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Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis

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Pathological and clinical studies implicate antibody-dependent mechanisms in the immunopathogenesis of multiple sclerosis. We tested this hypothesis directly by investigating the ability of patient-derived immunoglobulins to mediate demyelination and axonal injury *in vitro*. Using a myelinating culture system, we developed a sensitive and reproducible bioassay to detect and quantify these effects and applied this to investigate the pathogenic potential of immunoglobulin G preparations obtained from patients with multiple sclerosis ($n = 37$), other neurological diseases ($n = 10$) and healthy control donors ($n = 13$). This identified complement-dependent demyelinating immunoglobulin G responses in approximately 30% of patients with multiple sclerosis, which in two cases was accompanied by significant complement-dependent antibody mediated axonal loss. No pathogenic immunoglobulin G responses were detected in patients with other neurological disease or healthy controls, indicating that the presence of these demyelinating/axopathic autoantibodies is specific for a subset of patients with multiple sclerosis. Immunofluorescence microscopy revealed immunoglobulin G preparations with demyelinating activity contained antibodies that specifically decorated the surface of myelinating oligodendrocytes and their contiguous myelin sheaths. No other binding was observed indicating that the response is restricted to autoantigens expressed by terminally differentiated myelinating oligodendrocytes. In conclusion, our study identifies axopathic and/or demyelinating autoantibody responses in a subset of patients with multiple sclerosis. This observation underlines the mechanistic heterogeneity of multiple sclerosis and provides a rational explanation why some patients benefit from antibody depleting treatments.

Keywords: multiple sclerosis; demyelination; axonal injury; autoantibody

Abbreviations: MOG = myelin oligodendrocyte glycoprotein; PLP = proteolipid protein

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Introduction

The development of chronic disability in multiple sclerosis is associated with repeated episodes of inflammatory demyelination that lead to the formation of persistently demyelinated plaques of gliotic scar tissue and varying degrees of axonal loss (Steinman, 2001). Multiple sclerosis is generally considered an inflammatory T-cell-mediated autoimmune disease (Sospedra and Martin, 2005), but the efficacy of therapeutic strategies based on this concept is limited, indicating that additional mechanisms are involved in disease pathogenesis (Goodin *et al*., 2002). Circumstantial evidence suggests that these include antibody-dependent mechanisms, but formal evidence is lacking (Weber *et al*., 2011).

Immunopathological studies indicate that multiple effector pathways can contribute to lesion formation, the relative importance of which may vary between patients or during the course of the disease (Lucchinetti *et al*., 2000; Prineas *et al*., 2001). The involvement of antibodies in disease pathogenesis is supported by the clinical response of some patients to treatments known to inhibit antibody-mediated effects in other diseases (Vamvakas *et al*., 1995; Weinshenker, 2001; Keegan *et al*., 2002; Hauser *et al*., 2008; Hawker *et al*., 2009; Magaña *et al*., 2011), as well as the observation that actively demyelinating lesions are commonly associated with deposition of immunoglobulins and complement activation products (Lucchinetti *et al*., 2000; Breji *et al*., 2006). Local deposition of immunoglobulins and complement is also observed following antibody-mediated demyelination in animal models of multiple sclerosis (Storch *et al*., 1998), providing further credence for the hypothesis that autoantibody-dependent mechanisms are involved in the immunopathogenesis of multiple sclerosis (Genain *et al*., 1999; Raine *et al*., 1999). However, this interpretation has been questioned as complement and immunoglobulin deposition is observed in other neurological diseases (Barnett *et al*., 2009). The importance of resolving whether or not antibody-dependent mechanisms play an important role in multiple sclerosis is demonstrated by the major improvements in disease diagnosis and treatment that followed identification of pathogenic autoantibodies in myasthenia gravis and other autoimmune diseases (Leandro *et al*., 2002; Richman and Agius, 2003; Edwards and Cambridge, 2005; Jarius and Wildemann, 2010).

Unravelling the role of antibodies in multiple sclerosis is not only complicated by the mechanistic heterogeneity of the disease, but also the complexity of the disease associated autoantibody repertoire. Numerous autoantibody specificities are associated with multiple sclerosis, but their pathogenic relevance remains unclear (Kanter *et al*., 2006; Zhou *et al*., 2006; Mathey *et al*., 2007; Quintana *et al*., 2008; Derfuss *et al*., 2009). The archetypal example is myelin oligodendrocyte glycoprotein (MOG), which was first identified as a target for demyelinating autoantibodies in experimental autoimmune encephalomyelitis (Linington *et al*., 1988). Elevated titres of MOG-specific autoantibodies are present in patients with multiple sclerosis (Sun *et al*., 1991; Lindert *et al*., 1999; Gaertner *et al*., 2004; Lalive *et al*., 2006), but subsequent studies revealed this response, as detected by ELISA, western blot or radioimmunoassay (Xiao *et al*., 1991; Reindl *et al*., 1999; Lampasona *et al*., 2004), is not disease-specific. Only the introduction of cell-based assays that identify antibody responses, which recognize the native protein, clarified the situation (Haase *et al*., 2001; Zhou *et al*., 2006), identifying potentially pathogenic MOG-specific autoantibodies in cases of acute disseminated encephalomyelitis and paediatric multiple sclerosis but only rarely in adult onset disease (Zhou *et al*., 2006; McLaughlin *et al*., 2009; Lalive *et al*., 2011).

In order to determine if pathogenic autoantibody responses are present in patients with adult onset multiple sclerosis, we developed a sensitive bioassay to identify demyelinating and/or axopathic autoantibodies in clinical samples that required no prior knowledge of their antigen-specificity. This assay uses a highly reproducible myelinating culture system (Thomson *et al*., 2008) to quantify antibody-mediated, complement-dependent effects on myelin and axons *in vitro*. This approach was first described >40 years ago when myelinating tissue explants (Bornstein and Murray, 1958) were used to investigate the demyelinating potential of experimental autoimmune encephalomyelitis sera (Appel and Bornstein, 1964). These studies demonstrated that sera from animals immunized with CNS tissue homogenates not only contained demyelinating antibodies but also myelination-inhibiting and neuroelectric blocking factors (Seil *et al*., 1968, 1975); experiments that eventually identified MOG as a target for demyelinating autoantibodies in experimental autoimmune encephalomyelitis (Lebar *et al*., 1986; Linington and Lassmann, 1987; Schluesener *et al*., 1987). However, this approach was far less successful when used to identify disease-specific responses in patient sera (Caspary, 1977; Seil, 1977). This was due in part to the variability of myelination in the culture models available at the time, a problem compounded by the lack of objective techniques to quantify partial myelin loss. Nonetheless, demyelinating immunoglobulin G (IgG) responses were identified in a small percentage of patients (Grundke-Iqbal and Bornstein, 1980), although the nature of the serum factor(s) responsible for inhibiting electrical activity in the cultures remained controversial (Seil *et al*., 1976; Seil, 1977). Subsequent advances in tissue culture techniques, imaging and data analysis allowed us to build on these pioneering studies to develop an assay to quantify complement-dependent demyelination mediated by physiologically relevant antibody concentrations *in vitro*.

We have now used this bioassay to investigate the pathogenic potential of IgG autoantibodies in patients with multiple sclerosis, other neurological diseases and healthy controls. We identified demyelinating autoantibodies in approximately one-third of the patients with multiple sclerosis investigated in this study, but in none of the other donors. We also identified an axopathic response in two patients, confirming previous speculation that autoantibodies may contribute to the development of axonal pathology in multiple sclerosis (Mathey *et al*., 2007). Immunofluorescence microscopy and *in vitro* adsorption demonstrate that this pathogenic response targets myelin associated antigens expressed at the surface of highly differentiated myelinating oligodendrocytes. The confirmation that demyelinating and axopathic autoantibodies are present in some patients with multiple sclerosis may have significant implications for their clinical management, as plasma exchange, high-dose immunoglobulins and complement

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Table 1 Clinical data of multiple sclerosis patients, disease and healthy controls included in this study

Patient ID	Age (years)	Gender	Diagnosis	Disease duration (months)
MS 1	26	Female	Marburg's MS*	96
MS 2	42	Female	RRMS*	300
MS 3	45	Male	RRMS*	12
MS 4	49	Female	RRMS*	84
MS 5	29	Male	SPMS*	60
MS 6	49	Female	RRMS*	132
MS 7	31	Female	RRMS*	7
MS 8	52	Female	RRMS	120
MS 9	44	Female	RRMS	84
MS 10	46	Male	RRMS	60
MS 11	43	Female	RRMS	96
MS 12	43	Female	RRMS	84
MS 13	41	Female	RRMS	72
MS 14	59	Female	RRMS	72
MS 15	53	Female	RRMS	60
MS 16	34	Male	RRMS	84
MS 17	57	Female	PPMS	96
MS 18	56	Female	PPMS	120
MS 19	47	Male	PPMS	132
MS 20	46	Female	PPMS	84
MS 21	38	Male	PPMS	168
MS 22	44	Male	PPMS	84
MS 23	45	Female	PPMS	85
MS 24	57	Male	PPMS	96
MS 25	44	Female	PPMS	60
MS 26	53	Female	PPMS	36
MS 27	44	Female	PPMS	108
MS 28	42	Female	PPMS	60
MS 29	42	Female	PPMS	60
MS 30	63	Female	PPMS	48
MS 31	37	Male	PPMS	72
MS 32	54	Male	PPMS	48
MS 33	47	Male	PPMS	8
MS 34	50	Female	SPMS	324
MS 35	54	Male	RRMS*	N/A
MS 36	38	Female	RRMS*	N/A
MS 37	32	Female	RRMS*	N/A
OND 1	38	Male	SPN*	108
OND 2	49	Female	GBS*	6
OND 3	55	Female	GBS*	6
OND 4	24	Male	GBS*	6
OND 5	62	Male	CIDP*	6
OND 6	55	Female	CIDP*	240
OND 7	79	Male	CIDP*	48
OND 8	71	Male	ISAN*	1
OND 9	62	Female	MG*	60
OND 10	63	Female	CIDP*	96
HC 1	45	Female	Healthy control	–
HC 2	31	Female	Healthy control	–
HC 3	46	Male	Healthy control	–
HC 4	33	Female	Healthy control	–
HC 5	27	Female	Healthy control	–
HC 6	28	Female	Healthy control	–
HC 7	32	Female	Healthy control	–
HC 8	50	Female	Healthy control	–
HC 9	31	Female	Healthy control	–
HC 10	60	Male	Healthy control	–
HC 11	24	Female	Healthy control	–
HC 12	57	Male	Healthy control	–
HC 13	37	Female	Healthy control	–

CIDP = chronic idiopathic polyneuropathy; GBS = Guillian-Barré syndrome; ISAN = idiopathic sensory ataxic neuropathy; MG = myasthenia gravis; PPMS = primary progressive multiple sclerosis; RRMS = relapsing remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; SPN = sensory motor polyneuropathy.
*Denotes patients undergoing plasma exchange.

inhibitors are just some of the treatments available that are known to suppress antibody-mediated effects in other diseases.

