Identification of Pathogenic Autoantibody Responses in Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic disease of the human central nervous system (CNS) in which repeated episodes of inflammatory demyelination result in formation of persistently demyelinated plaques of gliotic scar tissue associated with varying degrees of axonal loss. MS is now considered a "complex trait" that is triggered in genetically susceptible individuals by environmental factors. The disease is also considered to contain an autoimmune component where both the adaptive and innate immune systems have been implicated in disease pathogenesis. There has been a steady accumulation of circumstantial evidence from both clinical and experimental studies that implicate a role for autoantibody dependent mechanisms. However this issue remains controversial in the absence of formal evidence that patients actually develop a pathogenic autoantibody response. The aim of this thesis was to resolve this question.

To do this we developed an *in vitro* bioassay based on a dissociated myelinating culture system from embryonic rat spinal cord. We demonstrated that this *in vitro* system could reproduce many features of *in vivo* myelinated axons. To validate this model as a viable screening assay characterised complement mediated autoantibody responses using a series of monoclonal antibodies and anti-sera. Due to their significance in the literature we focussed in particular on the MOG specific and Nfasc specific responses and comprehensively demonstrated that our bioassay offered a robust screening strategy in which to detect pathogenic antibody responses in the presence and absence of exogenous complement.

To determine whether we could use our model to detect pathogenic autoantibody responses in MS patients, we purified the IgG fraction from a cohort of MS patients (n=20), OND (n=10) and healthy controls (n=13). Using this patient purified IgG we demonstrated a MS specific demyelinating activity, which was present in ~50% of samples screened. However in 10% of patients demyelination occurred secondary to pronounced axonal injury. These effects were dependent on exogenous complement and were unique to the MS cohort. Pathogenic antibody responses tended to be most prevalent in those patients with an aggressive disease course. In addition to complement mediated CNS injury we also demonstrated that this pathogenic MS IgG could disrupt myelin formation in developing myelinating cultures. Attempts to define the specificity revealed that this was heterogeneous, however in one MS patient we discovered that Nfasc155 provided a dominant antigen for pathogenic autoantibody responses.

Together these data provide formal demonstration that MS is associated with pathogenic autoantibody responses. This has significant long term consequences for the clinical management of the disease.

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Declaration of Authorship

I declare that, except where referenced to others, this thesis is the product of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____

Printed name _____

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Abbreviations

2D	two dimensional
3D	three dimensional
Ab	antibody
AChR	acetylcholine receptor
ADCC	antibody dependent cellular cytotoxicity
ADEM	acute disseminated encephalomyelitis
AIS	axon initial segment
AnkG	ankyrin-G
APP	ß-amyloid precursor protein
AQA-4	aquaporin-4
BBB	blood brain barrier
BCA	bicinchoninic acid assay
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
Caspr	contactin associated protein
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CIDP	chronic idiopathic demyelinating polyneuropathy
CNPase	2',3'-cyclic nucleotide 3'-phosphohydrolase
CIS	clinically isolated syndrome
CNS	central nervous system
CO_2	carbon dioxide
CSF	cerebrospinal fluid
CV	coefficient of variance
DAPI	4'-6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DIV	days *in vitro*
DM^+	differentiation media with insulin
DM^-	differentiation media without insulin
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide Triphosphate
DRG	dorsal root ganglion
E	embryonic day
EAE	experimental allergic encephalomyelitis
EDSS	expanded disability status scale
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
Fab	fragment antigen binding
FBS	foetal bovine serum
Fc	fragment crystallisable
FNIII	fibronectin type III
FRS	fresh rat serum
G418	geneticin
GalC	galactocerebroside
GBS	Gullian-Barre Syndrome
gDNA	genomic deoxyribonucleic acid
GFAP	glial fibrillary acidic protein

HBSS	Hank's balanced saline solution
HC	healthy control
HRP	horse radish peroxidase
Hrs	hours
ICC	immunocytochemistry
ID	identification
IEF	isoelectric focussing
Ig	immunoglobulin
IL-	interleukin-
i.p	interperitoneal
IR	immunoreactivity
ISAN	idiopathic sensory ataxic neuropathy
kDa	kilodaltons
L-15	Leibovitz medium
M	molar
M.W	molecular weight
mAb	monoclonal antibody
MAC	membrane attack complex
MAG	myelin associated glycoprotein
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
MHC	major histocompatibility complex
MG	myasthenia gravis
min	minute(s)
MND	motor neurone disease
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
Nav	voltage-gated sodium channels
N-CAM	neural cell adhesion molecule
NeuN	neuronal nuclei
Nfasc155	155kDa isoform of neurofascin
Nfasc186	186kDa isoform of neurofascin
NF-L	neurofilament light chain
NK	natural killer
nm	nanometers
NMDA	N-methyl D-aspartate
NMJ	neuromuscular junction
NMO	neuromyelitis optica
NSM	neurosphere media
OCB	oligoclonal bands
OD	optical density
O/N	overnight
OPCs	oligodendrocyte progenitor cells
OPD	o-phenyldiamine
OSC	organotypic slice cultures
OSP	oligodendrocyte specific protein
P	postnatal day
pAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor

PFA	paraformaldehyde
PLL	poly-L-lysine
PLP	proteolipid protein
PM	plating media
PN	peripheral neuropathy
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
qRT-PCR	quantitative real time polymerase chain reaction
RA	rheumatoid arthritis
rcf	relative centrifugal force
rpm	revolutions per minute
RRMS	relapsing remitting multiple sclerosis
rrNfasc	recombinant rat neurofascin
RT	room temperature
T75	75 cm^3 tissue culture flask
TAG-1	transiently expressed axonal glycoprotein-1
TPX	therapeutic plasma exchange
TSH	thyroid stimulating hormone
SD	standard deviation
SD	Sprague Dawley
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M	standard error of the mean
SLE	systemic lupus erythematosus
SPMS	secondary progressive multiple sclerosis
SPN	sensory-motor polyneuropathy
Sulph	sulphatide
V	volts
VE	viral encephalitis
VGKC	voltage-gated potassium channels
VGCC	voltage-gated calcium channels

1 Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic disease of the human central nervous system (CNS) in which repeated episodes of inflammatory demyelination result in formation of persistently demyelinated plaques of gliotic scar tissue associated with varying degrees of axonal loss (review: Steinman et al., 2001). MS affects approximately 100,000 people in the UK and its incidence in Scotland (approximately 200 per 100,000) is among the highest worldwide (Handel et al., 2011). It is typically diagnosed between the ages 20 and 40 and is a leading cause of non-traumatic neurological disability in young adults. It not only severely compromises the quality of life of the patient and their families, but also has far wider adverse socioeconomic effects, costing the UK economy in excess of £1.5 billion (review: Kolbelt et al., 2000; review: Trisolini et al., 2010).

The macroscopic neuropathological changes associated with MS were first described by Carswell in 1838 (Murray, 2009), but it was only formally defined as a clinical entity thirty years later by Charcot who named it "*sclerose en plaques*" (Charcot, 1868). Over a century later it is accepted the diverse symptoms of MS are associated with perivascular inflammatory infiltrates, demyelination, axonal loss and astrogliosis in the brain and spinal cord (review: Frohman et al., 2006), but there is still no fully effective treatment for this devastating condition.

MS is now considered a "complex trait" that is triggered in genetically susceptible individuals by environmental factors (review: Sospedra and Martin, 2005). The complexity of the genetic component is evident from familial studies and more recently genome wide genetic screens. Monozygotic twins have a 20-35% increased risk of developing MS and first degree family members of patients have a 2 - 5% increased risk (Haines et al., 2002; Dyment et al., 2004). This can be attributed predominantly to the effects of specific HLA-DR and –DQ alleles that confer a greater risk of developing the disease (Haines et al., 2002; Barcellos et al., 2003), although an increasing number of other susceptibility genes are now being identified that include contribution from specific *IL7R* and

IL2R alleles (Hafler et al., 2007). However relatively low concordance for clinically definite MS in monozygotic twins implies additional environmental factors must be involved in its aetiology. Numerous environmental factors are implicated in the pathogenesis of MS including viral infections, smoking and vitamin D deficiency but as yet no single factor has been identified that acts a trigger for disease in genetically susceptible individuals (review: Handel et al., 2010).

The clinical classification of MS patients largely relies on the disease course [Figure 1.1]. The majority of MS patients develop initially a relapsing remitting form of the disease (RRMS), characterised by the appearance of new symptoms or the worsening of existing symptoms, followed by periods of recovery. Relapses vary in severity and duration and periods of remission may be relatively transient or last for many months or even years. If 10 to 20 years after the initial diagnosis, symptoms have not progressed and there is little disability, patients may be classified as having benign MS. However, most RRMS patients eventually develop secondary progressive MS (SPMS) which is characterised by a progressive worsening of symptoms in the absence of clearly defined acute relapses; an estimated 65% of RRMS patients will develop SPMS within 15 years of disease onset (Koch et al., 2008). The other major clinical variant is primary progressive MS (PPMS) which occurs in approximately 15% of cases of MS patients. This is characterised by a progressive increase in clinical deficits from onset of disease and lacks any superimposed relapses or remissions. PPMS differs from the other MS subtypes in a number of ways; men are as likely to develop primary progressive disease as women, disease onset is usually later in life (mid-30s to early 40s), initial disease activity is often in the spinal cord although there is later brain involvement and is characterised by severe atrophy and axonal degeneration (Review: Sospedra and Martin, 2005).

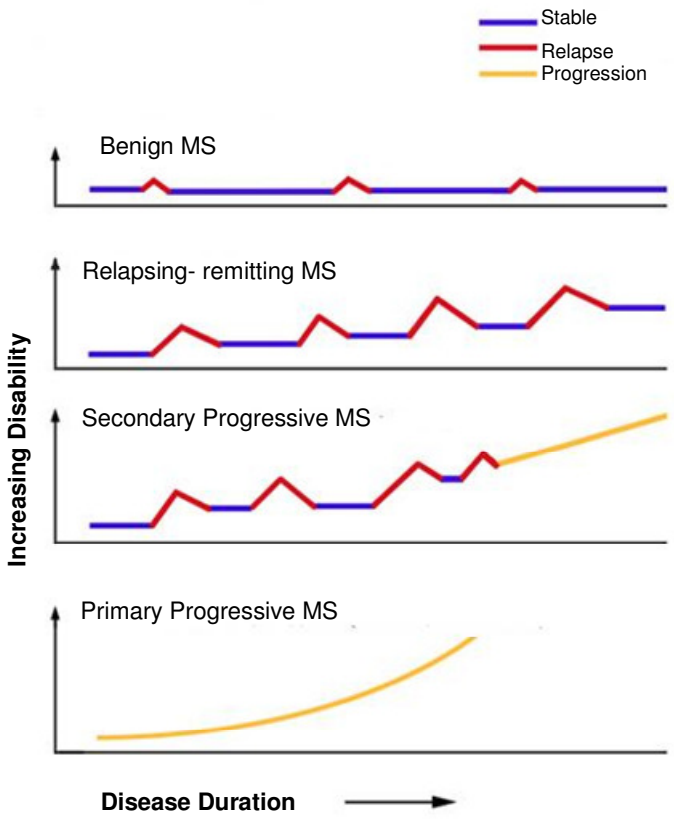


Figure 1.1: The four major MS subtypes
There are four major MS subtypes. The most common of which is relapsing remitting MS (RRMS). The majority of patients with RRMS go on develop secondary progressive disease characterised by the progressive worsening of symptoms. However if there is little to no disease progression or neurological deficit then the patient is classed as having benign MS. In ~15% of all MS cases present with primary progressive disease mediated by gradual deterioration without superimposed relapses or periods of recovery. Figure adapted from Lublin and Reingold (1996).

The introduction of sensitive magnetic resonance imaging (MRI) protocols has shown that the pathomechanisms responsible for disease progression in PPMS and SPMS differ from those associated with relapses in patients with RRMS. In early relapsing remitting disease the development of clinical deficits is associated with an ongoing inflammatory process in the CNS as defined by the presence of gadolinium enhancing lesions by MRI. Disease-modifying agents that significantly reduce the relapse rate also suppress the development of gadolinium enhancing lesions in RRMS. Intriguingly, not only is little or no inflammatory activity detected by MRI in the brains of patients with established progressive forms of the disease and SPMS, but disease modifying anti-inflammatory agents also appear to have no appreciable effect on the progressive accumulation of disability (Bradl and Lassmann, 2009). These

observations indicate that different mechanisms can contribute to the formation of chronically demyelinated plaques of scar tissue in the CNS, the end stage pathology of the disease. This interpretation is supported by immunopathological studies describing four distinct lesion types in different MS patients (Lucchinetti et al., 2000).

There are also a number of other diseases that mimic clinical, neuroradiological and pathological features of MS but which are clearly distinct clinical entities. These include acute disseminated encephalitis (ADEM), a post-infectious inflammatory demyelinating disease and neuromyelitis optica (NMO) an inflammatory demyelinating disease triggered by a primary autoimmune response to directed against aquaporin-4 which is expressed by astrocytes in the CNS. More problematic are patients diagnosed with clinically isolated syndrome (CIS) classified by the presence of a single demyelinating event at disease onset often will subsequently go onto develop RRMS but cannot be given this diagnosis at presentation (Polman et al., 2005).

Understanding how demyelination and axonal injury occur in MS is essential if we are to treat the disease efficiently, but our understanding of the pathomechanistic basis of MS remains limited despite fifty years of intense research. There is circumstantial evidence for the involvement of an autoimmune component, but whether MS is an autoimmune disease *per se* remains controversial. For many years it was assumed MS was a purely a $CD4^+$ T cell mediated disease. This hypothesis was based on the identification of particular HLA class II haplotypes as susceptibility genes and the demonstration that MHC class II restricted T cells specific for myelin antigens could induce experimental allergic encephalomyelitis (EAE), an MS-like disease in experimental animals (review: Zamvil and Steinmann, 1990). Over the past 75 years EAE has provided a powerful tool, enhancing our understanding of neuroinflammatory disease mechanisms (review: Gold et al., 2006). Two approved immunomodulating therapies for MS, glatiramer acetate and natalizumab, were developed from initial studies in EAE (Sela et al., 1990; Yednock et al., 1992). Nonetheless, the “T-cell centric” view of MS pathogenesis is now regarded as an oversimplification and it is now accepted that in addition to T cell dependent inflammation other pathomechanisms contribute to lesion formation.

