71	882	227	445	114.5	14	9	3.61	2.32
15	n.d	n.d.	n.d.	586	n.d.	10	8	n.d.	n.d.

n.d., not determined



Supplementary Figure 1. Monoclonal antibody clone 10H9 recognises breakdown products of NF-L. ***A****) NF-L was digested using cathepsins B and D enzymes and western blotting performed to detect breakdown products with 10H9 mAb. Samples were taken prior to (lane 1) and 10, 20, 40, 60, 120, 180, 240, and 300 min of enzymatic treatment (lanes 2-9 respectively).* ***B****) Signal intensity (x-axis) of the bands in each lane (molecular weight, y-axis) was analysed using Image J software.*



Supplementary Figure 2. Quantification of neuronal and myelin proteins in human grey and white matter shows higher concentration of neurofilament compared to myelin. A*) Isolated proteins from human grey (lane 1 and 2) and white (lane 3 and 4) matter homogenates were loaded on 10% polyacrylamide gels (10 µg) and transferred onto nitrocellulose membranes. Amounts of NF and MBP were detected.* ***B****) Signal intensity (x-axis) of NF and MBP (molecular weight, y-axis) were quantified using Image J software. In grey matter, the signal of neurofilament is 7.5 times stronger than MBP. In white matter, the signal of neurofilament is 3 times stronger than MBP.*

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