Materials and methods

Patient samples

Sera and plasma were collected from patients at the Southern General Hospital (Glasgow, UK), St. Josef-Hospital (Bochum, Germany), University of Heidelberg (Heidelberg, Germany) and the Ludwig-Maximilians-University Clinic Grosshadern (Munich, Germany). Each site collected samples using a protocol approved by their Institutional Review Board, and informed consent was obtained from all subjects. Samples were stored at −80°C in aliquots. We collected samples from 37 cases of clinically definite multiple sclerosis (female:male ratio 2:1; age range 26–59; mean age 48 years) as defined using the Poser or McDonald criteria (Poser *et al*., 1983; McDonald *et al*., 2001). Seven patients with multiple sclerosis (Patients MS1–7) were sampled during an acute steroid non-responsive relapse, which was treated by plasma exchange. Control samples were taken from 10 patients with other neurological diseases (female:male ratio 1:1; age range 24–79; mean age 56 years) and from 13 healthy donors (female:male ratio 3:1; age range 24–60; mean age 39 years; Table 1). Total IgG preparations were purified from each sample by protein G immunoaffinity chromatography using 1 ml HiTrap™ protein G fast flow columns (GE Healthcare) following the manufacturer's guidelines and stored at −80°C.

Assaying antibody-mediated demyelination and axonal loss *in vitro*

In vitro myelinating cultures were established as previously described (Sorenson *et al*., 2008). Briefly, a single cell suspension was prepared from embryonic Day 15.5 rat spinal cord (Sprague Dawley) and plated on to a confluent monolayer of neurosphere-derived astrocytes in plating media (50% Dulbecco's modified Eagle medium, 25% heat inactivated horse serum, 25% Hank's balanced salt solution with Ca^{2+} and Mg^{2+}, and 2 mM L-glutamine; Invitrogen) at a density of 150 000 cells/100 µl/13 mm diameter cover-slip. Cells were left to attach for 2 h at 37°C after which an additional 300 µl of plating media was added and 500 µl of differentiation medium, which contained Dulbecco's modified Eagle medium (4500 mg/ml glucose), 10 ng/ml

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biotin and 0.5% N1 hormone mixture [1 mg/ml apotransferrin, 20 mM putrescine, 4 μM progesterone, 6 μM selenium, 50 nM hydrocortisone and 0.5 mg/ml insulin (Sigma)]. Cultures were maintained at 37°C/7% CO_2 and fed three times a week by replacing half the culture medium with fresh differentiation media. Twelve days later insulin was omitted from the culture medium to promote myelination and after 28 days *in vitro*, these cultures were used as targets to investigate antibody-mediated, complement-dependent effects on myelin and axons. Immunoglobulins were added to cultures at the concentrations indicated in the text either in the presence or absence of 1% fresh rat sera as a source of complement. Additional cultures were treated with antibody alone or in combination with heat inactivated serum (56°C for 30 min) as additional controls. Each sample was analysed in triplicate using independent cultures.

Immunocytochemistry

The following antibodies were used. Mouse monoclonal antibodies: SMI-31 (phosphorylated neurofilament, Abcam), 8-18C5 (anti-MOG; Linington *et al*., 1988); Z2 (anti-MOG; Piddlesden *et al*., 1993), pan-NaV (Sigma), A12/18.1 (pan-Nfasc; Mathey *et al*., 2007), O4 (sulphatide, Millipore), NG2 (chondroitin sulphate proteoglycans, Millipore), TAG-1 (4D7 and 3.13C2; Derfuss *et al*., 2009) and anti-myelin proteolipid protein (PLP; cytoplasmic epitope common to PLP and DM20, Chemicon). Rabbit polyclonal antibodies: anti-aquaporin-IV (Abcam), anti-Caspr (Abcam), anti-neurofascin-155 (E. Meinl; Tait *et al*., 2000) and anti-neurofascin-186 (a kind gift from P. Brophy, University of Edinburgh). Species and isotype specific secondary antibodies labelled with Alexa Fluor 350®, Alexa Fluor 488® or Alexa Fluor 555® (Invitrogen). To visualize cell surface determinants on live cells, primary antibody or patient derived IgG fractions were applied for 30–45 min at 4°C after which cultures were washed in ice cold Dulbecco's modified Eagle medium before the appropriate secondary antibody was added for a further 30 min at 4°C. After extensive washing in Dulbecco's modified Eagle medium at 4°C cells were fixed in 4% paraformaldehyde for 15 min at room temperature. If required, cytoplasmic antigens were detected after permeabilization with 0.5% Triton X-100/phosphate-buffered saline for 10 min (Sigma) followed by 1 h in 1% bovine serum albumin/10% normal goat serum/0.3 M glycine at room temperature. Primary antibodies recognizing cytoplasmic or integral membrane proteins were then applied for 1 h at room temperature after which cover-slips were washed extensively in phosphate-buffered saline and the appropriate fluorochrome-conjugated secondary antibodies added for 45 min at room temperature (Alexa Fluor, Invitrogen). Unbound secondary antibody was removed by washing with phosphate-buffered saline followed by distilled water and mounted in Vectashield® (Vector laboratories).

Image capture and analysis

In each case, a minimum of 10 images (×10 magnification) were acquired from each of three cover-slips using an Olympus BX51 fluorescent microscope and Image-Pro software (Media Cybernetics). Axonal density was quantified using ImageJ software (NIH systems, version 1.41o) to obtain values for the areas of SMI-31 immunoreactivity and the total field. Axonal density was calculated by expressing the SMI-31$^+$ area as a percentage of the total field area. To calculate the percentage of myelinated axons, immunoreactivity associated with MOG$^+$ or PLP$^+$ myelin sheaths was also measured using ImageJ after they were manually highlighted using Adobe Photoshop Elements 4.0 (brush shape size 9). Percentage myelin was then calculated by dividing the area of highlighted myelin by axonal density. In order to expedite sample screening, quantification of myelination was automated using the pattern recognition based algorithm (BRAINS BATCH) and found that the values obtained were comparable to those obtained manually (Supplementary material).

To quantify cell numbers, a minimum of 30 images were taken from three cover-slips (×20 magnification) and immunopositive cells associated with a DAPI$^+$ nucleus were counted using the ImageJ cell counter function (NIH systems, version 1.41o). Cell counts were expressed as a percentage of the total number of DAPI$^+$ nuclei within the same focal plane.

Adsorption of myelin, neurofascin-155 and myelin oligodendrocyte glycoprotein antibodies

Myelin

Myelin was isolated from whole rat brain by differential sucrose gradient centrifugation (Norton and Podulso, 1973), washed repeatedly by centrifugation in phosphate-buffered saline containing protease inhibitors (protease inhibitor cocktail I; Sigma) and stored in phosphate-buffered saline at −80°C. To absorb myelin reactive antibodies, human IgG was incubated with myelin in the presence of protease inhibitors at a protein ratio 1:20 in phosphate-buffered saline for 16 h at 4°C. Myelin and bound immunoglobulin was pelleted by centrifugation (30 min at 12 000*g*) and IgG remaining in the supernatant stored at −20°C. Integrity of unbound immunoglobulin was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Neurofascin-155

To deplete the autoantibody-repertoire of components recognizing neurofascin-155 selected plasma samples were diluted with phosphate-buffered saline, filtered and brought to pH 7 before immunoaffinity chromatography at 4°C over recombinant rat neurofascin-155 (rrNfasc; 500 μg, R&D Systems) bound to an activated NHS (*N*-hydroxysuccinimide) column (GE Healthcare). After extensive washing with phosphate-buffered saline, bound immunoglobulins were eluted with 0.1 M glycine at pH 2.7 and immediately neutralized with 1 M Tris pH 9. IgG fractions depleted of rrNfasc reactivity were then prepared by protein G affinity chromatography. Protein concentrations were determined using a Nanodrop spectrophotometer (Thermo Scientific) and depletion of Nfasc reactive components of the antibody repertoire was confirmed by ELISA. After adsorption, fractions were readjusted to 100 μg/ml for use in the bioassay.

Myelin oligodendrocyte glycoprotein

Depletion the MOG specific autoantibody-repertoire in selected IgG samples was performed by solid phase immunoabsorption with recombinant rat MOG (refolded) bound to an activated NHS agarose column (GE Healthcare). After extensive washing with MOG, bound immunoglobulins were eluted with 0.1 M glycine pH 2.7 and immediately neutralized with 1 M Tris pH 9. Protein concentrations were determined using a Nanodrop spectrophotometer (Thermo Scientific) and depletion of MOG reactive components of the antibody repertoire was confirmed by ELISA. After adsorption, fractions were readjusted to 100 μg/ml for use in the bioassay. The efficacy of adsorption was tested using non-pathogenic patient derived IgG doped with 1 μg of Z2, which demonstrated that virtually all demyelinating activity is depleted post-adsorption.