There has been a steady accumulation of circumstantial evidence from both clinical and experimental studies that implicate a role for autoantibody dependent mechanisms. However, in the absence of formal evidence that patients actually develop a pathogenic autoantibody response, the clinical/pathological significance of autoantibodies remains controversial. This is the question addressed in this thesis. The following sections will therefore focus on reviewing the evidence that MS is associated with a pathologically significant autoantibody response, rather than on the details of purely T cell and/or monocyte dependent mechanisms that could contribute to disease development.

1.2 The broad spectrum of autoantibody mediated diseases

Early in the last century Ehrlich introduced the concept of "horror autotoxicus", the generation of antibodies against self antigens. However Ehrlich also described that "by certain contrivances" these self-reactive autoantibodies were unable to mediate disease. This led to the belief that autoimmune disease could not occur, a dogma that persisted well into the mid-1950s, when it was challenged by the discovery of pathogenic antibodies in experimental thyroid disease (Witebsky et al., 1957; Commentary: Silverstein, 2001). Over the following 50 years a broad spectrum of autoantibody mediated diseases have been identified that can affect almost every tissue in the body, either by targeting tissue-specific autoantigens, as is the case in Addison's disease and Myasthenia Gravis or recognition of tissue non-specific targets such double stranded DNA in patients with systemic lupus erythematosus (SLE) (Wellmann et al., 2005). A number of autoantibody mediated diseases of the CNS have also been identified including Morvan's syndrome and Neuromyelitis Optica [Table 1.1]. Although autoantibody mediated diseases are very diverse with respect to antigen specificity, target and mode of action they do exhibit a number of commonalities including gender bias (generally with a higher prevalence in females), genetic predisposition and the involvement of an environmental component in their aetiology.

Table 1.1: A selection of human autoimmune diseases mediated by autoantibodies (either completely or partly).

Disease	Self-antigen	Immune response
Organ-specific		
Addison's Disease	21 hydroxylase	Autoantibodies
Goodpasture's Disease	Type IV collagen	Autoantibodies
Grave's Disease	Thyroid stimulating hormone receptor (TSH-R)	Autoantibodies (stimulating)
Hashimoto's Thyroiditis	Thyroid stimulating hormone receptor (TSH-R) Thyroid proteins and cells	Autoantibodies (blocking)
Myasthenia Gravis	Acetylcholine receptor (AChR) Muscle specific Kinase (MUSK)	Autoantibodies T cells
Morvan's Syndrome	Voltage-gated potassium channel (VGKC)	Autoantibodies
Neuromyelitis Optica	Aquaporin-4 (astrocyte end foot)	Autoantibodies T cells
Neuromyotonia	Voltage-gated potassium channel (VGKC)	Autoantibodies
Systemic		
Systemic lupus erythematosus (SLE)	DNA, nuclear proteins, erythrocytes and platelets	Autoantibodies
Sjogren's Syndrome	Various antigens. Salivary gland, kidney , liver and thyroid are commonly targeted	Autoantibodies
Rheumatoid arthritis	Connective tissue, immunoglobulin	Autoantibodies Immune complexes

1.2.1 Criteria for recognising autoantibody mediated disease

Demonstrating that autoantibody-dependent mechanisms are involved in disease pathogenesis is complicated by the presence of pathologically irrelevant autoautoantibodies within the normal repertoire, and their potential expansion following tissue damage. So what criteria should be used to decide whether an autoantibody response plays a significant role in disease pathogenesis? Koch proposed a set of postulates to identify pathogens responsible for a specific disease. These were adapted by Witebsky and subsequently Rose (Witebsky et al., 1957; Rose and Bona, 1993) as criteria to identify pathogenic autoantibodies actively involved in disease development. For an antibody to be universally accepted as pathogenic most if not all of the following criteria must be fulfilled.

a: It must be present in most patients.

b: It must be present at the site of injury.

c: Passive transfer antibody should reproduce the clinical/pathological characteristics of the disease.

d: Immunisation with antigen induces disease and autoantibody production in experimental animals.

e: Demonstration of pathogenic antibody activity using target cells.

f: Reduction of antibody levels ameliorates the disease.

Table 1.2 summarises antibody mediated autoimmune diseases fulfilling these criteria for pathogenic antibodies, of which myasthenia gravis is the best known, whilst neuromyelitis optica, an inflammatory demyelinating disease reproducing some of the features of MS illustrates the difficulties of using the Witebsky/Rose criteria to define a pathologic autoantibody response in CNS disease.

Disease	Autoantibodies present in the majority of patients	Isolation of Ab from injury site	Transfer of disease	Induction of clinical disease with autoantigen	Disease modulation in response to B cell/ antibody directed therapies	References
Myasthenia Gravis (AChR)	**Yes**	**Yes**	**Maternal: Yes Experimental: Yes**	**Yes**	**Yes**	**Toyka et al., 1975 Lindstrom et al., 1976 Patrick and Lindstrom, 1973 Barnes et al., 1995**
NMO (AQP-4)	**Yes**	**Yes**	**Experimental: Yes**	**No**	**Yes**	**Lennon et al., 2004 Lennon et al., 2005 Kalluri et al., 2011 Bradl et al., 2010 Jarius et al., 2008**
Grave's Disease (TSH-receptor)	Yes	Yes	Maternal: Yes Human: Yes	Yes	ND	Shewring and Rees-Smith, 1982 Marazuela and J L Steegman, 2000 Weetmann, 1994 McKenzie and Zakarija, 1992 Witebsky et al, 1957 Roitt et al., 1956
Hashimoto's Disease (Thyroglobin, TSH-receptor, Thyroxine)	Yes	Yes	Maternal: Yes (TSH-R) Experimental: Yes	Yes	ND	
Goodpasture's syndrome (Type IV-collagen)	Yes	Yes	ND	Yes	Yes	Lerner et al., 1967 Johnson et al., 1978 Hudson et al., 1993
SLE (various)	Yes	Yes	Maternal: Yes	ND	Yes	Wellmann et al., 2005 Koyama et al., 2005 Lee et al., 2009 Review: Eisenberg, 2003

Table 1.2: A selection of autoimmune diseases which fulfil the some if not all criteria for pathogenic autoantibodies.

There are a number of autoimmune diseases both organ-specific (neurological and non-neurological) and systemic which can be described as pathogenic autoantibody mediated using a series of postulates which define pathogenic activity. *ND= not done or unknown*.

1.3 Myasthenia Gravis

Myasthenia Gravis (MG) is characterised by severe muscle weakness and fatiguability due to autoimmune destruction of the post synaptic membrane at the neuromuscular junction (NMJ) leading to a loss of muscle innervation. The disease can affect any muscle (except the heart) and can be fatal if the respiratory muscles become paralysed (known as myasthenic crisis). Research into the potential role of autoantibodies in MG pathogenesis began over 50 years ago when Simpson first introduced the concept of "antibodies to an endplate protein" (Simpson, 1960). This endplate protein was later identified as the acetylcholine receptor (AChR) (Patrick and Lindstrom, 1973; Lindstrom et al., 1976). MG is regarded the "textbook" example of an autoantibody mediated disease and one which satisfies all six criteria listed previously.

a: Autoantibodies are frequently present in patients.

Antibodies to AChR are present in ~80% of MG patients and were seldom found in healthy controls or in association with in other diseases (Lindstrom et al., 1976). A subset of MG which lack anti-AChR antibodies (~40%) have been shown to have high titres of antibodies against muscle specific kinase (MUSK) (Hoch et al., 2001; Evoli et al., 2003).

b: Detection of antibody at the site of injury.

Injury at the neuromuscular junction has been associated with deposition of IgG and complement activation products (C3 and MAC) at the post synaptic membrane (review: Engel, 1984). Injury is accompanied by a loss of AChR either via internalisation or via shedding (Fambrough et al., 1973).

c: Reproduction of disease features by passive transfer of autoantibodies.

Transfer of MG patient IgG confers a MG-like disease in mice. Mice with experimental MG reproduced clinical pathology with a marked loss of AChR expression at the NMJ. Electrophysiology confirmed that disruption of the NMJ was accompanied by a loss of muscle innervation (Toyka et al., 1977).

Antibodies are also able to mediate pathology via maternal transfer across the placenta. The majority of women with MG give birth to healthy babies or with minor transient MG. Unfortunately in a small number of cases children are born with arthrogryposis, a severe congenital disorder resulting in lifelong disability. Barnes et al. demonstrated that these severe birth defects were due to high maternal antibody titres against the foetal specific form of the AChR. Passive transfer of this patient serum into pregnant mice resulted in antibody-mediated arthrogryposis in the offspring (Barnes et al., 1995).

d: Immunisation with antigen induces disease and autoantibody production.

Rabbits injected with purified AChR develop experimental MG the clinical symptoms greatly improved upon intravenous injection of edrophonium (an acetyl-choline esterase inhibitor) (Patrick and Lindstrom 1973).

e: Demonstration of pathogenic antibody activity using target cells.

Serum or IgG purified from MG patients downregulates expression of AChR on cultured muscle cells (Review: Drachmann, 2003).

f: Reduction of antibody levels ameliorates the disease.

MG patients respond well to therapies such as plasma exchange or intravenous IgG that remove pathogenic immunoglobulins from the blood which correlates with a marked improvement in clinical symptoms (Newsom-Davies, 1979).

Robb et al, observed a reduction in clinical severity in MG in response to immune suppression using azathioprine and prednisolone which corresponded to a marked reduction in AChR antibody titre (unpublished observations by Robb et al., communicated by Dr C. Buckley, European School of Neuroimmunology, October 2010).

1.4 Neuromyelitis Optica

Neuromyelitis optica (NMO) was first described over a century ago by Allbutt who reported a patient with a "sympathetic disorder of the eye" after an acute episode of myelitis (Allbutt, 1870). The disease was further characterised by Devic (1894) and is now also known as Devic's disease. NMO is classified as an inflammatory demyelinating disease characterised by a selective involvement of the spinal cord and optic nerve and which typically spares the brain. MRI studies of the spinal cord reveal extensive central longitudinal lesions which results in severe disability and respiratory failure in those patients with lesions affecting the brain stem whilst optic neuritis may be unilateral or bilateral. It was assumed for many years that NMO was simply a rare anatomical variant of MS. In fact an optic-spinal variant of MS, which accounts for 15-40% of all MS cases in Japan (Misu et al., 2002; Kira et al., 2003) shares many common features with NMO. However the identification of NMO-Ig as a specific biomarker for NMO lead to it being identified as a distinct disease entity (Lennon et al., 2004; [Table 1.3]. The antigenic target of NMO-Ig is aquaporin-4 (AQP-4) (Lennon et al., 2005). AQP-4 is the major water channel expressed by astrocytes in the CNS where it is preferentially localised within astrocytic end feet at the blood-brain barrier. Knockout studies in mice demonstrate AQP-4 is involved in maintaining the integrity of the blood brain barrier and loss of this protein causes the blood brain barrier to become hyper-permeable allowing free translocation of molecules in to the CNS (Zhou et al., 2008). NMO is the first proven example of an autoantibody mediated disease of the CNS that results in extensive loss of myelin, despite not fulfilling all of the Witesbsky/Rose citeria.

- Passive transfer of disease by NMO-Ig requires permeablisation of the blood brain barrier (Bradl et al., 2009).

- Active immunisation of rodents with AQP-4 does not induce disease (Kalluri et al., 2011).

These points demonstrate the problems of strictly applying Witebsky/Rose criteria to CNS disorders.

Table 1.3: The key features of NMO and MS
Although MS and NMO are inflammatory demyelinating diseases of the CNS, comparison of the key features of both diseases reveals significant differences in their pathology and aetiology.

	Neuromyelitis Optica	Multiple Sclerosis
Definition	Optic Neuritis and transverse myelitis	Any white matter tract involvement
Course	Relapsing (~70%) Monophasic (~30%)	Relaping (~85%)
Median age of onset	40s	20s
Gender ratio (F:M)	5:1 (relapsing) 1:1 (monophasic)	2:1
MRI Brain	Usually normal	Periventricular white matter lesions
MRI Spinal Cord	Longitudinally extensive central lesions	Multiple small peripheral lesions
Oligoclonal Bands in the CSF	Usually absent (~30%)	Usually present (~90%)
NMO IgG	Seropostive (>70%)	Seronegative

a: Autoantibodies are frequently present in patients.

The presence of NMO-IgG in the serum of patients of NMO patients was first described in 2004 by Lennon et al. using an indirect immunofluorescence staining by applying patient serum to murine CNS tissue. The staining pattern of NMO-IgG is unique, outlining CNS microvessels, pia, subpia and Virchow-Robin spaces. From a cohort of 45 patients with clinically definite NMO, 73% were seropositive for NMO IgG. In contrast, NMO-IgG was only detected in 2% of classic MS patient samples screened (n=20). Interestingly, in the small group of Japanese patients with Asian optic-spinal MS, 58% were seropositive for NMO-IgG (Lennon et al., 2004). A range of AQP-4 specific assays are also now available and that identify AQP-4 specific autoantibodies in most patients with clinically confirmed NMO (McKeon et al., 2009).

b: Detection of antibody at the site of injury.

Binding of NMO-IgG to AQP-4 to astrocytes at the blood brain barrier has been demonstrated in both murine (Lennon et al., 2005) and primate CNS sections (Vincent et al., 2008). In each case NMO-IgG binding patterns show a linear perivascular localisation in the white matter and granular layer. This staining pattern is unique to NMO-IgG and a subset of patients with optic-spinal MS but is not observed in classic MS samples (Lennon et al., 2004; Roemer et al., 2007; Vincent et al., 2008). Histopathological analysis of NMO autopsy samples shows perivascular inflammatory demyelinating lesions associated with extensive deposition of immunoglobulins and complement activation products at the perivascular rim or in a rosette pattern surrounding vessels. These lesions correlate to significant vascular fibrosis (Lucchinetti et al., 2002). Lesion formation is accompanied by loss of AQP-4 expression irrespective of lesion localisation or the extent of necrosis or demyelination. In early spinal cord lesions, demyelination is preceded by loss of AQP-4. In MS, significant AQP-4 loss was only detectable in inactive MS lesions (Roemer et al., 2007).

c: Reproduction of disease features by co-transfer of autoantibodies.

In MG the antigenic target is readily accessible to circulating antibody, but this is not the case for CNS antigens, which are sequestered behind the blood brain barrier (BBB). It is well established that the BBB will normally inhibit entry of antibody into the CNS, and as a consequence circulating CNS specific autoantibodies will cause no clinical or pathological deficits in healthy animals (Litzenburger et al., 1998). However, the pathogenic potential of NMO associated autoantibodies can be demonstrated either by direct injection in to the CNS (Saadoun et al., 2011: *in press*) or passive transfer into animals with EAE (Bradl et al., 2009). In the latter case NMO patient-derived autoantibodies not only exacerbated clinical disease but also resulted in formation of NMO-like lesions characterised by immune complex deposition, AQP-4 and astrocyte loss and inflammatory cell infiltrates. Similar observations were made in adoptive transfer studies using patient-derived AQP-4 specific monoclonal antibodies (Bennett et al., 2009).

Transfer of immunoglobulins purified from NMO patients into rats with EAE mediated by encephalogenic MBP specific T cells augments clinical disease. Associated histopathology demonstrates the formation of NMO-like lesions characterised by immune complex deposition, AQP-4 and astrocyte loss accompanied by extensive immune cell infiltrates (Bradl et al., 2010). These observations replicate those reported previously when transferring an AQP-4 specific monoclonal antibody into rats with T cell mediated EAE (Bennett et al., 2009).

d: Immunisation with antigen induces disease and autoantibody production.

There is as yet no formal AQP-4 based in vivo animal model of NMO, although some rodent models of MOG-induced EAE do exhibit notable NMO-like pathology (i.e. lesions restricted to spinal cord and optic nerve) (Stefferl et al., 1999; Krishnamoorthy et al., 2006; Bettelli et al., 2006). Immunisation of mice with a full length AQP-4 peptide or peptides corresponding to immunogenic T cell epitopes did not induce spinal cord injury or optic neuritis (Kalluri et al., 2011). This lack of observed pathology may be due to dependence on conformational epitopes similar to that described in some forms of MOG induced EAE (Stefferl et al., 1999). Although active immunisation with AQP-4 peptides is insufficient to induce injury, it has been reported that immunisation is capable of producing high titre AQP-4 anti-sera in rodents (Review: Graber et al., 2008).

e: Demonstration of pathogenic antibody activity using target cells.

Binding of NMO-IgG to astrocytic AQP-4 can be shown both *in vivo* and *in vitro* using cultured astrocytes. In active NMO lesions there is a loss of perivascular GFAP+ astrocytes (Misu et al., 2007), but the exact mechanism leading to astrocyte loss remains unclear, although incubation of rat astrocytes *in vitro* with NMO-Ig mediates complement dependent lysis (Kinoshita et al., 2009). Using an in vitro BBB model Vincent et al. demonstrated that incubation of astrocytes in culture with NMO-IgG caused internalisation of AQP-4 and enhanced BBB permeability (Vincent et al., 2008). However, it still remains unclear how autoantibodies that attack the end feet of astrocytes trigger demyelination. Incubation of co-cultures of astrocytes and oligodendrocytes with NMO-IgG resulted in major astrocyte dysfunction and a marked loss of oligodendrocytes.

This observation was reproduced in ex vivo optic nerve tissue preparations. One potential mechanism may due to increased glutamate cytotoxicity, as incubation of treated co-cultures with a NMDA receptor antagonist partially protected oligodendrocyes (Marignier et al., 2010).

f: Reduction of antibody levels ameliorates the disease.

Antibody depleting therapies such as plasma exchange are useful in the clinical management of NMO (Keegan et al., 2002) as is B cell depletion (Cree et al., 2005). Immune modulation using rituximab, azathioprine and cyclophosphamide is associated with reduced AQP-4 antibody titres and reduced relapse rates. Conversely, a three fold increase in AQP-4 antibody titres was detected shortly before relapse (Jarius et al., 2008).

In conclusion, the demonstration that antibody-dependent mechanisms were involved in NMO and the identification of AQP-4 as a target for this response radically improved the clinical management of NMO. If it can be shown that autoantibody mediated mechanisms contribute to the pathogenesis of MS, it is likely that this will result in a similar improvements in disease diagnosis and treatment.

1.5 Antibody involvement in multiple sclerosis- the supporting evidence

1.5.1 The presence of oligoclonal bands in the CSF

It has been known since the 1940s that MS is associated with elevated levels of intrathecal immunoglobulins within the CSF (Kabat et al., 1948). Resolution of patient CSF Ig by agarose gel isoelectric focusing (IEF) produces an oligoclonal banding pattern completely distinct from the polyclonal "smear" obtained when analysing serum IgG (Johnson et al., 1977). Oligoclonal bands (OCB) are present in the CSF in a majority of MS patients and are considered a diagnostic hallmark of the disease. Despite their presence in the majority of patients, the banding pattern and specificity varies (Andersson et al., 1994). The significance of this heterogeneity remains unclear. There is evidence to suggest that OCBs may be useful relevant prognostic biomarkers as OCB-negative MS patients tend to follow a benign disease course (Farrell et al., 1985). Conversely, in a retrospective study, elevated intrathecal IgG synthesis and number of OCBs were correlated with a progressive disease course (Avasarala et al., 2001), although a more recent study failed to validate this observation (Koch et al., 2007) therefore their true value is difficult to determine. In RRMS the presence of oligoclonal IgM in the CSF of RRMS patients is indicative of an increased probability of developing secondary progressive disease (Villar et al., 2002) and enhanced intrathecal IgM production was associated with rapid disease progression (Villar et al., 2003). However once again, other groups failed to replicate these findings as they were not confirmed by an independent study (Schneider et al., 2007), so the value of these CSF measurements as informative biomarkers remains controversial and their clinical relevance obscure.

1.5.1.1 Source of oligoclonal bands

B cells and antibodies do not normally cross the intact BBB to any significant extent. However, at sites of inflammation not only are B cells are recruited into the CNS, but passive diffusion of serum proteins into the lesion is also greatly increased due to localised damage to the BBB. B cells were first described in the

CNS of MS patients by Prineas, who detected ectopic lymphoid-like structures in some autopsy samples (Prineas, 1979). These follicle-like aggregates of B cells are reported to be a relatively common finding in SPMS, where they are localised within the meninges. Moreover, their presence correlates with a more aggressive disease course (Serafini et al., 2004; Magliozzi et al., 2007). The presence of these follicles, together with the observation that synthesis of individual OCB can persist for many years, suggests the CNS in MS provides niches that support sustained B cell survival and differentiation (review: Meinl et al., 2006). Unfortunately there is as yet no formal evidence that OCB IgG is actually derived from B cells within these aggregates. However, analysis of the IgG CSF proteome revealed that this overlapped with the IgG transcriptome obtained from a single representative of clonally expanded CSF B cell populations in individual patients (Obermeier et al 2008). This formally demonstrates that at least some OCB components are derived from clonally expanded CNS B cell populations in MS. However it must be stressed that OCB are not specific for MS, and ectopic B cell follicles are also present at sites of chronic inflammation in other autoimmune diseases such as rheumatoid arthritis (Revew: Aloisi and Pujol-Borrell, 2006).

1.5.1.2 Potential specificities of OCBs in MS

As stated above, the presence of OCB in CSF is not unique to MS, but is also common in infectious diseases of the CNS such as measles encephalitis (Vandvik and Norrby, 1973), Lyme neuroborreliosis (Halperin et al., 1989) and neurosyphilis (Pedersen et al., 1982). In these cases OCB are generally directed against the causative pathogen, which lead to numerous attempts to identify a pathogen-specific OCB response in MS patients. Some OCB associated with MS do actually recognise common pathogens such as the measles, rubella and varicella-zoster viruses (Sindic et al., 1994) or Chlamydia pneumoniae (Derfuss et al., 2001). However these specificities are normally associated with minor OCB components and are generally considered to represent a sample of the patient's immunological/infectious history (Reiber et al., 1998). The failure of these studies to identify prominent intrathecal B cell responses to pathogens stimulated speculation that OCB associated with MS were directed against CNS autoantigens. However, no study has identified a verified autoimmune target (see Owens et al., 2009; reviewed in Sospedra and Martin, 2005).

1.5.2 Evidence from histopathogical studies of MS lesions

One of the most recent influential contributions to our understanding of the pathogenesis of MS was an immunopathological study performed by Lucchinetti and colleagues. The study was the product of extensive international collaboration that allowed the authors access to a large collection of biopsy and autopsy samples from actively demyelinating MS lesions (Lucchinetti et al., 2000). Although this study was extensively criticised as the majority of biopsy samples came from patients with clinically or radiologically atypical disease who may not therefore be representative of "classic" cases of MS (Poser et al., 2000), nevertheless, it is still of great interest and significance. Lesions were classified using a number of criteria including the phenotype of cells infiltrating the lesion, presence of complement activation products (C9neo) and immunoglobulin deposition [Table 1.4]. Type I lesions were characterised by extensive demyelination associated with infiltrating macrophages/microglia, whilst the most common type II was characterised by deposition of immunoglobulins and C9neo at the site of myelin injury. The least common lesion patterns observed were type III and type IV lesions. These lesions were not associated with immune mediated demyelination via macrophages or antibodies/complement but were reminiscent of viral or toxin induced models of demyelination. Interestingly, signs of remyelination were reported only in association with immune mediated/ inflammatory lesion (types I and II).

Most importantly, this study demonstrated that while MS is not only clinically but also pathologically heterogeneous, in any one patient the pattern of demyelination in active lesions seems to be homogeneous. This observation has major implications for the clinical management of MS. In particular, the study highlighted the possibility that humoral mechanisms play a significant role in lesion formation in the majority of patients (as high as 50%); suggesting that targeting this arm of the immune response could provide significant clinical benefits.

This study has been extremely influential in developing our understanding of MS, but its findings remain controversial. The concept that demyelination in MS may be mediated by several different effector pathways was challenged by Breij et

al. who analysed actively demyelinating lesions in autopsy material from 39 patients. In this study all cases of active demyelination were associated with complement activation products, immunoglobulins and phagocytic macrophages. This supports the hypothesis that humoral mechanisms play a significant role in demyelination and calls into question the involvement of other effector mechanisms (Breij et al., 2006). However deposition of immunoglobulins and complement within CNS many not be unique to MS lesions. Analysis of autopsy samples from 25 MS and 24 OND patients failed to identify any specific association between MS and deposition of immunoglobulins and complement activation products in the CNS. The authors interpreted their observations as evidence that white matter injury in general is associated with complement activation irrespective of the identity of the disease (Barnett et al., 2009).

Table 1.4: Heterogeneous characteristics of MS lesions as described by Lucchinetti et al. (2000).

	Type I	Type II	Type III	Type IV
Demyelination	Centred around veins	Centred around veins	Not centred around veins. Concentric rings of demyelination seen in ~30%	Sometimes centred around veins
Oligodendrocytes within lesion	Many	Many	Few (apoptotic)	Few
Complement & immunoglobulin deposition	None	Much	None	None
Macrophage infiltration	Much	Much	Little	Little
Signs of remyelination	Yes	Yes	No	No
Reported frequency	~20%	~55%	~25% (Type IV is rare and reliability remains unclear)	

1.5.3 The efficacy of B cell/ antibody targeted therapies

Most therapeutic agents routinely used in MS such as corticosteroids, interferons and Tysabri target the inflammatory component of the disease. These treatments provide a significant clinical benefit in patients with RRMS but do not specifically target antibody/humoral pathomechanisms. However, as our understanding of MS pathogenesis has advanced there is more interest in exploring the potential role of antibodies and B cells as therapeutic targets.

1.5.3.1 Plasma exchange

Plasma exchange is an extracorporeal therapy designed to remove and replace plasma from the patient's blood. For many years it has been used with great success in treating a wide range of autoantibody mediated diseases such as myasthenia gravis, SLE and Guillain-Barre syndrome. However, plasma exchange is not a widely accepted therapy for MS. This is despite clinical studies dating back to the 1990s that suggest ~45% of MS patients with acute steroid non-responsive exacerbations disease may benefit from therapeutic plasma exchange (TPX; Rodriguez et al., 1993; Weinshenker et al, 2001). In view of the reported pathological heterogeneity of MS it is unlikely that TPX would be beneficial in all patients. This concept is supported by a recent retrospective study which demonstrated the therapeutic potential of plasma exchange correlated with the presence of immunoglobulin and complement activation products in biopsied lesions (Keegan et al., 2005). C9neo immunoreactivity is a defining feature of pattern II type lesions as defined by Lucchinetti, Lassmann and colleagues and only these patients benefited significantly from TPX (Keegan et al., 2005).

1.5.3.2 B cell depletion therapies

Following these observations suggesting that humoral pathomechanisms may drive disease activity in at least some patients, several clinical studies were initiated to investigate whether depletion of B cells might also prove beneficial (Review: Cross and Waubant, 2011). The agent of choice for these studies was Rituximab, a monoclonal chimeric antibody that selectively targets and depletes CD20+ B cells and which is already licensed to treat lymphoma and rheumatoid arthritis. A recent phase II trial of Rituximab in relapsing-remitting MS

indicated substantial clinical benefit (Hauser et al., 2008). A single infusion of Rituximab resulted in a rapid and sustained depletion of B cells (> 24 weeks) associated with dramatic reductions in both the total number of MRI lesions and the number of newly appearing gadolinium enhancing lesions in the brain compared to controls. This decrease in lesion activity/load was accompanied by a reduction in clinical disease activity as determined by the relapse rate. This rapid response was unexpected CD20 is not expressed by plasma cells and it occurred before there was any significant effect on serum immunoglobulin levels (Hauser et al. 2008). An independent study also failed to demonstrate any effect of Rituximab on CSF IgG levels, rate of synthesis or removal of oligoclonal bands (Cross et al., 2006). These observations indicate that the pathogenic role of B cells in MS extends beyond their role as a source of autoantibodies, but at present how B cell depletion modulates the inflammatory response in the CNS remains unclear. Rituximab has also been trialled in PPMS and in parallel with the findings in RRMS it also suppressed inflammatory disease activity as determined by MRI (Hawker et al., 2009).