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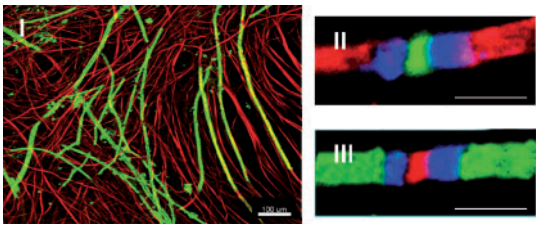
A



B

Ab specificity	Species	Isotype	% Axonal loss	% Myelin loss
MOG (Z2)	mouse	IgG2a	2 ± 2.2	**100 ± 0.9****
MOG (8-18C5)	mouse	IgG1	1.2 ± 3.2	**98 ±1.2****
Sulphatide (O4)	mouse	IgM	1.5 ± 1.8	**100 ±2.9****
PLP	rabbit	IgG	0 ± 2.0	0 ± 3.7
MBP	mouse	IgG2a	0 ± 3.6	0 ± 2.5
TAG -1 (4D7)	mouse	IgM	1.4 ± 2.6	0 ± 2.9
TAG-1 (3.13C2)	mouse	IgG1	2.3 ± 1.2	2 ± 1.3
Nfasc186	rabbit	IgG	**15 ±3.6***	**97 ±2.4****
Nfasc 155/186 (A12/18.1)	mouse	IgG2a	**40 ±3.2****	**100 ±0.8****
Myeloma Protein **(MOPC1)**	mouse	IgG1	0 ± 2.4	0± 2.0
Myeloma Protein (UPC10)	mouse	IgG2a	1 ± 3.0	0 ± 2.2

Figure 1 Myelinating cultures reproduce the major characteristics of *in vivo* CNS-myelinated axons. [**A(I)**] *In vitro* myelinating cultures consist of a network of SMI-31$^+$ axons (red, phosphorylated neurofilament staining) some of which are myelinated by PLP$^+$ oligodendrocytes (green; ×20 magnification; scale bar = 100 μm). These myelin internodes are interspaced by nodes of Ranvier characterized by axonal expression of Nfasc186/NaV bounded by Caspr/Nfasc155 at the paranode. [**A(II)**] Caspr, blue; PLP, red; Nfasc186, green (×100 magnification, scale bar = 10 μm); [**A(III)**] Nfasc155, blue; PLP, green; NaV, red (×100 magnification, scale bar = 10 μm). (**B**) To validate using these myelinating cultures as a target for pathogenic antibody responses, we used a panel of antibodies against a number of targets previously implicated in multiple sclerosis. In each case, cultures were treated overnight with 10 μg/ml antibody in the presence of 1% fresh rat serum as a source of complement. Axonal density and myelination were quantified using computer aided analysis of immunofluorescent images. The pathogenic activity of each antibody was highly selective and dependent on its targets localization. Treatment with antibodies recognizing antigens accessible at the myelin/oligodendrocyte surface such as MOG (Z2/8-18C5) or sulphatide (O4) results in complete and selective demyelination with no significant axonal injury. In contrast, antibodies recognizing intracellular epitopes such as myelin basic protein (MBP) or the C-terminus of PLP were unable to induce myelin loss. Targeting axons using an antibody against Nfasc186 mediates significant axonal loss and secondary demyelination. These antibody-mediated effects were complement-dependent as heat inactivation of serum abolished all antibody mediated effects. Antibody activity is also antigen specific as demonstrated using isotype controls (MOPC1, UPC10). Percentage values for axonal loss and demyelination were calculated in relation to untreated cultures (mean ± SD, $n = 3$; $^*P < 0.05$, $^{**}P < 0.001$, *t*-test).

Enzyme-linked immunosorbent assay

ELISA was performed using 96-well polyvinyl ELISA plates (Costar) coated overnight with myelin (10 μg protein/ml), rat MOG or rrNfasc (1.0 μg/well) at 4°C in phosphate-buffered saline. After blocking with 1% bovine serum albumin/phosphate-buffered saline for 1 h at 37°C, dilutions of sera or human immunoglobulins were added and binding was detected using appropriate anti-human immunoglobulin horseradish peroxidase conjugates (Dako). All samples were analysed in triplicate.

Results

Detection of demyelinating autoantibodies *in vitro*

Myelinating cultures were generated from dissociated embryonic rat spinal cord, as previously described (Sorenson *et al.*, 2008). Oligodendrocytes in these cultures elaborate multiple myelin sheaths associated with formation of Caspr$^+$/neurofascin-155$^+$ paranodal junctional complexes and sequestration of neurofascin-186 and voltage-gated sodium channels at nodes of Ranvier (Ratcliffe *et al.*, 2001; Charles *et al.*, 2002; Sorenson *et al.*, 2008; Fig. 1A). To confirm these cultures reproduce the molecular organization and accessibility of autoantigens present on myelinated axons *in vivo*, they were incubated with antibodies specific for myelin and axoglial antigens in the presence or absence of rat serum as a source of complement (Fig. 1B). Recognition of antigens exposed at the myelin/oligodendrocyte surface, such as MOG or sulphatide, resulted in complement-dependent demyelination but no significant axonal loss. In contrast, antibody binding to neurofascin-186 on the axonal surface resulted in axonal loss, accompanied by a complete but secondary demyelination. Antibodies directed against antigens associated with the cytoplasmic face of the myelin membrane such as myelin basic protein and PLP were unable to mediate any detectable effect *in vitro* due to the inaccessibility of the antigen. The same was true for transient axonal glycoprotein-1 (TAG-1), an antigen sequestered within the juxtaparanodal domain of myelinated fibres (Traka *et al.*, 2003), but in this case, access of antibody to its target is restricted by the diffusion barrier provided by the paranodal junctional complex (Rios *et al.*, 2003). Heat inactivation abolished the ability of rat serum to support these antibody-mediated effects indicating they were complement-dependent.

The ability of these myelinating cultures to provide a sensitive bioassay to detect demyelinating autoantibodies was established using the MOG-specific monoclonal antibody Z2. A lag of ~3 h

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occurred between addition of antibody and serum and significant loss of myelin sheaths, although this was preceded by earlier antibody/complement mediated oligodendrocyte injury associated with deposition of membrane attack complex (Supplementary material). Thereafter, loss of myelin sheaths as determined by immunoreactivity for PLP co-localized on SMI-31$^+$ axons was complete within 4 h. Complement-mediated antibody-dependent demyelination in this system had no effect on axonal density even after 16 h. Half maximal loss of myelin was obtained at an antibody concentration of 50 ng/ml (~300 pM). At this concentration, monoclonal antibody Z2 mediated 50.2 ± 6.5% [mean ± standard deviation (SD), $n = 3$; $P < 10^{-5}$) demyelination, but no significant axonal loss as determined by SMI-31 immunoreactivity (0.4 ± 0.26%, $P > 0.5$; Fig. 2).

Identification of demyelinating and axopathic autoantibodies in patients with multiple sclerosis

We investigated sera from patients with multiple sclerosis ($n = 37$), patients with other neurological diseases ($n = 10$) and healthy controls ($n = 13$) for the presence of pathogenic autoantibodies using purified IgG to avoid potential artefacts due to myelinotoxic serum components (Table 1). Purified IgG (100 μg/ml) was added in the presence of 1% rat serum and SMI-31 immunoreactivity and myelination determined 16 h later. Additional cultures were treated with fresh rat serum alone, as well as IgG in the presence of heat-inactivated serum to control for complement- and/or antibody-independent effects. Complement-dependent demyelinating IgG responses were detected in sera from half the multiple sclerosis cases, but none of the patients with other neurological diseases or healthy control sera (Table 2). In two patients with multiple sclerosis, this demyelinating antibody response was accompanied by significant axopathic activity (Patient MS5, axonal loss = 34 ± 5.7%; Patient MS14, axonal loss = 22 ± 3.5%) confirming previous speculation that antibody-dependent mechanisms may contribute to the development of axonal pathology in some patients (Derfuss *et al*., 2010). Repeating the study at an IgG concentration of 1 mg/ml did not identify additional seropositive donors (Supplementary material). Dose response experiments revealed that IgG mediated, complement-dependent demyelination and axonal loss were significantly reduced at concentrations <100 μg/ml and were no longer detectable at 1 μg/ml (Fig. 3). Extrapolating from results obtained using the MOG-specific monoclonal antibody Z2 these results indicate these human IgG preparations contain ~5–50 ng of demyelinating antibody/100 μg IgG, which would equate to a serum concentration of ~3–30 nM.

Although occasional cases of primary progressive multiple sclerosis were seropositive for a demyelinating response, the frequency of seropositive cases was significantly higher in patients with relapsing remitting multiple sclerosis (Fishers Exact Test, $P = 0.0103$). To investigate the effect of plasma exchange on this pathogenic response, we identified a further three seropositive cases of relapsing remitting multiple sclerosis for which samples were available from both the initial and final cycles of plasma exchange. This revealed that, at least in this small patient group, plasma exchange significantly reduced demyelinating IgG activity (Table 3).

The demyelinating response is directed against terminally differentiated oligodendrocytes

Previous studies suggested that in addition to targeting myelinated axons, autoantibodies may also target oligodendrocyte progenitor cells (Niehaus *et al*., 2000; Lilly *et al*., 2004). To determine if this was the case, live, unfixed myelinating cultures were stained with representative IgG preparations and their distribution determined by immunofluorescence microscopy. When incubated in the absence of complement at 4°C IgG (100 μg/ml), demyelinating activity decorated the surface of PLP$^+$ myelin sheaths as well as contiguous oligodendrocyte processes and cell bodies (Fig. 4A). No other cell type was recognized. No specific staining was observed in seronegative patients (Fig. 4B). We were unable to observe any specific staining that co-localized with neuronal/axonal structures. However, this may be obscured by the myelin reactive component that aligns with the ensheathed axon.

These observations suggest that the specificity of the demyelinating autoantibody response is restricted to antigens expressed at the surface of terminally differentiated myelinating oligodendrocytes. However, as some myelin-associated antigens (such as O4) are expressed by oligodendrocyte progenitor cells prior to myelination, these cells may also be targeted by the pathogenic autoantibody response (Niehaus *et al*., 2000; Kanter *et al*., 2006). We therefore investigated the ability of patient-derived IgG preparations to mediate a complement-dependent decrease in the number of early (NG2$^+$) oligodendrocyte progenitor cells, as well as later O4$^+$ oligodendrocyte progenitor cells and pre-myelinating oligodendrocytes (Table 4; Trapp *et al*., 1997). There was no significant effect on the NG2$^+$ population indicating that these cells are not targeted by the pathogenic autoantibody response. In contrast, IgG preparations with demyelinating activity reduced the number of O4$^+$ cells by 20–40%. A similar reduction was seen in cultures treated with a MOG-specific monoclonal antibody (Z2) indicating that this reflects a loss of mature MOG$^+$ oligodendrocytes that continue to express the O4 marker. Treatment with the O4 monoclonal antibody itself not only induced complete demyelination but virtually eliminated O4$^+$ cells from the cultures and reduced the NG2$^+$ population by ~40% indicating loss of a population of NG2$^+$/O4$^+$ progenitors. The survival of large numbers of O4$^+$ cells in cultures treated with patient-derived IgG preparations indicates that contrary to previous studies (Ilyas *et al*., 2003; Kanter *et al*., 2006) sulphatide, the O4 antigen was not a dominant target for the pathogenic autoantibody response in this cohort of patients.

Intra-patient heterogeneity in the specificity of the pathogenic response

Immunofluorescence microscopy indicates the target(s) of the demyelinating response is restricted to terminally differentiated oligodendrocytes and their contiguous myelin sheaths. Currently,

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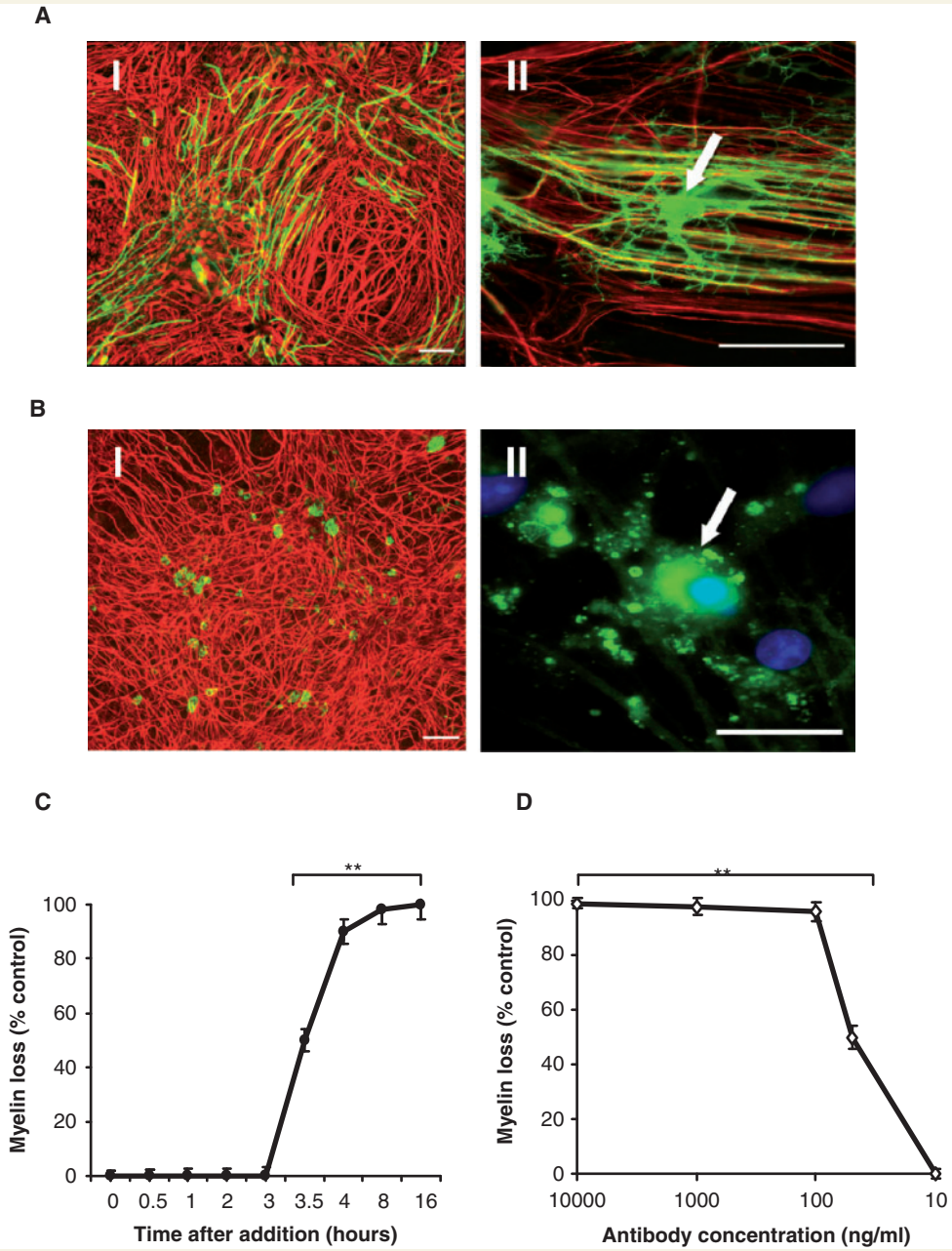

Figure 2 Targeting MOG induces selective demyelination without causing axonal injury. [**A**(I)] Overnight treatment of myelinating cultures with 10 μg/ml Z2 (MOG-specific monoclonal antibody) in the absence of complement does not induce axonal injury as visualized by intact SMI-31 (phosphorylated neurofilament) staining (red) or demyelination visualized with PLP (green; ×10 magnification; scale bar = 100 μm). High magnification images show oligodendrocytes are unaffected after overnight treatment with Z2 in the absence of a source of complement [**A**(**II**)]; ×60 magnification; scale bar = 50 μm). [**B**(**I**)] In contrast, antibody treatment in the presence of fresh rat sera as a source of complement (1%)-induced complete demyelination and loss of oligodendrocytes but leaving an intact bed of axons (×10 magnification; scale bar = 100 μm). [**B**(**II**)] Antibody-mediated demyelination is associated with oligodendrocyte lysis (PLP, green; nucleus, blue; ×100 magnification; scale bar = 50 μm). (**C**) Demyelination occurs rapidly after antibody addition. Significant demyelination was first detected after 3.5 h and by 4 h demyelination is virtually complete. Percentage values for axonal loss and demyelination were calculated in relation to cultures treated with equivalent antibody concentrations in the presence of heat inactivated complement (mean ± SD, $n = 3$; $^{**}P < 0.001$, *t*-test). (**D**) Myelinating cultures provide a highly sensitive screening strategy in which to detect pathogenic autoantibody responses. Significant demyelinating activity is detectable at antibody concentrations ⩾50 ng/ml (~300 pM). Percentage values of demyelination were calculated in relation to cultures treated with equivalent concentrations of an isotype control antibody (mean ± SD, $n = 3$; $^{**}P < 0.001$, *t*-test).

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Table 2 IgG derived from patients with multiple sclerosis mediates complement dependent demyelination and axonal injury *in vitro*