1.6 Myelinated axons- the target of autoantibodies in MS

The studies outlined above suggest antibody-dependent mechanisms contribute to disease development in some patients with MS, but the specificity of this response remains obscure. As demyelination is the most obvious pathological feature of MS it was assumed that any clinically significant autoantibody response would be directed against a CNS myelin-specific autoantigen. This concept was supported by numerous studies in EAE that identified several myelin associated antigens as targets for autoantibody mediated demyelination in vivo (review: McLaughlin and Wucherpfennig, 2008). The spectrum of potential targets is actually wider, as it is now recognised that axon-specific autoantibodies may also contribute to disease development (Mathey et al., 2007; review: Derfuss et al., 2010). Yet of the numerous potential targets identified over the past four decades, none were ultimately found to be MS specific [Table 1.5]. Indeed, it is rash to assume that all autoantibody responses are pathogenic, as an increasing body of evidence demonstrates that some components of the autoantibody repertoire can actually stimulate remyelination in vivo (review: Schwab et al., 2004; Reindl et al., 2003; Mi et al., 2007).

Nonetheless, it remains possible that an MS-specific autoantibody response will be identified and that this in turn will provide new diagnostic/prognostic tools to improve disease management. This section reviews this concept, focusing on recent advances in our understanding of the MOG-specific autoantibody response.

Table 1.5: Antibodies to myelin and other CNS autoantigens implicated in MS
Decades of MS research have yielded a number of potential targets for autoantibodies in MS. These antibody responses have varying functions in disease. Some are described as being involved in mediating disease pathogenesis (MOG, Nfasc, GalC) and whereas others are thought to be involved in promoting remyelination and repair (Lingo-1, heat shock proteins, Nogo-A). Antibody responses have also been reported as potential prognostic markers (neurofilament, phosphatidyl choline).

Antigen	Significance in MS	References
Alpha-B-crystallin	Ab present in MS patient sera and CSF, potential link between antibody titre and relapse rate	Agius et al., 1999 Celet et al., 2000 Vojdani et al., 2003
Alu repeats	OPC derived B cell epitope. Abs found in MS CSF and sera (~50)	Archelos et al., 1998
AN-2 (NG-2)	OPC surface antigen, abs found in MS patient CSF	Niehaus et al., 2000
CNPase	Abs present in MS patient sera and CSF (~74)	Walsh and Murray 1998
GalC	Abs found in RRMS (~40) patient serum but much less in CIS (~8)	Menge et al., 2005 Menon et al., 1997
Gangliosides	GD1a, GM3 and sulphatide abs present in sera and CSF of MS patients. More frequent in patients with chronic progressive disease	Acarin et al., 1996 Sadatipour et al., 1998 Kanter et al., 2006
Glycopeptides	Abs against CSF114(Glc) & Glc(alpha1,4)Glc(alpha) present in MS patient sera	Lolli et al., 2005 Schwartz et al., 2006
HSP 60 & HSP90	Antigens expressed by OPCs, antibodies may inhibit remyelination	Selmaj et al., 1992 Cid et al., 2005
Lingo-1	Nogo receptor interacting protein. Potential role in axonal survival and remyelination.	Mi et al., 2007
MAG	Abs present in MS patient CSF, potential correlation with disease progression	Baig et al., 1991 Moller et al., 1989
MBP	Antibodies present in sera and CSF of MS patients (~25-58)	Schmidt et al., 2001 Egg et al., 2001 Reindl et al., 1999 Olsson et al., 1990 Cruz et al., 1987
MOG	The major focus of MS autoantigen research. Abs present in MS lesions. Antibodies present in sera and CSF of MS patients (~0-80). Serum anti-MOG abs in CIS patients predictive for chronic disease	Genain et al., 1999 Berger et al., 2003 Kuhle et al., 2007
Neurofascin	Axoglial adhesion molecule. Abs to both Nfasc155 and Nfasc186 isoforms are present in MS serum.	Mathey et al., 2007
Neurofilament	Abs present in MS patient sera and CSF; titre correlates with disease progression and MRI activity.	Ehling et al., 2004 Silber et al., 2002
Nogo-A	Neurite outgrowth inhibitor. Abs in serum and CSF of patients with RRMS and other acute neurological diseases.	Reindl et al., 2003
OSP	Abs found in CSF of MS patients. Does not bind to native antigen.	Bronstein et al., 1999 Aslam et al., 2010
Phosphatidylcholine	Oligoclonal IgM present in MS patient CSF, potential correlation with disease progression	Villar et al., 2002 Villar et al., 2005
PLP	Abs and specific B cells in the CSF of MS patients	Sun et al., 1991a Warren et al., 1994
Proteosome	Protein complex involved in processing and chaperone function. Abs present in serum and CSF of MS patients (~66).	Mayo et al., 2002
Transaldolase	Abs present in MS patient serum and CSF	Banki et al., 1994

1.6.1 Myelin oligodendrocyte glycoprotein (MOG)

Myelin oligodendrocyte glycoprotein (MOG) is a CNS-specific autoantigen sequestered at the outer surface of the myelin sheath and oligodendrocyte (Linington et al., 1988; Brunner et al., 1989; Kroepfl et al., 1996). MOG was first identified as a dominant target for demyelinating autoantibodies in animals with EAE induced by immunisation with CNS tissue homogenates in Freunds complete adjuvant (Lebar et al., 1986). Subsequently passive transfer experiments demonstrated that anti-MOG antibodies augments disease severity and leads to extensive demyelination in animals with (Linington et al., 1988; Genain et al., 1995). These properties are attributed to the accessibility of the extracellular IgV-like domain of MOG to antibody in the extracellular milieu (Gardinier et al., 1992). Demyelination mediated by MOG-specific antibodies in these EAE models is associated with co-deposition of immunoglobulin and complement activation products (Genain et al., 1999; Raine et al., 1999; Storch et al., 1998), reproducing the immunopathology of pattern II MS lesions as defined by Lucchinetti and colleagues (Lucchinetti et al., 2000). It should be noted that transfer of antibodies recognising intracellular MBP epitopes does not induce widespread demyelination in EAE (Schluesener et al., 1987).

These animal experiments stimulated a plethora of studies in which MOG-specific antibody responses were investigated in MS and other neurological diseases. To date there are hundreds of papers relating to the MOG-specific response in MS were published the results of which may be summarised as being generally conflicting and controversial [Table 1.6]. Some studies report that MS is associated with elevated serum or CSF MOG-specific antibody titres (Sun et al., 1991; Lalive et al., 2006; Lindert et al., 1999; Gaertner et al., 2004) whilst others find no significant differences between MS patients and patients with other neurological inflammatory diseases or healthy controls (Haase et al., 2006; Reindl et al., 1999; Lampsona et al., 2004; Xiao et al., 1991;). The frequency of seropositive patients in these studies ranges from 0 to 88% in MS and 0 to 67% in healthy controls depending on the source of antigen and assay protocol. The observation that MOG-specific antibodies were pathogenic in EAE also stimulated attempts to attribute some clinical significance to these responses detected in patients. A potential prognostic role was first reported by Berger et al. who

studied a cohort of 103 CIS patients and reported that those with high levels of serum anti-MBP and anti-MOG IgM were most likely to suffer relapses earlier and more frequently than seronegative patients (Berger et al., 2003). However these observations could not be reproduced in subsequent report using an identical protocol (Kuhle et al., 2007).

In retrospect the lack of reproducibility between studies reflects a failure to accept that any autoantibody response that may play a primary role in disease pathogenesis has to recognise its target as it exists in vivo. In other words any *in vitro* assay to detect such responses should reproduce as closely as possible the targets three-dimensional structure, post-translational modifications and membrane topology as they occur *in vivo*. Unfortunately this was not the case in the majority of published studies, as these either relied on using denatured recombinant MOG expressed in *E.coli* or synthetic peptides in ELISA or Western blot based assays. The importance of retaining the native 3-D structure of MOG if one is to detect pathogenic i.e. demyelinating antibody responses is now apparent from the crystal structure of MOG complexed with the Fab fragment of a demyelinating MOG-specific mAb. In both rodent and marmoset models of EAE demyelinating MOG-specific antibody responses are directed against conformational/discontinuous epitopes (Bourquin et al., 2000; Brehm et al., 1999; von Budingen et al., 2004). A demyelinating response which is not observed when animals are immunised with linear MOG derived peptides. These observations are consistent with the published crystal structure of the MOG/anti-MOG Fab complex. This demonstrated the demyelinating MOG-specific mAb 8-18C5 recognised a discontinuous and conformation-dependent epitope focused on the FG loop of the proteins Ig-V like fold (Breithaupt et al., 2003). Subsequently it was confirmed this conformation-dependent epitope is the immunodominant target of the demyelinating MOG-specific response in several strains of mice and the Dark Agouti rat (Breithaupt et al., 2008). In vitro it is also essential to retain the correct conformation of MOG to detect pathogenic MOG-specific antibodies targeting the oligodendrocyte surface (Lolli et al., 2005; Marta et al., 2005).

To identify potentially pathogenic responses in MS patients a number of groups adopted cell based assay systems that use mammalian cell lines manipulated to express MOG on their surface (Haase et al., 2006; Gaertner et al., 2004; Lalive et al., 2006; Zhou et al., 2006). Theoretically these cell based assays will detect antibodies against native MOG embedded in a membrane which will go undetected by other methods. Crucially this approach detects MOG-specific antibodies in only a small percentage of patients indicating that it is unlikely to be the dominant target for demyelinating antibodies in MS. A conclusion supported by the failure of fluid phase assays using correctly folded and glycosylated recombinant MOG preparations to detect high affinity MOG-specific antibodies in patient sera (Lampsona et al., 2004; O'Connor et al., 2005).

Nonetheless MOG-specific antibodies are present in acutely demyelinating MS lesions where they are associated with myelin debris (Genain et al., 1999), and can be recovered from MS autopsy samples (O'Connor et al., 2005). In the latter study in 50% of the MS cases these antibodies were highly specific for MOG compared to 13% in non-MS controls and exhibited higher affinities for MOG than antibodies isolated from the patients serum or CSF. The clinical significance of these MOG-specific autoantibody responses is still to be determined in classical forms of MS, but there is increasing evidence that the presence of antibodies to the native antigen may define a specific subset of patients with paediatric inflammatory demyelinating disease.

Table 1.6: Overview of selected publications investigating serum antibody responses (IgG) to MOG in patients with adult-onset MS

There have been numerous contributions over the past 20 years to determine the frequency of antibody responses against MOG in patients with adult-onset MS. Some studies have reported elevated MOG specific autoantibody responses in MS patients but other reports suggest no significant difference between MS and other neurological diseases or healthy controls. The reported frequencies of α-MOG responses in MS patients range from 0-82% depending on the method of detection and antigen source used in each study.

Study	Assay	N	% MOG+ patients
Sun et al., 1991b	**ELISPOT** *MOG purified from human myelin*	**MS:** 16	**MS:** 50 **HC:** 0
Xiao et al., 1991	**ELISA** *MOG purified from human myelin*	**MS:** 30 **ONND:** 30	**MS:** 0 **ONND:** 0
Reindl et al., 1999	**Western Blot** *Recombinant human MOG Ig domain expressed in E.coli*	**MS:** 130 **ONID:** 32 **ONND:** 30 **RA:** 10	**MS:** 38 **ONID:** 53 **ONND:** 3 **RA:** 10
Lindert et al., 1999	**Western Blot** *Recombinant human MOG Ig domain expressed in E.coli*	**MS:** 37 **Controls:** 37	**MS:** 54 **Controls:** 22
Egg et al., 2001	**Western Blot** Recombinant human MOG Ig domain expressed in *E.coli*	**MS:** 261	**MS:** 35
Haase et al., 2001	**ELISA** *synthetic MOG peptides* **FACS** *recombinant human MOG expressed on mammalian cells*	**MS:** 17 **HC:** 9	**MS:** 82 **HC:** 67 **MS:** 6 **HC:** 0
Gaertner et al., 2004	**ELISA** *recombinant human native MOG expressed by mammalian cells*	**MS:** 75 **Non-MS:** 20	**Elevated in MS compared to controls**
Lampsona et al., 2004	**Liquid phase radioimmunoassay** recombinant human MOG *in vitro* translation	**MS:** 87 **Non-MS:** 59	**MS:** 6 **Non-MS:** 4
O'Connor et al., 2005	**Liquid phase radioimmunoassay** *recombinant human MOG (refolded in vitro by mammalian cells)*	**MS:** 14 **Non-MS:** 8	**MS:** 3 **Non-MS:** 0
Lalive et al., 2006	**FACS** recombinant human MOG expressed by mammalian cells	**MS:** 92 **CIS:** 36 **HC:** 37	**Elevated in CIS and RRMS but less frequent in SPMS and absent in PPMS**

1.6.1.1 MOG antibody responses in paediatric demyelinating diseases

Paediatric MS defined as having disease onset before the age of 16 is thought to represent between 2 to 5% of all MS cases (Chitnis et al., 2009), although it's precise incidence is unclear. In the majority of cases (>90%) the disease follows a relapsing remitting disease course, but tends to be more aggressive than its adult-onset equivalent being characterised by higher relapse rates (Gorman et al., 2009) and rapid accumulation of neurological disability (Renoux et al., 2007). Unlike adult onset disease, paediatric MS has no strong gender bias (pre puberty) and is present across many ethnic groups (review: Banwell et al., 2011).

In contrast to previous reports on adult-onset MS, paediatric MS is associated with a far higher frequency of patients seropositive for MOG-specific autoantibodies as determined using cell based assays. This was demonstrated beautifully by McLaughlin et al. (2009) using a FACS based assay with MOG transfectants to compare the frequency of anti-MOG antibody responses in paediatric and adult-onset MS patients with appropriate age matched controls for each group. Within the adult cohort there was no significant difference between those donors with or without MS (4% and 5% respectively), whereas. 21% of paediatric MS cases were seropositive compared to only 6% of age matched OND controls (McLaughlin et al., 2009).