Patient ID	Diagnosis	+ Active complement		Heat inactivated complement	
		% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss
MS 1	Marburg MS*	2 ± 2.9	95 ± 3.5**	0 ± 2.4	5.2 ± 5.5
MS 2	RRMS*	3 ± 2.6	96 ± 4.0**	2 ± 3.4	4.9 ± 5.0
MS 3	RRMS*	4 ± 3.0	97 ± 2.2**	2 ± 1.9	3.2 ± 2.7
MS 4	RRMS*	2 ± 2.1	98 ± 1.3**	4 ± 2.9	0 ± 3.3
MS 5	SPMS*	34 ± 5.7**	92 ± 2.2**	1 ± 3.5	2 ± 2.4
MS 6	RRMS*	2 ± 3.0	0 ± 0	0 ± 3.7	0 ± 0.1
MS 7	RRMS*	3 ± 3.7	94 ± 2.0**	3 ± 3.7	4.9 ± 2.4
MS 8	RRMS	0 ± 2.2	0 ± 4.6	0 ± 2.4	0 ± 2.6
MS 9	RRMS	0 ± 2.0	0 ± 3.7	0 ± 2.8	0 ± 1.9
MS 10	RRMS	0 ± 2.3	0 ± 4.0	0 ± 2.9	0 ± 4.0
MS 11	RRMS	0 ± 2.1	95 ± 2.0**	0 ± 3.4	0 ± 2.0
MS 12	RRMS	0 ± 2.5	93 ± 1.7**	0 ± 4.8	0 ± 1.7
MS 13	RRMS	3 ± 2.4	1.5 ± 4.1	3 ± 2.6	0 ± 1.1
MS 14	RRMS	22 ± 3.5**	96 ± 3.2**	2 ± 3.5	0 ± 3.2
MS 15	RRMS	0 ± 2.1	3 ± 4.2	1 ± 2.3	3.4 ± 3.1
MS 16	RRMS	0 ± 2.2	94 ± 2.9**	1.6 ± 1.2	4.0 ± 1.9
MS 17	PPMS	0 ± 2.9	1.6 ± 4.0	1.4 ± 2.8	3.6 ± 1.4
MS 18	PPMS	0 ± 2.3	0 ± 4.5	2.5 ± 2.7	0 ± 4.5
MS 19	PPMS	0 ± 2.0	1.2 ± 4.3	4 ± 2.0	1.4 ± 4.5
MS 20	PPMS	1 ± 0.6	4.6 ± 4.0	1.9 ± 2.6	3.2 ± 4.6
MS 21	PPMS	3.1 ± 1.8	4.2 ± 3.1	2.9 ± 2.1	4.2 ± 3.1
MS 22	PPMS	1.7 ± 0.9	3.9 ± 1.6	1.6 ± 1.1	3.9 ± 1.7
MS 23	PPMS	0 ± 1.6	2.6 ± 1.5	1.9 ± 1.2	3.4 ± 2.1
MS 24	PPMS	3 ± 1.8	5.3 ± 2.1	2.1 ± 1.8	2.8 ± 2.6
MS 25	PPMS	2.8 ± 1.8	3.8 ± 1.9	3.6 ± 1.7	4.1 ± 3.8
MS 26	PPMS	4.3 ± 2.1	5.5 ± 3.6	4.1 ± 3.4	5.5 ± 3.6
MS 27	PPMS	3.4 ± 1.9	4.8 ± 3.1	3.7 ± 2.6	4.5 ± 2.8
MS 28	PPMS	3.2 ± 1.7	2.8 ± 1.4	4.3 ± 1.7	6.5 ± 4.9
MS 29	PPMS	4.5 ± 1.4	3.9 ± 1.7	5.2 ± 2.2	3.4 ± 2.7
MS 30	PPMS	5.5 ± 3.2	2.6 ± 1.6	5.1 ± 2.3	4.6 ± 3.7
MS 31	PPMS	5.1 ± 1.9	4.5 ± 2.6	3.4 ± 2.8	4.1 ± 3.2
MS 32	PPMS	4.0 ± 3.1	60 ± 7.2**	3.2 ± 2.6	3.9 ± 1.2
MS 33	PPMS	1.7 ± 1.0	66 ± 6.9**	2.7 ± 1.6	3.7 ± 2.8
MS 34	SPMS	3.5 ± 2.9	3.4 ± 2.4	3.3 ± 1.9	3.7 ± 2.6
OND 1	SPN*	1.5 ± 2.5	2.4 ± 1.5	0 ± 3.5	0 ± 3.6
OND 2	GBS*	0.8 ± 1.4	0 ± 3.4	0 ± 3.3	0 ± 4.2
OND 3	GBS*	0 ± 4.1	3.3 ± 1.2	0 ± 3.2	0 ± 2.2
OND 4	GBS*	1.5 ± 1.6	0 ± 2.2	0 ± 2.5	0 ± 2.5
OND 5	CIDP*	2.5 ± 1.5	0 ± 6.0	0 ± 2.8	0 ± 2.6
OND 6	CIDP*	0 ± 2.4	0 ± 1.3	0 ± 3.9	0 ± 2.9
OND 7	CIDP*	0 ± 3.0	0 ± 2.4	0 ± 2.0	0 ± 2.8
OND 8	ISAN*	1.1 ± 2.3	0 ± 4.0	0 ± 3.3	0 ± 4.4
OND 9	MG*	1.6 ± 3.7	2 ± 5.2	0 ± 4.8	0 ± 2.6
OND 10	CIDP*	0 ± 5.1	0 ± 3.2	0 ± 2.2	0 ± 3.8
HC 1	–	0.2 ± 2.5	1.3 ± 6.7	2.2 ± 2.5	2.3 ± 5.4
HC 2	–	1.2 ± 2.4	1.8 ± 5.2	3.4 ± 2.4	2.3 ± 3.1
HC 3	–	0.5 ± 2.6	1.1 ± 5.0	1.5 ± 2.6	3.1 ± 2.0
HC 4	–	0 ± 3.1	1.2 ± 4.3	1.3 ± 2.1	3.2 ± 3.2
HC 5	–	0.5 ± 4.4	1.8 ± 6.9	2 ± 2.6	2.8 ± 4.4
HC 6	–	0.5 ± 5.9	1 ± 3.8	2.2 ± 3.9	3 ± 3.3
HC 7	–	0 ± 2.2	0 ± 4.1	0 ± 3.1	0 ± 3.1
HC 8	–	0 ± 2.1	0.6 ± 4.4	0 ± 3.0	0 ± 3.4
HC 9	–	0.3 ± 3.0	0.7 ± 4.3	1.3 ± 1.0	0 ± 5.3
HC 10	–	0.4 ± 2.9	0.4 ± 3.8	0 ± 2.6	0 ± 4.1
HC 11	–	0 ± 2.7	0 ± 4.2	0 ± 1.8	0 ± 3.5
HC 12	–	0 ± 2.6	1.1 ± 4.1	0 ± 3.2	0 ± 4.2
HC 13	–	0.6 ± 4.0	0 ± 5.1	0 ± 3.0	0 ± 1.5

Myelinating cultures (28 days *in vitro*) were incubated with 100 μg/ml IgG with 1% fresh rat serum as a source of complement for 16 h. To control for complement independent effects heat inactivated complement was used. Percentage values for axonal loss and demyelination were calculated in relation to untreated cultures [(mean ± SD), ($n = 3$) (*$P < 0.05$, **$P < 0.001$, *t*-test)]. CIDP = chronic idiopathic polyneuropathy; GBS = Guillian-Barré syndrome; ISAN = idiopathic sensory ataxic neuropathy; MG = myasthenia gravis; PPMS = primary progressive multiple sclerosis; RRMS = relapsing remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis; SPN = sensory motor polyneuropathy.

*Denotes patients undergoing plasma exchange.

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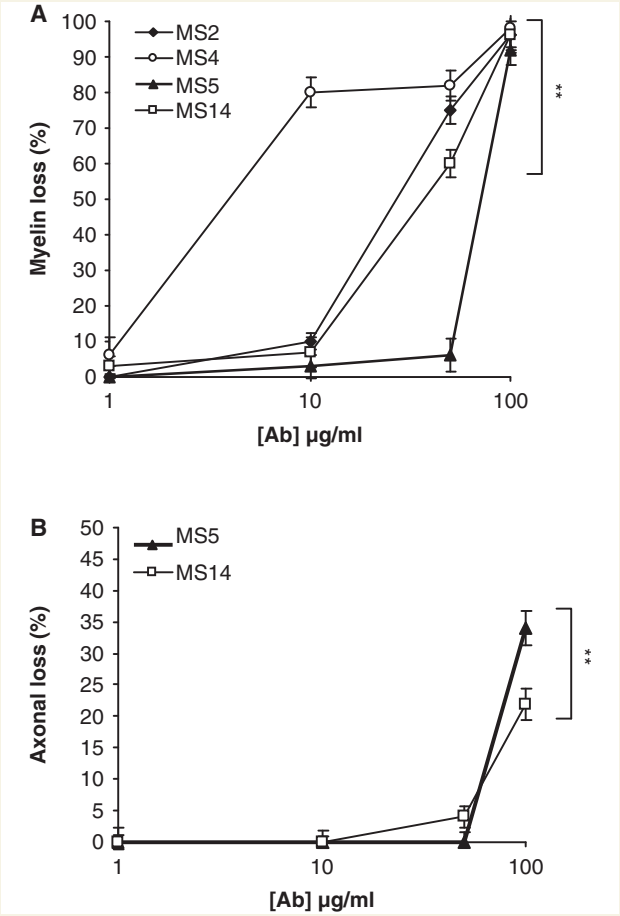

Figure 3 Dose dependence of multiple sclerosis derived autoantibody-mediated pathogenesis. IgG from four patients with multiple sclerosis (Patients MS2, MS4, MS5 and MS14) known to contain axopathic and/or demyelinating activity was added to myelinating cultures at 100, 50, 10 and 1 µg/ml in the presence of 1% fresh rat serum as a source of complement.
(**A**) Demyelinating activity in all cases was significantly reduced at 50 µg/ml and was no longer detected at 1 µg/ml.
(**B**) Axopathic activity present in samples from Patients MS5 and MS14 was not detected at IgG concentrations <100 µg/ml. Percentage values for axonal loss and demyelination were calculated in relation to cultures treated with equivalent antibody concentrations in the presence of heat inactivated complement (mean ± SD, $n = 3$; $^{**}P < 0.001$, *t*-test).
Ab = antibody.

the only antigen known to fulfil these criteria and induce a demyelinating autoantibody response in experimental animals is MOG (Iglesias *et al*., 2001). One approach to explore the specificity of this pathogenic response is solid phase immunoabsorption. We therefore investigated the effects of treating pathogenic IgG preparation with isolated rat myelin, or recombinant MOG conjugated to agarose beads. Incubating purified IgG preparations with myelin significantly reduced or completely abolished their demyelinating activity confirming the target(s) were myelin associated. However, depletion of the MOG specific repertoire had no effect on the demyelinating activity of these samples, indicating that MOG is not a dominant target for the demyelinating response in these patients (Table 5). The observation that two patients developed axopathic autoantibodies in addition to a demyelinating response indicates that the specificity of the pathogenic autoantibody response varies between patients. We previously identified neurofascin, an axo-glial antigen present on both myelin and the axolemma as a potential target for axopathic autoantibody responses (Mathey *et al*., 2007). We investigated this further using recombinant rat neurofascin-155 (rrNfasc155) in attempts to absorb demyelinating and axopathic components of the autoantibody repertoire. Immunoaffinity chromatography over immobilized rrNfasc155 had no effect on the demyelinating activity of the majority of the IgG preparations, but completely eliminated both the demyelinating and axopathic activity associated with the IgG preparation from one patient (Table 5). These observations provide further evidence that Nfasc-specific autoantibodies may contribute to disease pathogenesis but it is apparent that in the majority of cases, neurofascin-155 is not the dominant target of the pathogenic autoantibody response.