Intriguingly the major differential diagnosis for paediatric MS is acute disseminated encephalomyelitis (ADEM) and these patients also exhibit elevated responses to MOG in both cell and fluid phase based immunoassays. Early in disease it is extremely difficult to distinguish between the two diseases. ADEM is a demyelinating disease of the CNS which usually has a monophasic disease course and is most prevalent in children but can also occur in adults. The incidence of ADEM is estimated at approximately 1 per 100,000 and in 50 - 75% of cases, disease onset occurs shortly after a viral or bacterial infection or vaccination (review: Menge et al., 2005) which lead to speculation that MOG-specific responses in these patients may occur by virtue of molecular mimicry. Cross reactive pathogenic antibody responses between viral and myelin antigens have been previously demonstrated experimentally involving mimicry between MOG and rubella virus (Besson-Duvanel et al., 2001).

Over the past five years significant progress has been made in cataloguing MOG-specific antibody responses in children with MS and ADEM [Table 1.7]. The reported frequencies vary from 2 to 21% for paediatric MS and 18 to 54% for ADEM. These variations probably reflect technical differences in the assays employed by different groups of researchers, including the selection of the threshold used to define a positive response. However it is becoming increasingly clear that not only does this particular antibody response define a specific subset of young patients with MS, but may also provide a useful diagnostic biomarker to help differentiate patients with ADEM from those with other acute encephalopathies (Lalive et al., 2011). Yet it has still to be demonstrated whether these MOG-specific antibodies actively contribute to disease pathogenesis by exacerbating demyelination, as would be anticipated from studies in EAE.

Table 1.7: Overview of selected publications investigating serum antibody responses (IgG) to MOG in paediatric MS and ADEM

Within the last 5 years there have been a small number of reports demonstrating that children with MS or ADEM have elevated titres of MOG specific autoantibodies. The pathogenic significance of these autoantibody responses has not been yet demonstrated.

Study	Assay	N	% MOG+ patients
O'Connor et al., 2007	**Liquid phase radioimmunoassay** *Tetramers of human MOG extracellular domain (refolded in vitro by mammalian cells)*	**MS:** 19 **ADEM:** 56 **CIS:** 32 **VE:** 58 **HC:** 75	**MS:** 5 **ADEM:** 18 **CIS:** 0 **VE:** 0 **HC:** 1
McLaughlin et al., 2009	**FACS** *native human MOG expressed on mammalian cells*	**Paediatric** **MS:** 131 **OND:** 34 **NND:** 37 **Adult** **MS:** 254 **CIS:** 30 **HC:** 86	**Paediatric** **MS:** 21 **OND:** 6 **NND:** 0 **Adult** **MS:** 4 **CIS:** 7 **HC:** 5
Brilot et al., 2009	**FACS** *native human MOG expressed on mammalian cells*	**ADEM:** 9 **CIS:** 28 **HC:** 30	**ADEM/CIS:** 40 **HC:** 0
Di Pauli et al., 2011	**Immunofluorescence** *native human MOG expressed on mammalian cells*	**MS:** 89 **CIS:** 38 **ADEM:** 34 **OND:** 58 **HC:** 47	**MS:** 2 **CIS:** 8 **ADEM:** 44 **OND:** 2 **HC:** 0
Lalive et al., 2011	**FACS** *native human MOG expressed on mammalian cells* **ELISA** *recombinant human MOG extracellular domain*	**MS:** 22 **ADEM:** 11 **VE:** 7 **HC:** 13	**MS:** 5 **ADEM:** 27 **VE:** 0 **HC:** 0 **MS:** 14 **ADEM:** 55 **VE:** 0 **HC:** 0

1.6.2 Myelin lipids as MS autoantigens

Myelin proteins are generally considered the most probable target for pathogenic autoantibodies in MS. However, ~70% of the myelin sheath is composed of lipid (Norton, 1984) and historically lipid-specific autoantibodies have also been investigated as causative agents in the context of immune-mediated demyelination, in particular antibodies recognising myelin associated glycosphingolipids such as galactocerebroside and sulphatide.

1.6.2.1 Galactocerebroside

Galactocerebroside (GalC) is a major component of the myelin sheath, accounting for about 25% of myelin lipid. GalC is integrated into the outer surface of the membrane bilayer where it is accessible to antibody in the extracellular milieu. Historically there are a number of publications implicating GalC-specific antibodies in the pathogenesis of immune mediated demyelination in EAE (Raine et al., 1981; Fierz et al., 1988) but their significance in MS remains obscure (Rostami et al., 1987; Kanter et al., 2006). However, it was recently reported that GalC-specific antibodies provide a stage specific biomarker in that they were detected preferentially in patients with RRMS compared to CIS, SPMS, PPMS and other neurological diseases (Menge et al., 2005).

1.6.2.2 Sulphatide

In addition to GalC, myelin is also enriched in sulphatide (3-O-sulphogalactosyl ceramide; Sulph). This glycolipid is present in the outer leaflet of the myelin bilayer where it can bind Sulph-reactive antibodies if present in the extracellular compartment. Intriguingly not only are elevated levels of Sulph-specific antibodies present in the CSF of patients with MS compared to controls, but active immunisation with Sulph or the passive transfer of a Sulph-reactive mAb exacerbates EAE (Kanter et al., 2006). The later observation confirms that Sulph can provide a physiological target for autoaggression, but as yet whether Sulph-reactive antibodies detected in patients are pathogenic remains unproven.

1.6.2.3 Other lipid specificities associated with MS

The presence of oligoclonal IgM bands has been described as a potential prognostic marker in MS (Villar et al., 2002). These IgM antibodies recognise a number of myelin lipids, in particular phosphatidylcholine were most frequently observed in patients with an aggressive disease course (Villar et al., 2005). However as is the case for both GalC- and Sulph-specific responses there is no conclusive evidence that these responses actively contribute to immune mediated demyelination. Indeed with respect to antibodies present in CSF repeated studies have failed to demonstrate these are able to bind to oligodendrocytes (Lubetzki et al., 1986) or any major myelin antigen (Owens et al., 2009).

1.6.3 Axonal/ neuronal autoantigens

Although MS was long described as a demyelinating disease characterised by primary oligodendrocyte loss and demyelination in which there was extensive sparing of axons, it is now recognised that MS lesions are often associated with profound axonal loss. Acute axonal injury as demonstrated by axonal swellings and enhanced immunoreactivity for beta-amyloid precursor protein (APP) is most pronounced in regions of active inflammation and demyelination and is associated with varying degrees of axonal transection (Ferguson et al., 1997; Trapp et al., 1998). The mechanisms involved are still being elucidated but there is now evidence suggesting these may involve an autoantibody-mediated component. Screening MS sera for reactivity to cell surface antigens expressed by neuronal cell lines identified neuronal reactivity in the sera of at least 70% of PPMS patients and in 25% of those with RRMS (Lily et al., 2004). The antigen specificity of these responses was not reported, but other studies have reported neurofilament, ganglioside and neurofascin-specific responses in some MS patients.

1.6.3.1 Neurofilament

It is suggested that autoantibodies recognising against the neurofilament light chain (NF-L) provide a biomarker for axonal injury. Increases in NF-L-specific antibody titres are present in the serum of patients with PPMS (Ehling et al., 2004; Silber et al., 2002) and correlate with increased disability as scored using expanded disability status scale (EDSS; Silber et al., 2002) and brain atrophy as visualised by MRI (Eikelenboom et al., 2003).

1.6.3.2 Gangliosides

Gangliosides are glycosphingolipids which decorate the surface of both CNS and PNS axons. It is reported that pathogenic autoantibody responses against gangliosides are associated with Bickerstaff's encephalitis (GQ1b) (Odaka et al., 2001), Miller-Fisher syndrome (GQ1b) (Chiba et al., 1992) and are also present in patients with Gullian-Barre syndrome (review: Willison and Yuki, 2002). In MS the situation is less clear, but some patients do develop responses to GD1a (Acarin et al., 1996) and GM3 (Sadatipour et al., 1998). These anti-ganglioside responses tend to be most frequent in patients with progressive disease (Sadatipour et al., 1998), but their pathophysiological relevance, if any, remains unknown.

1.6.3.3 Neurofascin

Neurofascin (Nfasc) is cell adhesion molecule of the L1 Ig superfamily which exists as two major isoforms, Nfasc186 and Nfasc155, in myelinated fibres. These are structurally and functionally distinct [Figure 1.2]. Nfasc186 is a neuronal product localized at the nodes of Ranvier and axonal initial segments (AIS) where it interacts with voltage gated sodium channels and cytoplasmic proteins such as ankyrin G and ßIV-Spectrin (Zhang et al., 1998a; Ratcliffe et al., 2001). In contrast, Neurofascin-155 (Nfasc155) is an oligodendroglial protein sequestered at septate-like junctions formed where the paranodal loops of the myelin sheath contact the axonal surface. Here Nfasc155 interacts with an axonal Caspr-Contactin complex to form electron dense assemblies characteristic of the paranodal junctional complex (Tait et al., 2000; Charles et al., 2002).

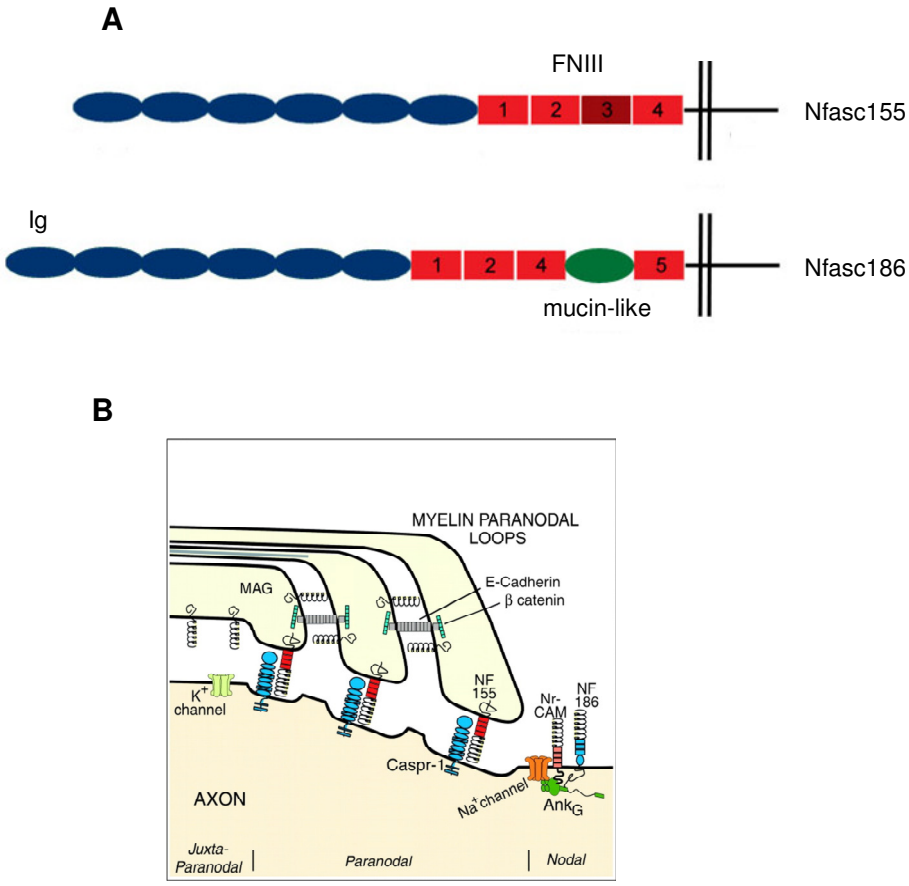


Figure 1.2: Nfasc exists as two structurally and functionally distinct isoforms.
A: Nfasc 155 (upper) consists of six immunoglobulin (Ig) and 4 fibronectin (FN) III domains. Nfasc186 (lower) shares the same structure as Nfasc155 apart from the presence of an alternate FNIII domain and a mucin like domain. Image modified from Tait et al., 2000. **B:** Nfasc155 is expressed by the oligodendrocyte at the paranode where it interacts with caspr-1/contactin. Nfasc186 is expressed by the axon at the node of Ranvier forming interactions with the voltage gated sodium channels and ankyrin G. Image taken from Trapp and Kidd, 2000.

Nfasc was identified as a candidate autoantigen in MS using a proteomics approach (Mathey et al., 2007) in which immunoreactivity of serum samples with myelin glycoproteins was screened by western blotting. Approximately 20% of patients tested had significant antibody responses to a 150-180kDa protein which was subsequent identified as Nfasc. Further characterisation by ELISA revealed that ~30% of patients had high titres against the extracellular domain of Nfasc155 and that this response was most pronounced in patients with chronic progressive disease. However, it should be noted that this response as detected by ELISA was not MS-specific as similar responses could also be identified in some patients with other neurological diseases, as well as healthy controls. The authors demonstrated that this Nfasc-reactive antibody repertoire contained a cross reactive component that recognised the extracellular domain of Nfasc186 and Nfasc155 expressed on transfected cells. Co-transfer studies in EAE using a pan-Nfasc specific mAb (A12/18.1) revealed the dominant pathological target for antibodies with this specificity in the inflamed CNS was Nfasc186 expressed at the node of Ranvier. *In vivo* antibody recognition of Nfasc at the node exacerbated disease severity in EAE and was associated with complement deposition and axonal injury. *In vitro* studies demonstrated acute conduction block mediated by A12/18.1 in hippocampal slice cultures was complement-dependent (Mathey et al., 2007). However, as yet these findings have not yet been reproduced using patient-derived Nfasc-specific antibodies therefore the exact role of this response in MS remains undefined.

In conclusion autoantibodies recognising axonal antigens can induce primary axonal injury in the CNS, but whether this is a significant factor in the pathogenesis of MS is still a matter of speculation.

1.7 To what extent are autoantibodies involved in the pathogenesis of MS?

The simple answer to this question is that we still do not know.

Application of the Rose-Witesby to MS [Table 1.8] suggests that if a myelin-specific autoantibody response is involved then this is only the case in a subset of patients. Specifically, it is important to note myelin-specific autoantibody responses are not MS specific, but are also observed in many other neurological disorders. Similarly there is no consensus as to the pathological significance of complement activation products in the CNS. With respect to third postulate "*Induction of disease by passive transfer of antibodies*" as far as I am aware there is no report of maternal transfer of disease in MS, as reported myasthenia gravis. Moreover, the only study in which passive transfer of Ig is reported to exacerbate demyelination *in vivo* is flawed (Zhou et al., 2006). Although the baseline burden of disease in this study was minimal, passive transfer of patient Ig failed to exacerbate clinical disease severity and although a minimal level of demyelination was detected the authors failed to confirm their supposition this pathology was mediated by a MOG-specific autoantibody response. The technical difficulties associated with this approach are apparent from studies investigating the pathogenic potential of AQP-4 specific autoantibodies in NMO (Bradl et al., 2009). In this case reproducible exacerbation of disease severity and its pathological correlates in EAE was dependent on achieving circulating anti-AQP-4 titres similar to those seen in patients.

In summary we are left in a quandary in that we suspect antibody-dependent mechanisms contribute to disease pathogenesis in a subset of patients but in the absence of any defined target we can neither identify these patients nor address the crucial question, "Is this response clinically relevant?" One solution to this problem is to develop an *in vitro* assay that will identify those patients with a demyelinating or axopathic serum autoantibody response without requiring prior knowledge of its specificity. The next section reviews contributions made in this direction over the past 70 years.

Table 1.8: To what extent do anti-myelin antibodies in MS patients fulfil the Rose-Witesby postulates?

Postulate	Demonstrated in MS?	References
Antibodies frequently present in MS patients	Yes/No *NB: reports are conflicting*	Table 1.6 Review: Reindl et al., 2006
Detection of antibody at the site of injury	Yes (anti-myelin/anti-MOG)	Genain et al., 1999 O'Connor et al., 2005
Induction of disease by passive transfer of antibodies	Experimental: Yes (patient derived anti-MOG)	Zhou et al., 2006
Immunisation with antigen induces disease and antibody production	Yes (EAE)	Review: Gold et al, 2006
Reduction of antibody levels ameliorates the disease	Yes (TPX & Rituximab)	Keegan et al., 2005 Review: Cross et al., 2011
Demonstration of pathogenic activity using target cells	Inconclusive formal demonstration lacking	

1.8 *In vitro* studies of demyelinating disease

1.8.1 Complement fixation assays

In vitro studies of autoantibody responses associated with MS began over 80 years ago. In 1934 Sachs and Steiner conducted a novel study in which they measured the ability of MS brain extracts to activate complement in patient sera with brain extracts from patients with arteriosclerosis as a negative control. They defined a sample as positive if the serum selectively reacted with the MS brain extract but not the control. Using this assay almost half the MS patients they screened were positive. In contrast complement fixing antibodies were seldom detected in OND (<5%) or healthy (<5%) controls (Sachs and Steiner, 1934). Over the next 30 years, attempts to confirm and expand these findings led to conflicting reports and divided opinions [Table 1.9]. Some laboratories reproduced these observations, the most prominent being the study by Raskin which used a similar complement fixation assay but where Sachs and Steiner used a single antigen source, this study used MS and control brain extracts but also purified brain proteolipid fractions (Raskin, 1955). The results of the two studies were in broad agreement in that complement fixing activity was consistently higher in patients with MS compared to controls. The studies are remarkable as they provide the first indication that MS may be associated with a serological response to CNS antigens. However, this complement fixing activity was not MS-specific, an observation that suggests this approach to identify a clinically relevant response in patient sera was not ideal. Indeed later studies criticised several technical aspects of these assays and using somewhat modified approaches were unable to reproduce the initial findings (MacLeod et al., 1962).

Table 1.9: A selection of studies investigating anti-CNS serological responses using an *in vitro* complement fixation assay

Study	Assay	N	% positive patients
Sachs and Steiner, 1934	**Complement fixation assay** *MS brain extract*	**Definite MS:** 289 **Suspected MS:** 173 **OND:** 110	**Definite MS:** 42 **Suspected MS:** <25 **OND:** 3
Frick, 1951	**Complement fixation assay** *MS brain extract*	**MS:** 50 **OND:** 50	**MS:** 50 **OND:** 6
Raskin, 1955	**Complement fixation assay** *MS brain extract* *MS, normal and OND brain proteolipid extracts*	**MS:** 120 **HC:** 60 **OND:** 41	**MS:** 83 **HC:** 38 **OND:** 43
MacLeod et al., 1962	**Complement fixation assay** *Alcoholic MS brain extract*	**MS:** 120 **OND:** 34	**MS:** 0 **OND:** 0

1.8.2 Tissue culture studies of demyelination

1.8.2.1 EAE and serum anti-myelin activity

It was recognised in the late 1950's that tissue culture models that reproduce the topology and organisation of the target tissue may provide an elegant method to demonstrate the presence of pathogenic autoantibodies in vitro. In the context of demyelinating diseases this approach was first used by Appel and Bornstein who demonstrated sera from rabbits with EAE induced by immunisation with CNS tissue homogenates in Freunds adjuvant mediated complement dependant demyelination in rodent cerebellar cultures (Bronstein and Appel, 1961). In a follow up study they demonstrated that demyelinating activity in EAE sera was found in the IgG2 fraction and could be abolished by absorption with brain tissue, but not with liver, lung or kidney tissue. Moreover demyelination in this model was associated with antibodies binding to the myelin and oligodendrocyte surface (Appel and Bronstein, 1964). Demyelination induced using EAE sera not only spared axons but could be followed by remyelination, as demonstrated by removal of the pathogenic sera after the initial demyelinating

event (Bornstein and Appel, 1961; Raine and Bornstein, 1970). Antibody-mediated demyelination in this and similar model systems is complement dependent (Grundke-Iqbal et al., 1981) and associated with the formation of membrane attack complex (C5b-C9) (Liu et al., 1983). In addition to mediating complement-dependent effects, in the absence of complement these myelin-reactive antiserum also block myelin formation *in vitro* (Bornstein, 1970).

To identify the antigen(s) responsible for these pathological effects studies were performed using sera from animals immunised with purified CNS myelin antigens. In the case of MBP (Lebar et al., 1976; Seil et al., 1968; Kies et al.1973; Seil et al., 1975), PLP (Agrawal et al., 1984; Mithen et al., 1980; Seil and Agrawal, 1980) and MAG (Seil et al., 1981) no *in vitro* demyelinating or myelination inhibiting responses were detected, whilst anti-sera from animals immunised with GalC (Fry et al., 1974; Hruby et al., 1977) or GM1/GM4 gangliosides (Roth et al., 1985) were demyelinating and in the absence of complement also blocked myelin formation.

A similar approach *in vitro* was used to investigate the pathogenic potential of anti-sera raised against the axolemma enriched fraction derived from rat brain. These sera inhibited neuronal outgrowth and mediated destruction of mature axons in embryonic mouse spinal cord-dorsal root ganglion cultures. However axopathic activity was not detected in the spinal cord and was confined to axons within the dorsal root ganglion and the outgrowth zone (Bourdette et al., 1986; 1988). Similarly an anti-serum to the ganglioside GM1 did not have axopathic activity in vitro (Bourdette et al., 1989). Extensive investigation of the axopathic potential of serum antibodies is lacking but is certainly warranted given the level of axonal injury observed in MS lesions.

1.8.2.2 The identification of an *in vitro* demyelinating factor within MS serum

The ability of sera from some patients with acute MS to mediate demyelination *in vitro* was first reported almost 50 years ago (Bronstein and Appel., 1965). Subsequent studies have in general supported this initial observation [Table 1.10] (Review: Caspary et al., 1977). However, its clinical relevance remained

controversial largely because of the presence of demyelinating activity some healthy controls but also in a high proportion (~60%) of OND patients, in particular those with motor neurone disease (MND) (Bronstein and Appel., 1965; Hughes and Field 1967). Attempts to reconcile this observation with the presence of secondary demyelination in MND patients proved controversial (Wolfgram and Myers, 1973; Horwich et al., 1974).

It was assumed that this demyelinating activity was due to the presence of anti-myelin antibodies. This concept is supported by reports that the demyelinating activity is associated with the serum IgG fraction (Dowling et al., 1968; Wolfgram and Duquette, 1976) and the demonstration that absorption of IgG (IgG1, IgG2 and IgG4) using *Staphylococcus aureus* protein A results in a small but significant reduction in serum demyelinating activity (Grundke-Iqbal and Bornstein, 1979). However interpretation of all these studies is complicated by reports that some sera contain myelin-toxic factors that are both IgG and complement independent (Bradbury et al., 1984). Nonetheless, ultrastructural studies of *in vitro* demyelination mediated by samples from some MS patients revealed that this phenomenon reproduces many of the features observed using an anti-myelin serum from animals with EAE (Raine et al., 1970; Raine et al., 1973). Attempts to define the specificity of this demyelinating activity suggest it may be directed against oligodendrocytes rather than myelin (Wolfgram and Duquette, 1976). This concept was supported by the identification of antibodies that recognised cultured oligodendrocytes were identified in MS patients but not in sera from patients with OND or healthy controls (Abramsky et al., 1977). However, this staining of oligodendrocytes was later shown to be nonspecific (Traugott et al., 1979).

In retrospect these studies demonstrate that myelinating cultures can provide a tool to identify pathogenic antibody dependent effects *in vitro*, but their application as a screening tool not only requires standardised methods to generate purified Ig preparations from human sera, but also object methodologies to quantify myelin and/or axonal loss.

Table 1.10: A selection of studies demonstrating the presence of a factor in MS serum that can mediate demyelination *in vitro*.

Study	Assay	N	% positive patients
Bradbury et al., 1985	**Co-cultures of embryonic mouse cervical spinal cord with dorsal root ganglia**	**MS:** 53 **OND:** 53 **HC:** 20	**MS:** 74 **OND:** 68 **HC:** 22
Lumsden, 1971	***In vitro* demyelination of neonatal rat cerebellum slice cultures**	**MS:** 128 **OND:** 93 **HC:** 55	**MS:** 80 **OND:** 19 **HC:** 0
Hughes and Field, 1967	***In vitro* demyelination of neonatal rat cerebellum slice cultures**	**MS:** 25 **MND:** 26 **HC:** 34	**MS:** 84 **MND:** 62 **HC:** 24
Bornstein and Appel, 1965	***In vitro* demyelination of neonatal rat cerebellum slice cultures**	**MS:** active: 37 active?: 30 inactive: 33 **MND:** 15 **HC:** 28	**MS:** active: 68 active?: 40 inactive: 0 **MND:** 60 **HC:** 7

1.9 Aims of this thesis

A large body of circumstantial evidence implicates the involvement of antibody-dependent mechanisms in the development of MS in some patients. If this is the case then it has important clinical implications. Complement inhibitors, plasma exchange and intravenous immunoglobulins are just some of the strategies now used to inhibit pathogenic events triggered by autoantibodies in other human diseases, and which in theory could also benefit some patients with MS.

This defines the aim of my thesis which was to provide a definitive answer to the question: "Is MS associated with a pathogenic autoantibody response?"

To achieve this goal it was necessary to:

- Determine whether myelinated axonal segments within an *in vitro* myelinating culture model faithfully reproduce the antigenic profile and molecular organisation of myelinated fibres *in vivo*.

- Validate these cultures as a screening tool to detect and quantify demyelinating and/or axopathic autoantibody responses *in vitro*.

- Use the resulting bioassay to determine the frequency of demyelinating/axopathic IgG responses cohorts of patients with MS, OND and healthy controls.

2 Materials and methods

2.1 Biochemical techniques

2.1.1 ELISA

ELISA was performed using 96-well polyvinyl ELISA plates (Costar). Plates were coated with 10µg/ml of the appropriate antigen diluted in PBS overnight at 4°C. The following day plates were washed with ELISA wash buffer, blocked with 1% BSA in PBS for 1 hour at 37°C and after further extensive washing incubated with primary antibody or serum for 1 hour at 37°C. Plates were washed again and secondary antibody conjugated to horse-radish peroxidase (HRP) was applied for 45 min at 37°C. Unbound conjugate was removed by washing and bound HRP was visualised using o-phenyldiamine (OPD) following incubation at RT in the dark and quenched with 4M H_2SO_4. Absorbance was read at 492nm using a 96-well plate reader Tecan Sunrise plate reader with Magellan software (Tecan, Switzerland).

Table 2.11 ELISA secondary antibodies used in this study.

Host	Isotype	Dilution	Conjugate	Source
Goat	Rat IgG	1/3000	HRP	Southern Biotech (USA)
Goat	Mouse IgG	1/3000	HRP	Sigma (UK)
Goat	Rabbit IgG	1/3000	HRP	Southern Biotech (USA)
Goat	Human IgG	1/5000	HRP	Dako (Denmark)
Goat	Human IgM	1/5000	HRP	Dako (Denmark)
Goat	Human IgG1	1/5000	HRP	Zymed labs (USA)
Goat	Human IgG2	1/5000	HRP	Zymed labs (USA)
Goat	Human IgG3	1/5000	HRP	Zymed labs (USA)
Goat	Human IgG4	1/5000	HRP	Zymed labs (USA)

2.1.2 SDS-PAGE

SDS-PAGE was performed using the BioRad® Protean II apparatus according to manufacturer's guidelines. Gels were cast the previous day and kept overnight at 4°C. The resolving and stacker gels were prepared to give the appropriate percentage. The resolving gel was poured between two clean gel plates and overlaid with 100% butanol. This was removed once the resolving gel had polymerised and the stacker gel mix was layered onto which a comb was inserted to form sample wells. Samples were prepared by adding of sample buffer and boiling for 5 min before loading. Gels were ran at 200V (constant voltage) until the bromophenol blue dye front had reached the end of the resolving gel (~55 min).

2.1.3 Bicinchoninic acid assay

To accurately determine protein concentrations a bicinchoninic acid assay (BCA) was performed using the Pierce ® BCA protein assay kit following manufacturer's standard protocol.

First a series of diluted BSA samples were prepared at 2mg/ml, 1.5mg/ml, 1mg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and 25µg/ml concentrations. Standards were applied to a 96 well microplate (Greiner) in triplicate. Samples to be measured were diluted in series and applied also in triplicate. To generate the working reagent 50 parts BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) was mixed with 1 part BCA reagent B (4% cupric sulfate) mixed thoroughly. After adding the working reagent to each well; the plate was incubated at 37°C for 30 min. Absorbance was measured at 562nm using a 96-well plate reader Tecan Sunrise plate reader with Magellan software (Tecan, Switzerland).