Discussion

In this report, we demonstrate that a subset of patients with multiple sclerosis develop autoantibody responses that can mediate demyelination and/or axon damage *in vitro*. These pathogenic effects were complement-dependent, and the demyelinating response was directed against antigen expressed on the surface of terminally differentiated myelinating oligodendrocytes and contiguous myelin sheaths. These observations provide formal proof that the disease associated autoantibody repertoire contains

Table 3 Analysis of longitudinal samples taken after treatment by plasma exchange

Patient ID	PLEX intervals (days)	IgG (at initial PLEX)		IgG (at final PLEX)	
		% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss
MS 35	0, +2, +7	–	95 ± 0.8**	–	0 ± 7.7
MS 36	0, +1, +4, +6	–	94 ± 1.6**	–	0 ± 6.1
MS 37	0, +2, + 3, +6, +7	–	92 ± 2.1**	–	15 ± 5.4

Longitudinal samples were taken from three patients with relapsing remitting multiple sclerosis after multiple rounds of plasma exchange (PLEX). In each case, 500 µg IgG was added to myelinating cultures (28 days *in vitro*) with 1% fresh rat serum as a source of complement for 16 h. Percentage values for axonal loss and demyelination were calculated in relation to untreated cultures [mean ± SD ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.001$, – not significant, *t*-test)].

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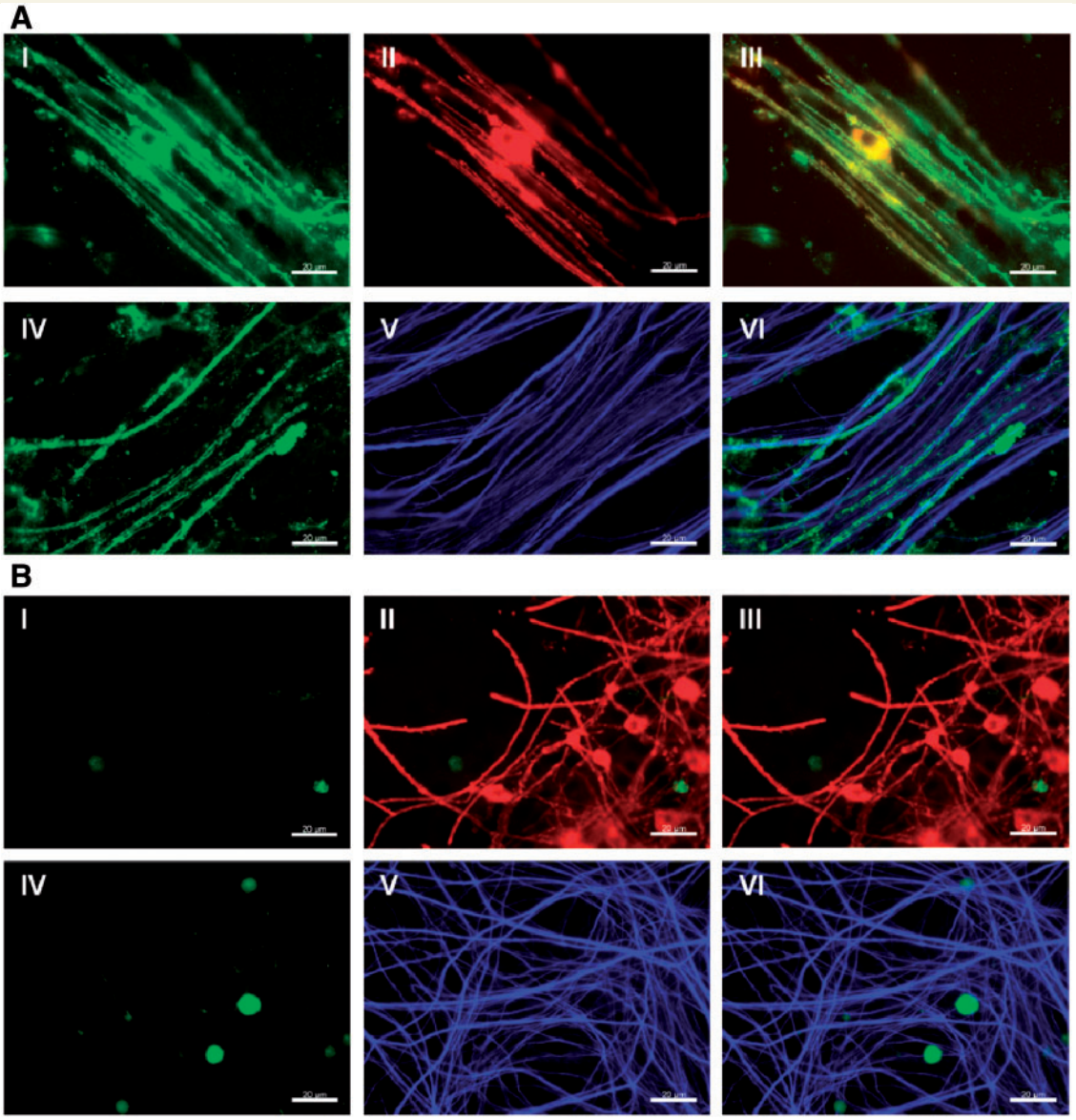

Figure 4 Patient derived IgG preparations with demyelinating activity decorate the surface of myelin sheaths and contiguous oligodendrocytes. [**A**(**I–III**)] Live staining of myelinating cultures with patient derived IgG preparations with *in vitro* demyelinating activity revealed binding to surface of PLP^+ myelin sheaths and oligodendrocytes (human IgG green; PLP, red) and align with $SMI\text{-}31^+$ axons [**A**(**IV–VI**)] (human IgG, green; SMI-31, blue; ×60 magnification; scale bar = 20 μm). [**B**(**I–III**)] Live staining of myelinating cultures with human IgG preparations with no axopathic or demyelinating activity fails to identify any specific recognition of PLP^+ myelin sheaths and oligodendrocytes (human IgG green; PLP, red) or [**B**(**IV–VI**)] $SMI\text{-}31^+$ axons (human IgG, green; SMI-31, blue; ×60 magnification; scale bar = 20 μm).

specificities that can participate in the development of demyelinating lesions.

In this proof of principal study, demyelinating/axopathic autoantibodies were detected in approximately a third of patients with multiple sclerosis ($n = 34$), but not in patients with other neurological diseases ($n = 10$) or healthy controls ($n = 13$). Moreover, they were detected more frequently in patients with relapsing remitting multiple sclerosis than primary progressive disease ($P < 0.02$, Fishers-exact test), in particular, in those patients with an acute steroid resistant relapse ($P < 0.05$, Fishers-exact test). These observations not only support the concept that multiple sclerosis is mechanistically heterogeneous (Lucchinetti *et al.*, 2000), but also indicate, contrary to previous speculation (Pender, 2004; Vyshkina and Kalman, 2008), that autoantibodies do not play a dominant role in the pathogenesis of primary progressive multiple sclerosis. The absence of demyelinating or axopathic autoantibodies in patients with other neurological diseases suggests they are a specific feature of multiple sclerosis. However, this interpretation must be treated with caution as recent studies identified potentially pathogenic MOG-specific autoantibodies in

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Table 4 Demyelination mediated by multiple sclerosis IgG preparations does not eliminate the majority of oligodendrocyte progenitor cells

Patient ID	Axonal injury	Demyelination	IgG binding (ICC)	% Loss of cells		
				NG2$^+$	O4$^+$	PLP/DM20$^+$
MS1	–	+	+	–	40 ± 10.1**	77 ± 9.7**
MS2	–	+	+	–	20 ± 18.2**	65 ± 11.7**
MS3	–	+	+	–	35 ± 15.3**	74 ± 8.6**
MS4	–	+	+	–	36 ± 12.4**	72 ± 6.7**
MS5	+	+	+	–	38 ± 10.8**	71 ± 5.9**
MS6	–	–	–	–	–	–
MS7	–	+	+	–	25 ± 16.7*	68 ± 8.8**
OND1	–	–	–	–	–	–
OND2	–	–	–	–	–	–
OND3	–	–	–	–	–	–
OND4	–	–	–	–	–	–
HC1	–	–	–	–	–	–
HC2	–	–	–	–	–	–
HC3	–	–	–	–	–	–
HC4	–	–	–	–	–	–
Z2	–	+	+	–	37 ± 9.9**	85 ± 10.1**
O4	–	+	+	42 ± 6.7**	90 ± 8.8**	95 ± 6.2**

Quantification of the effects of multiple sclerosis patient derived IgG preparations on O4$^+$, NG2$^+$ and PLP/DM20$^+$ cell numbers in myelinating cultures. The IgG preparations were used at an end concentration of 100 μg/ml in the presence of 1% rat serum as a source of complement. O4 and Z2 monoclonal antibodies were used at a concentration of 10 μg/ml.
Percentage values for axonal loss and demyelination were calculated in relation to untreated cultures [mean ± SD (n = 3; *P < 0.05, **P < 0.001, – not significant compared to controls, t-test)]. ICC = immunocytochemistry.

Table 5 The intra-patient heterogeneity of pathogenic autoantibody responses

Patient ID	IgG pre-adsorption		IgG post-myelin adsorption		IgG post-MOG adsorption		IgG post Nfasc155 adsorption	
	% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss
MS1	–	95 ± 3.5**	–	10 ± 2.2	–	92 ± 4.6**	–	93 ± 4.5**
MS2	–	96 ± 4.0**	–	21 ± 3.2*	–	99 ± 3.4**	–	97 ± 3.0**
MS3	–	97 ± 2.2**	–	6 ± 3.8	–	95 ± 5.2**	–	97 ± 2.0**
MS4	–	98 ± 1.3**	–	25 ± 4.2*	–	94 ± 4.4**	–	94 ± 1.8**
MS5	34 ± 5.7**	92 ± 2.4**	15 ± 1.3*	8 ± 4.2	40 ± 6.6**	100 ± 1.9**	–	0 ± 1.2
MS6	–	–	–	–	–	–	–	–
MS7	–	94 ± 2.0**	–	33 ± 3.8**	–	96 ± 4.8**	–	92 ± 2.2**

Multiple sclerosis patient-derived IgG (100 μg/ml) was incubated with purified rat myelin (2 mg/ml), refolded recombinant rat MOG or recombinant rat neurofascin-155 (Nfasc155). Non-adsorbed IgG was added to myelinating cultures (28 days *in vitro*) at 100 μg/ml.
Percentage values for axonal loss and demyelination were calculated in relation to untreated cultures [mean ± SD (n = 3; *P < 0.05, **P < 0.001, – not significant compared to controls, t-test)].

patients with acute disseminated encephalomyelitis and neuromyelitis optica (Zhou *et al*., 2006; Brilot *et al*., 2009; McLaughlin *et al*., 2009; Mader *et al*., 2011).