2.2 Molecular biology techniques

2.2.1 RNA extraction

RNA was extracted using the RNeasy® plus micro kit (Qiagen) in accordance with the manufacturer's standard protocol.

Cells were lysed using the supplied lysis buffer and further disrupted using a 21 gauge needle and a 1ml syringe. Genomic DNA was removed from the lysate by binding to a gDNA eliminator column and centrifuging at 8000g for 0.5min. The flow through was retained and mixed with 70% ethanol. RNA was then isolated using RNeasy spin columns. After loading columns were washed repeatedly with supplied wash buffers and 80% ethanol at 8000g. After the final wash step columns were transferred to new collection tubes can centrifuged at 10,000g for 5 min. The RNA was then eluted with 14µl Nuclease free water and stored at -80°C until use.

2.2.2 Primer design

Primers were designed using primer3 (http://frodo.wi.mit.edu/primer3/) software. Nfasc155 primers were designed for the unique fibronectin domain (FN3) (NM_001160313.1). Primers for Nfasc186 were designed to recognise the sequence encoding the mucin-like domain (NM_001160314.1) sequences were taken from NCBI National Centre for Biotechnology Information (National Institute for Health, USA). Binding to target sequence was confirmed by BLAST (National Institute for Health, USA).

Primers were ordered from Sigma-Aldrich and were made up 10µM with DEPC-treated water on arrival.

2.2.3 cDNA synthesis

RT-PCR was performed using the Superscript™ III First-Strand Synthesis System (Invitrogen, UK) following the manufacturers protocol.

5µg total RNA was incubated with 10mM dNTP mix and 50ng/µl random hexamers. Primers were annealed by incubation at 65°C for 5 min. The reaction was cooled by placing on ice for a minimum of 1 min. Superscript™ III reverse transcriptase (200 U/µl) was then added to the reaction in the presence of 10x RT buffer, 25mM $MgCl_2$, 0.1M Dithiothreitol (DTT) and RNAse OUT™ (40U/µl). The reaction was well mixed, collected by brief centrifugation and incubated firstly for 10min at 25°C followed by 50min at 50°C. The reaction was terminated by incubation at 85°C for 5min and chilled on ice. After brief centrifugation RNAse H was added to remove any residual RNA and incubated for 20min at 37°C. cDNA was stored at -20°C until further use.

2.2.4 Q-PCR

Q-PCR reactions were prepared using SYBR® green master mix (2X) (Applied Biosystems, USA) containing pre mixed SYBR green dsDNA reporter dye, PCR buffer, dNTPs and Taq polymerase. Therefore each reaction consisted of 1X SYBR green reaction mix, 10µM each primer and 100ng cDNA and made up to 20µl volume with dH_2O. Reactions were set up in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosystems, USA).

Q-PCR reactions were run and monitored using an Applied Biosystems 7900HT Fast Real-Time PCR system with Sequence Detection Systems software (version 2.3).

2.2.4.1 Cycling conditions

95°C for 10min, (95°C for 15s, 60°C for 1min) x 40 cycles, 72°C for 5 min then melt curve analysis in a temp range of 75-99°C in 1°C increments.

2.2.4.2 Quantification

Changes in gene expression were quantified using the comparative C_t method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). This method compares the C_t values of the samples of interest to those of a baseline or control sample. The C_t values of both the sample and the control are first normalised to a suitable housekeeping gene using the following equation.

$$\Delta\Delta Ct = \Delta C_t \text{ sample} - \Delta C_t \text{ reference}$$

From the ΔΔCt value we can calculate the fold change in gene expression:

$$\text{Fold change} = 1/(2^{-\Delta\Delta Ct})$$

2.3 Myelin absorption studies

2.3.1 Myelin purification

Crude myelin was purified from whole rat brain using an abbreviated method based on that described by Norton and Podulso (1973).

Wistar rats (female ~3 weeks old) were sacrificed by overdose of CO_2, decapitated and the entire brain was removed, weighed and stored at -80°C. Tissues were homogenised with a Dounce homogeniser in approximately 20 volumes (w/v) of 0.32M sucrose (10 strokes). The homogenate was layered over 25ml of 0.85M sucrose in SW38 centrifuge tubes (Beckmann) and centrifuged at 75,000 g for 60 min. The layers of crude myelin formed at the interface of the two sucrose solutions were collected with a Pasteur pipette. Myelin layers were pooled and suspended in water by homogenisation and the final volume brought up to 180ml. This suspension is centrifuged 75,000 g for 15 min the resulting supernatants were discarded. Crude myelin pellets were subjected to osmotic shock by suspension in 180ml of water. The solution was centrifuged at 12,000 g for 10 min and the supernatant was discarded. This step was repeated once more ensure the removal of most small membrane fragments.

In order to obtain purified myelin, pellets were combined and resuspended in 0.32M sucrose. The suspension was then layered over 0.85M sucrose and centrifuged at 75,000 g for 30 min. The myelin was washed three times with water by centrifugation at 12,000 g for 10 min. The pellet was resuspended in dH_2O containing a protease inhibitor cocktail (Sigma, UK). Purified myelin was stored at -80°C and its purity was assessed by SDS-PAGE. Myelin protein concentration was measured using a BCA assay as described above.

2.3.2 Myelin adsorption

For myelin adsorption experiments 1µg monoclonal antibody or 100µg human IgG was incubated overnight with 2mg total myelin protein in 1ml PBS at 4°C. Myelin and bound immunoglobulins were pelleted by centrifugation at 12,000 g and the resulting supernatant was tested for residual antibody binding by ELISA and pathogenic activity using the myelinating culture model.

2.4 Cell Culture Techniques

2.4.1 Monoclonal antibody production

2.4.1.1 A12/18.1, Z2 and 8-18C5 hybridoma

Hybridoma cells were cultured in CELLine® cell culture flasks (BD biosciences) following manufacturer's guidelines. The media compartment was filled with 200ml of the appropriate media and the cell compartment was inoculated with $2x10^6$ cells/ml diluted in media. Supernatant was harvested from the cell compartment after 7 days and twice a week thereafter. Supernatants were centrifuged at 450 g and stored at -20 °C. Antibody production was monitored by ELISA and purified from pooled supernatants by protein G chromatography as described below.

2.4.2 Transfected cell lines

Hela cells were transfected to express the extracellular domains of Nfasc155 and Nfasc186 (Mathey et al., 2007).

Transfected HeLa cells were grown in the presence of G418 to prevent the development on non-expressing cell populations. Cells were cultured in T75cm^3 flasks and passaged 1:2 once the cells reached ~70% confluency. For antibody treatment and immunocytochemistry 100,000 cells were plated onto uncoated 13mm coverslips and were allowed to attach overnight.

2.4.3 Neurosphere derived astrocytes

Neurospheres were generated based on protocols described by Reynolds and Weiss (1996) and Zhang et al. (1998b) and differentiated into astrocytes as described by Sorenson et al. (2008).

2.4.3.1 Isolation of the corpus striatum from postnatal rat brain

Neurospheres were produced from the corpus striatum of P1 SD rats (less than 36 hours old). Postnatal pups were killed by i.p. injection of euthathal in accordance to UK Home Office guidelines.

The brain was removed from the skull and placed in a petri dish in a dorsal orientation and separated into the two cerebral hemispheres by cutting at the corpus callosum with a scalpel (no. 22 blade). The two hemispheres were then positioned in a sagittal orientation [Figure 2.1]. The region containing the striatum was isolated from the entire hemisphere by making an initial cut at the frontal tip of corpus callosum followed by a second at the lateral ventricle [Figure 2.1A]. The isolated section was then placed rostrally and the caudate nucleus of the striatum carefully removed using curved forceps (Dumont no. 5) and placed into a bijou containing 1ml L-15 media [Figure 2.1B].

2.4.3.2 Production of neurospheres from rat striatum

Isolated stria were dissociated by gentle trituration with a glass Pasteur pipette and centrifuged at 140 g for 5 min. The resulting pellet was resuspended in 2ml NSM, added to an uncoated T75cm^2 cell culture flask (Corning) and the volume made up to 20mls with NSM. The flasks were supplemented with EGF (Peprotech) at a final concentration of 5ng/ml to promote sphere formation. Neurospheres were maintained at 37°C/7% CO_2 and fed twice per week by addition of 5ml NSM supplemented with 5ng/ml EGF. After approximately 7 days the neurospheres were used to generate astrocytes.

2.4.3.3 Generation of astrocyte monolayers from neurospheres

Glass coverslips (13mm) (VWR) were coated with 13.3µg/ml poly-l-lysine (Sigma-Aldrich, Dorset, UK) for a minimum of 1 hour at 37°C. After coating the coverslips were washed extensively with sterile water and placed into a 24 well plate (one coverslip/well) and left to air dry before use.

Neurospheres were transferred into 50ml falcon tubes and centrifuged at 140 g for 5 min. The pellet was resuspended in 2ml DMEM + 10% FBS by gentle

trituration with a glass Pasteur pipette. The total volume was made up to 12ml with DMEM +10% FBS and 0.5ml was added to each well (~50,000 cells/ coverslip) after which additional media was added to obtain a total volume of 1ml/ well. Astrocytes were maintained at 37°C/7% CO_2 until confluent (~7 days).

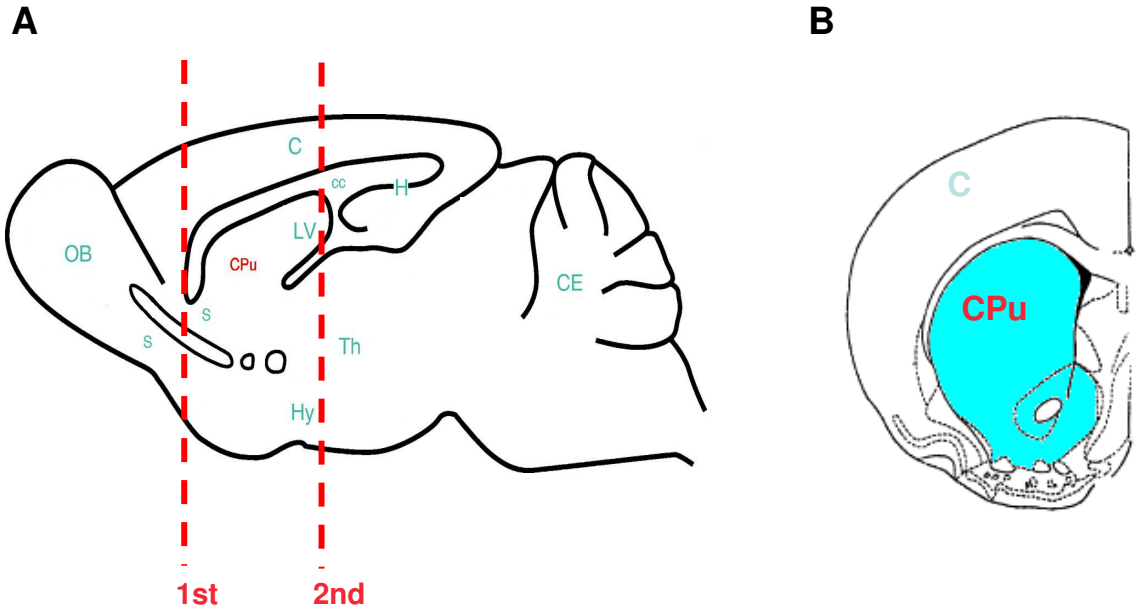


Figure 2.1: Isolation of the striatum from postnatal rat brain

A. Sagittal section of rat brain from P1 SD rats. To isolate the region containing the striatum two cuts were made. The first at the frontal tip of the corpus callosum and the second at the lateral ventricle (red dashed lines). **B.** When the isolated section is placed in a rostral orientation the striatum is easily visible (highlighted in blue) and can be carefully removed with curved forceps. Figures adapted from Budantsev et al., 2007 and Gammie et al., 2004. *Abbreviations: C, cerebral cortex; cc, corpus callosum; CE, cerebellum; CPu, caudate-putamen complex (striatum); H, hippocampus; Hy, hypothalamus; LV, lateral ventricle; OB, olfactory bulb; S, septum; Th, thalamus.*

2.4.4 Dissociated spinal cord cultures

Myelinating spinal cord cultures were generated as described previously (Sorenson et al., 2008).

2.4.4.1 Isolation of embryonic rat spinal cord

SD rats were time mated and the day of plugging denoted as day E0.5. *In vitro* myelinating cultures were generated from E15.5 embryos.

The pregnant female was sacrificed by overdose of CO_2 followed by cervical dislocation in accordance to UK Home Office guidelines. The abdominal skin and fur were sterilized using 70% ethanol. The uterus was exposed by making a V-shaped incision at the lower abdomen cutting through the skin and abdominal wall and was carefully removed and placed into ice cold HBSS.

Embryos were dissected from the gravid uterus and decapitated approximately 3mm rostral to the cervical flexure. The cranial 5 to 6mm of skin covering the spinal cord was gently removed and the spinal cord extruded carefully into 1ml HBSS (without Ca^{2+} and Mg^{2+}). Great care was taken to remove all meninges and dorsal root ganglion.

2.4.4.2 Production of myelinating spinal cord cultures

Isolated spinal cords were mechanically dissociated by light chopping with a sterile scalpel (no. 22 blade) and collected in a 1ml of sterile HBSS (without $Ca^{2+}$and Mg^{2+}). The tissue was further dissociated by addition of 100µl 10x trypsin and 100µl 1% collagenase for 15 min at 37°C. Enzymatic activity was stopped by adding 2ml SD solution (soybean trypsin inhibitor with DNAse I) after which the tissue was titurated using a glass Pasteur pipette.

The suspension was then centrifuged at 200 g for 5 min after which the supernatant was removed and the cell pellet resuspended in 2ml plating media (PM). A live cell count was performed using a haemocytometer and trypan blue and the cells were subsequently diluted in PM to a concentration of $1.5x10^6$ cells/ml.