The high frequency of demyelinating/axopathic autoantibodies found in patients with acute steroid resistant relapses suggests they would benefit from antibody depleting treatments such as plasma exchange (Keegan *et al*., 2005). This was not tested directly in this study, but we were able to assess the longitudinal effect of plasma exchange on the demyelinating antibody response in three patients. Unexpectedly, this was associated with a rapid reduction in the demyelinating activity in the IgG fraction that occurred over a period of 9–18 days. This may indicate that the demyelinating IgG response in these patients is a transient phenomenon similar to that described for MOG-specific autoantibody responses in acute disseminated encephalomyelitis (Pröbstel *et al*. 2011); alternatively, it may reflect a response to the high-dose corticosteroid pulses, these patients received before plasma exchange. Further studies are now required to resolve this point and to establish the kinetics of demyelinating/axopathic IgG responses in treated and untreated patients.

The apparent absence of pathogenic autoantibodies in Guillain–Barré syndrome and chronic inflammatory demyelinating

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polyneuropathy reflects the tissue specificity of the disorders that have little or no CNS involvement (Kamm and Zettl, 2012). Tissue damage in variants of Guillain–Barré syndrome is mediated by anti-ganglioside antibodies (Rinaldi and Willison, 2008), but even if present in the samples we analysed, their specific targets were not expressed in these myelinating CNS cultures.

Although we identified complement-dependent, demyelinating and axopathic IgG responses in approximately a third of patients, this is probably an underestimation of the frequency of pathogenic autoantibody responses associated with multiple sclerosis. Historical studies suggest that pathogenic autoantibodies may also be present that block (re)myelination or directly influence neuronal activity (Caspary, 1977; Seil *et al*., 1977; Archelos and Hartung, 2000; Huizinga *et al*., 2008). Moreover, whilst the use of rodent cultures provides a pragmatic solution to the problem of generating reproducibly myelinated cultures from human tissue, they will not detect pathogenic autoantibodies unless these exhibit a significant degree of species cross-reactivity. Fortunately, the amino acid sequences of many myelin and myelin-associated antigens are highly conserved across species suggesting that in most cases there will be some degree of cross-reactivity, but whether this is sufficient to demonstrate pathogenicity can only be resolved experimentally. However, experimental studies demonstrate that with respect to MOG, a *bono fide* target for demyelinating antibodies in experimental animals, there is significant overlap between human and rodent epitopes recognized by the disease associated anti-MOG response in acute disseminated encephalomyelitis and paediatric multiple sclerosis (McLaughlin *et al*., 2009; Pröbstel *et al*., 2011). Conversely, the bioassay may generate false positives if patient sera contain antibodies that cross-react with rodent antigens, but not their human orthologues. It also remains to be established whether this culture model will identify pathogenic effects mediated by autoantibody responses against aquaporin-4, the primary target for pathogenic autoantibodies in neuromyelitis optica (Lennon *et al*., 2004; Bradl *et al*., 2009). This may be the case as astrocytes in the cultures do express aquaporin IV, but we have not yet tested this experimentally as our focus was on identifying autoantibody responses that mediated primary demyelination and/or axonal loss (Supplementary material).

Clinical and experimental studies have identified a number of potential targets for pathogenic autoantibody responses in multiple sclerosis including antigens expressed by oligodendrocyte precursors such the NG2 proteoglycan, myelin components such as MOG, PLP and sulphatide (the 'O4' antigen) and axonal antigens such as neurofacin-186 (Niehaus *et al*., 2000; Kanter *et al*., 2006; Mathey *et al*., 2007; Meinl *et al*., 2011). However, in this study, demyelinating IgG preparations did not bind to any structures other than the surface of myelin sheaths and contiguous oligodendrocytes. This indicates that this pathogenic response is specific for antigens expressed by myelinating oligodendrocytes that are not expressed during earlier stages of oligodendrocyte differentiation. This was confirmed by investigating the effects of pathogenic IgG preparations on oligodendrocyte progenitor cell numbers as defined by expression of $NG2^+$ and $O4^+$, an approach that demonstrated that these cells were not targeted by a pathogenic autoantibody response in these patients (Fig. 5).

The obvious candidate for the demyelinating response was MOG, as potentially demyelinating MOG-specific autoantibodies are not only present in paediatric inflammatory demyelinating disorders, but also occasionally in cases of adult onset multiple sclerosis (Zhou *et al*., 2006; McLaughlin *et al*., 2009). However, whilst the demyelinating activity of the patients IgG preparations could be absorbed using isolated myelin, immunoaffinity chromatography over immobilized refolded MOG had no effect indicating that MOG is not a dominant target for demyelinating autoantibodies in those patients investigated in this study.

The observation that in one patient isolated myelin absorbed both demyelinating and axopathic components of the IgG repertoire suggested this response may be directed epitopes common to neurofascin-155 (a paranodal component of the myelin sheath) and neurofascin-186 expressed at the surface of myelinated axons at the node of Ranvier and axonal initial segment. In most cases, depleting patient-derived IgG preparations of neurofascin-155 reactive autoantibodies had no effect on their pathogenic potential *in vitro*. However, in one patient, immunoaffinity chromatography over immobilized neurofascin-155 completely eliminated the ability of this patient's IgG to mediate demyelination and axonal loss *in vitro*. This experiment not only confirmed that neurofascin can occasionally provide a dominant target for an axopathic/demyelinating response in multiple sclerosis (Mathey *et al*., 2007), but also suggests the specificity of the pathogenic response will vary between patients. Defining the specificity of these pathogenic autoantibodies is now essential if we are to understand the aetiology of these responses and investigate their clinical significance.

The standard approach to demonstrate the latter is passive transfer of patient antibody into animals with acute experimental autoimmune encephalomyelitis. This has been used extensively to demonstrate acute pathogenic effects mediated by monoclonal antibodies or high titre polyclonal antisera, but proved of limited use in studies investigating the pathogenic potential of immunoglobulins from patients with multiple sclerosis (Zhou *et al*., 2006). Our results provide insight as to why this may be the case. Extrapolating from results obtained using the demyelinating MOG-specific monoclonal antibody Z2, we estimate that patient sera contain the functional equivalent of 5–50 ng Z2/100 μg total IgG (~3–30 nM in serum). This is similar to the range reported for autoantibodies in other autoimmune diseases [e.g. myasthenia gravis (α-acetylcholine receptor antibodies) ~10–30 nM; Lindstrom *et al*., 1976; Vincent *et al*., 1985]. However, $\geqslant$100 μg/animal of MOG-specific monoclonal antibody is normally required to obtain a significant acute exacerbation of disease severity and/or pathology in experimental autoimmune encephalomyelitis (Linington *et al*., 1988; Mathey *et al*., 2007). This equates to injecting ~100 mg of patient IgG, which is not only far higher than that normally used, but also likely to cause adverse physiological effects in addition to any CNS specific deficit. Future *in vivo* studies will require approaches that enable us to examine the effects of chronic exposure to physiologically relevant autoantibody titres, a problem that will be easier to address once the specificity of the pathogenic response is known.

In summary, we demonstrate that a subset of patients with multiple sclerosis develops disease-associated demyelinating and

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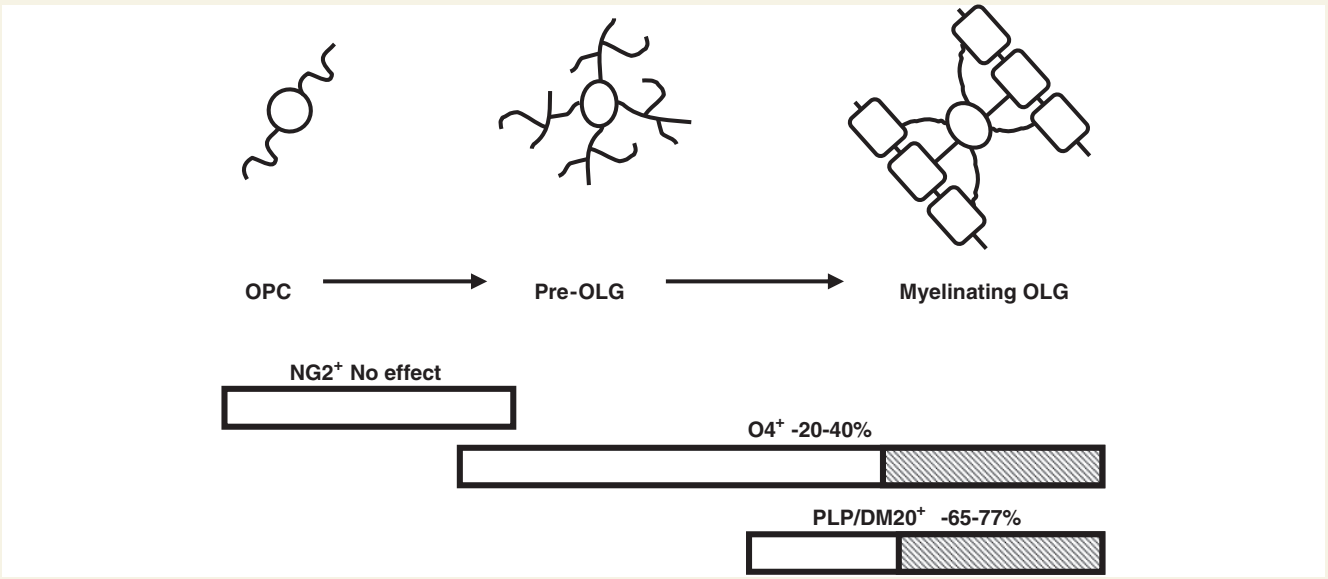

Figure 5 The autoantigen(s) recognized by pathogenic multiple sclerosis IgG are expressed by mature and myelinating oligodendrocytes. Schematic representation of the oligodendrocyte lineage differentiation pathway. NG2 is only expressed by oligodendrocyte progenitor cells (OPC) early in differentiation. Sulphatide (O4) is expressed later by oligodendrocyte progenitor cells, pre-oligodendrocytes, mature oligodendrocytes (OLG) and myelin. PLP is expressed on mature oligodendrocytes and myelin, however, it must be noted that DM20, an isoform of PLP, is expressed earlier by a population of pre-oligodendrocytes. From our data, we can determine that the pathogenic antibodies detected in multiple sclerosis IgG are directed against antigens expressed on mature oligodendrocytes and myelin as antibody treatment spared the majority of $NG2^+$ and $O4^+$ cells. The shaded area represents the relative proportion of cells lost in responses to treatment with multiple sclerosis patient derived IgG.

axopathic autoantibody responses that target antigens expressed by highly differentiated myelinating oligodendrocytes. Our findings provide (i) formal evidence that demyelinating autoantibodies are present in some patients with multiple sclerosis; (ii) support for the concept that autoimmune responses directed against axo-glial antigens contribute to the development of axonal pathology; and (iii) demonstrate heterogeneity within the pathogenic autoantibody repertoire associated with multiple sclerosis.

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Supplementary material

Supplementary material is available at *Brain* online.

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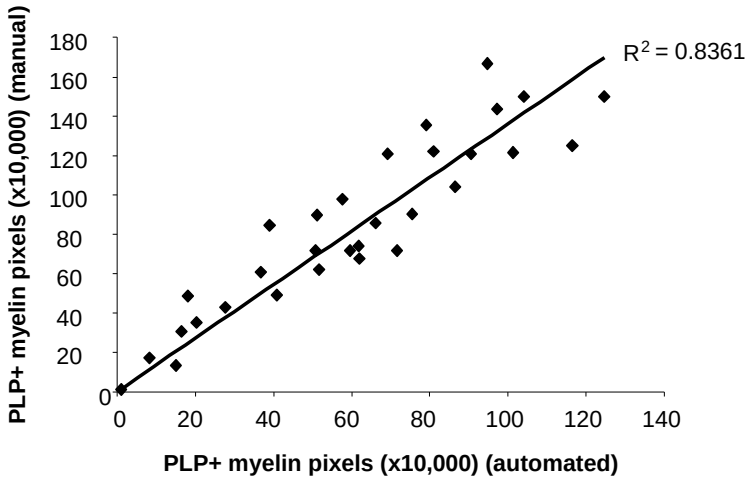
A

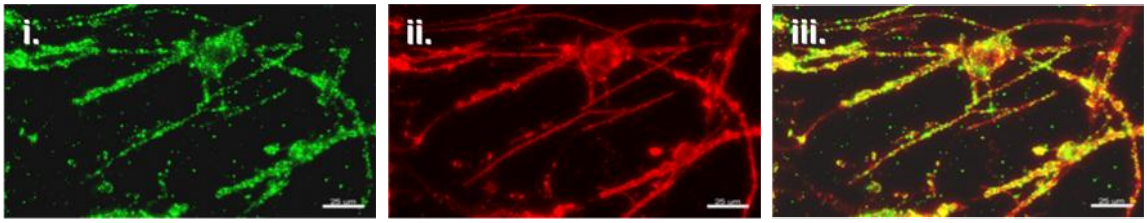
	10µg/ml Z2	50ng/ml Z2	1ng/ml Z2
Manual Quantification			
% Myelinated axons	0.197 ± 0.03**	3.45 ± 1.2*	6.98 ± 2.9
Coefficient of variance (%)	15.2	34.8	41.5
Automated Quantification			
% Myelinated axons	0.105 ± 0.01**	3.02 ± 0.8*	5.34 ± 2.2
Coefficient of variance (%)	9.5	26.5	41.2

B



Supplementary data S1: Myelin Quantification.
A: Myelinating cultures (28 DIV) were treated with a series of concentrations of Z2 (anti-MOG mAb) in the presence of fresh rat serum as a source of complement (as shown in figure 2D). Myelin/oligodendrocytes were visualised by PLP immunocytochemistry. Quantification of myelination was performed manually as described previously (Sorenson et al., 2008) or automatically using a newly developed BRAINS BATCH algorithm. Values are shown as mean ± standard deviation, (n = 3) (*p<0.05, **p<0.001, compared to untreated controls, T-test).

B: Comparison of individual pixel counts across 30 images taken from untreated cultures analysed manually or using the automated program by linear regression. Pixels values obtained were comparable between the two methods (R^2= 0.831).



Supplementary data S2: MOG specific antibody mediated complement dependent myelin injury occurs within two hours via MAC deposition on the surface of oligodendrocytes and myelin. Myelinating cultures were treated with 10µg/ml 8-18C5 in the presence of complement. Two hours after antibody addition MAC (poly-C9, Dako, Denmark) (green) (i) can be detected on the surface of PLP+ oligodendrocytes and myelin (red) (ii-iii). (60X magnification; scale bar 25µm).

A

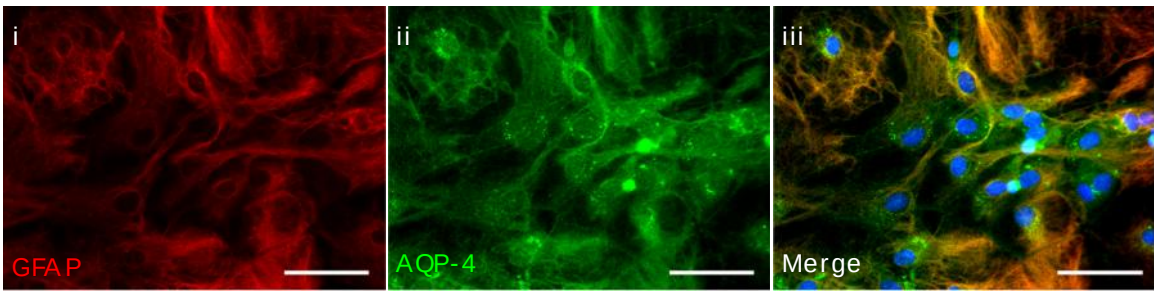
Patient ID	Diagnosis	% Axonal loss (±SD)	% Myelin loss (±SD)
MS1	Marburg MS	1 ± 1.9	**100 ± 4.5****
MS2	RRMS	0 ± 1.0	**100 ± 3.0****
MS3	RRMS	0 ± 2.8	**100 ± 2.4****
MS4	RRMS	0 ± 2.8	**100 ± 2.2****
MS5	SPMS	**30 ± 6.4****	**100 ± 1.0****
MS6	RRMS	1 ± 3.0	0 ± 1.2
MS7	RRMS	4 ± 3.3	**100 ± 1.0****
MS8	RRMS	1 ± 2.9	0 ± 3.9
MS9	RRMS	4 ± 2.8	0 ± 2.6
MS10	RRMS	4 ± 2.3	2 ± 4.4
MS11	RRMS	3 ± 1.1	**100 ± 1.5****
MS12	RRMS	1 ± 2.2	**100 ± 1.3****
MS13	RRMS	3 ± 2.4	3 ± 2.1
MS14	RRMS	**18 ± 1.3***	**100 ± 1.6****
MS15	RRMS	0 ± 2.1	3 ± 2.8
MS16	RRMS	2 ± 2.8	**100 ± 1.2****
MS17	PPMS	1 ± 2.4	2.6 ± 2.2
MS18	PPMS	1 ± 2.3	1 ± 4.0
MS19	PPMS	2 ± 1.8	2 ± 3.3
MS20	PPMS	2 ± 1.6	5 ± 3.1

B

Patient ID	Diagnosis	% Axonal loss (±SD)	% Myelin loss (±SD)
OND1	SPN	0 ± 1.6	1.5 ± 2.3
OND2	GBS	2 ± 1.8	1 ± 1.2
OND3	GBS	1.2 ± 2.6	1 ± 1.8
OND4	GBS	0 ± 1.0	0 ± 1.6
OND5	CIDP	0 ± 2.2	0 ± 2.4
OND6	CIDP	1 ± 2.5	0 ± 3.2
OND7	CIDP	4.4 ± 1.2	0 ± 1.0
OND8	ISAN	1 ± 1.4	2 ± 1.2
OND9	MG	0 ± 2.2	0 ± 1.2
OND10	CIDP	0 ± 1.9	0 ± 1.2

Supplementary data S3: Addition of patient derived IgG at 1mg/ml reveals no further pathogenic samples in comparison to addition at 100µg/ml.

A: Summary of pathogenic potential of the IgG fraction from the 20 MS patient samples tested. 50% of the samples tested mediated complete demyelination. In two patients this was also accompanied by a loss of axons ranging from 18% to a loss of 30% of the total number of axons. **B:** Summary of pathogenic potential of the IgG fraction from the 10 OND patient samples tested. No significant loss of axons or glia was detected even at 1mg/ml. There was no difference in the activity of samples at 1mg/ml compared to 100µg/ml. Values shown are from one representative data set performed three times in triplicate ± standard deviation (*$p<0.05$, **$p<0.01$; T-test).



Supplementary data S4: Astrocytes present in rat myelinating cultures express aquaporin-4.
Astrocytes within myelinating cultures (28 DIV) were visualised by glial acidic fibrillary protein (GFAP, red) (i) immunocytochemistry and co-stained for aquaporin-4 (AQP-4, green) (ii) indicating that the astrocytes with our system express significant levels of AQP-4 (merge, iii) (40X magnification, scale bar 50μM).