Dissociated spinal cord cells (150,000 cells/ coverslip) were carefully plated onto coverslips supporting a monolayer of neurosphere derived astrocytes. Three coverslips were placed into a 35mm petri dish. Cells were left to attach for approximately 2 hrs at 37°C after which 500µl DM+ and 250µl PM was carefully added to each petri dish. Cultures were maintained for 28-30 days at 37°C/7% CO_2 and fed three times per week with DM by removing half of the media and replacing it with fresh media. After 12 DIV insulin was omitted from the culture media to promote myelination.

2.5 Immunocytochemistry

2.5.1 Antibodies

Primary antibodies used throughout this work are detailed below [Table 2.2]. Secondary antibodies; Alexa Fluor® 350 (blue), Alexa Fluor® 488 (green) and Alexa Fluor® 565 (red) were purchased from Invitrogen (Paisley, UK) and used at a dilution of 1 in 400 throughout.

2.5.2 Live staining of extracellular antigens

Primary antibodies were diluted in ice cold DMEM and applied to coverslips for 30 min at 4°C after which the coverslips were washed in DMEM and the appropriate fluorochrome conjugated secondary antibody was added for 30 min at 4°C. The coverslips were then washed in DMEM and fixed with 4% PFA for 15 min (RT) and either co-labelled for intracellular antigens as described below or mounted in Vectashield (Vector labs). However in some cases as noted in the text PFA fixation was performed immediately after incubation with primary antibody or serum.

2.5.3 Staining of intracellular antigens

(a) Cells were fixed with 4% PFA, washed in PBS and then permeabilised with 0.5% Triton X-100 for 10min (RT) and blocked in blocking buffer for 60 min (RT). Fixed and permeablised cells were incubated overnight at 4°C with primary antibody diluted in blocking buffer. After washing with PBS an appropriate fluorochrome conjugated secondary antibody was added for 45 min (RT). Coverslips were then washed in PBS followed by dH_2O and mounted in Vectashield (Vector labs).

(b) In order to conserve particular epitopes it was necessary to fix and permeablise the cells using methanol rather than Triton X-100. In this case cells were fixed in 100% methanol for 10 min at -20°C, washed with PBS, blocked with blocking buffer for 60 min (RT). Primary antibodies were diluted in blocking buffer at the appropriate dilution and applied for 60 min at RT or 4°C overnight.

The cells were then washed with PBS and the corresponding fluorochrome conjugated secondary antibody added for 45 min (RT). Coverslips were then washed in PBS followed by dH_2O and mounted with Vectashield (Vector labs).

Table 2.2: Primary antibodies used in this study

Antibody	Species	Isotype	Dilution	Fixation	Notes
Oligodendrocyte /Myelin					
8-18C5 (MOG)	Mouse	IgG1	1/200	Live	Linington et al., 1988
AA3 (PLP)	Rat	IgG	1/100	PFA + Triton	Yamamura et al., 1991
GalC	Rabbit	IgG	1/50	PFA + Triton	Sigma (UK)
MBP	Mouse	IgG2a	1/100	PFA + Triton	Chemicon (Europe)
NG2	Rabbit	IgG	1/200	Live	Millipore (Europe)
O4	Mouse	IgM	1/200	Live	Millipore (Europe)
PDGFRα	Rabbit	IgG	1/500	Methanol	Abcam (UK)
PLP	Rabbit	IgG	1/200	PFA + Triton	Antisera
PLP	Mouse	IgG2a	1/100	PFA + Triton	Chemicon (Europe)
Z2	Mouse	IgG2a	1/200	Live	Piddlesden et al., 1993
Node of Ranvier					
A12/18.1	Mouse	IgG2a	1/100	Live	Prof. M Rasband (USA)
AnkyrinG	Mouse	IgG1	1/100	Methanol	Zymed labs (USA)
Caspr	Rabbit	IgG	1/1000	Methanol	Abcam (UK)
Nfasc155	Rabbit	IgG	1/200	Live/ Bouins	Prof. E Meinl (Germany)
Nfasc186	Rabbit	IgG	1/300	Live	Prof. P. Brophy (UK)
Pan NaV	Mouse	IgG1	1/500	PFA	Sigma (UK)
Pan Nfasc	Rat	IgG	1/250	Live	Dr E. Mathey
4D7 (TAG-1)	Mouse	IgM	1/100	Methanol	Derfuss et al., 2009
3.1C12 (TAG-1)	Mouse	IgG1	1/100	Methanol	Derfuss et al., 2009
Neurons/Axons					
NeuN	Mouse	IgG1	1/100	Methanol	Millipore (Europe)
PGP9.5	Rabbit	IgG	1/500	Methanol	Abcam (UK)
SMI-31	Mouse	IgG1	1/1000	PFA + Triton	Abcam (UK)
Miscellaneous					
CD11b/c	Mouse	IgG2b	1/200	Live	Abcam (UK)
ED1	Mouse	IgG1	1/200	Methanol	Abcam (UK)
GFAP	Rabbit	IgG	1/500	PFA + Triton	Dako (Denmark)
MAC (C5b-C9)	Mouse	IgG2a	1/100	PFA	Dako (Denmark)
MHC-II	Mouse	IgG1	1/200	Live	Abcam (UK)

2.5.4 Identification of Nfasc localisation in vitro

2.5.4.1 Nfasc155

Nfasc155 readily accessible for antibody binding was detected by live staining as described above using a rabbit polyclonal antibody specific for the unique FN-III domain of Nfasc155.

To visualise Nfasc155 staining within paranodal structures it was necessary to disrupt the paranode using Bouins fixative. Cells were fixed with 4% PFA for 10 min, washed in PBS, fixed with Bouins fixative for 3 min and washed extensively with PBS. Cells were blocked with blocking buffer for 60 min at RT. Primary antibodies were diluted in blocking buffer at the appropriate dilution and applied for 60 min at RT or 4°C overnight. The cells are then washed with PBS and the corresponding fluorochrome conjugated secondary antibody was added for 45 min at RT. The coverslips were washed in PBS followed by dH_2O and mounted with Vectashield (Vector labs).

2.5.4.2 Nfasc 186/ Nfasc 155

Nfasc186 and Nfasc155 readily accessible for antibody binding was detected by live staining as described above using a monoclonal antibody (A12/18.1) or a rat anti-sera, which recognises shared epitope(s) on both Nfasc155 and Nfasc186.

2.5.5 Immunocytochemistry to detect complement activation

The reagent routinely used in our department to visualise MAC is a mAb generated against human C5b-C9 recognising human poly C9. It has been previously demonstrated that this antibody recognises rat poly C9 (Kallio et al., 2000) however we needed to confirm this using our rat serum and antibodies. To do this I used transfected cell lines expressing high levels of antigen at the cell surface and incubation of transfectants with 10µg/ml antibody in the presence of 1% FRS mediated complement cell death and MAC deposition on the cell surface within 1 hour of addition.

2.5.5.1 Antibody treatment of transfectants

Transfected HeLa cells were treated with antibody at concentration indicated in the text for 30 min and kept at 4°C to minimise antibody internalisation. Fresh rat serum was then added to the cells and the cells were brought up to 37°C and incubated until fixation.

Transfectants were fixed with 4% PFA. Blocked for 1 hr in 1% BSA/ 10% normal goat serum/ 0.3M glycine at room temperature. To detect residual antibody bound to the surface secondary antibody was added (Alexa Fluor 488, Invitrogen) for 45min at room temperature. Cells were washed and mounted in Vectashield (Vectorlabs) with DAPI to visualize the nuclei.

2.6 Image Capture and Analysis

2.6.1 Image Capture

All images for cell counting, quantification of axonal density and myelination were taken using an Olympus BX51 fluorescent microscope (Olympus). A minimum for thirty images were analysed per condition in each experiment. Images were taken in a semi-random fashion i.e. ten random images were taken per coverslip from myelinated regions.

2.6.1.1 Quantification of axonal density

Neurites were visualised by phosphorylated neurofilament staining (SMI-31) and a minimum of ten images were taken per coverslip (10X magnification).

Using Image J (version 1.41o, National Institute of Health, USA) the image was split into three separate images corresponding to the red, green and blue channels and the channel corresponding to SMI-31 staining was then converted to a black and white image. Axonal density was calculated as the number of SMI-31+ pixels expressed as a percentage of the total number of pixels per field of view [Figure 2.2A].

2.6.1.2 Quantification of total myelin/ oligodendrocyte density

Oligodendrocytes and their associated sheathes were visualised by staining for an oligodendrocyte/ myelin marker (e.g. MOG or PLP) and a minimum of ten images were taken per coverslip (10x magnification).

Using Image J (version 1.41o, National Institute of Health, USA) the image was split into three separate images corresponding to the red, green and blue channels and the channel corresponding to oligodendrocyte/ myelin staining was then converted to a black and white image. Oligodendrocyte/myelin density was calculated as the number of positive pixels expressed as a percentage of the total number of pixels per field of view [Figure 2.2A].

2.6.1.3 Quantification of myelination

As there is no specific myelin marker that differentiates completely between oligodendrocytes and myelin sheathes these were manually highlighted using Adobe Photoshop elements® (blue) [Figure 2.2B] and measured using a macro within Image J (written by John Annan) (Appendix). To provide a measure of the extent of myelination the number of myelin positive pixels was divided by the number of SMI-31 pixels in the same field.

2.6.1.4 Cell counting

In order to quantify cell populations within cultures a minimum of 30 images were taken from three coverslips (20x magnification) and immunopositive cells associated with a $DAPI^+$ nucleus were counted using the ImageJ cell counter function (version 1.41o). Cell counts were expressed as a percentage of the total number of $DAPI^+$ nuclei in the image at the same plane of focus.

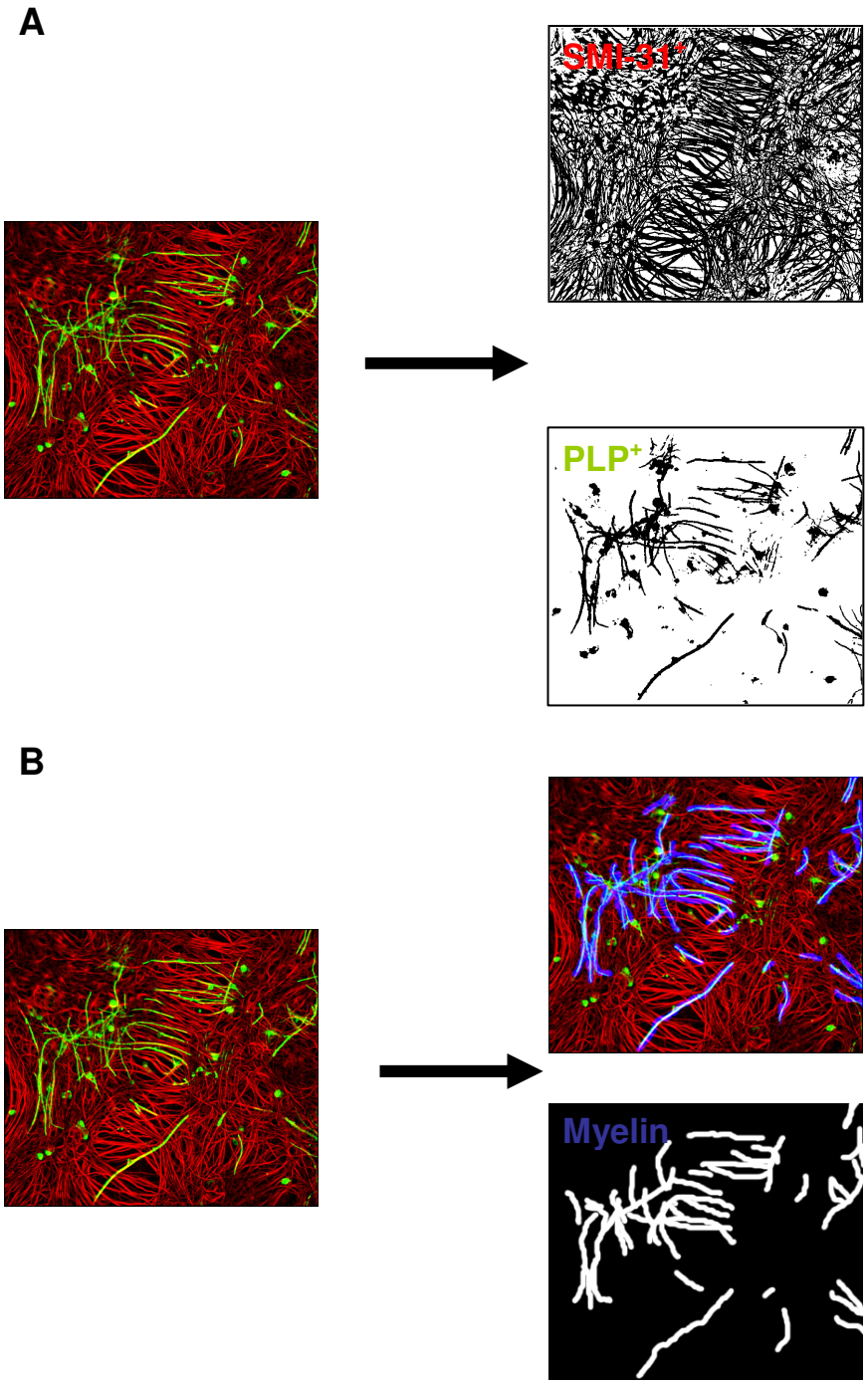


Figure 2.2: Quantification of axonal density and myelination using Image J.
A. To calculate axonal density images were split into their individual colour channels using Image J software. The channel corresponding to SMI-31 (red) was converted into a black and white image and the axonal density was calculated as the number of SMI-31+ pixels within a field of view (10X magnification) and expressed as a percentage. The same approach was adopted in order to calculate the total density of myelin/oligodendrocytes by focusing on the green channel. **B.** To calculate the percentage myelination it was necessary to exclude staining on oligodendrocyte cell bodies and to focus solely on the myelin sheathes. To achieve this, myelin sheathes were manually highlighted using Adobe Photoshop ® (Adobe systems) (blue). The highlighted area was quantified using a macro within Image J. For each image percentage myelination was expressed as the total number of myelin pixels/ total number of SMI-31 pixels (based on Sorenson et al, 2008).

2.7 Immunoglobulin Purification

2.7.1 Protein G chromatography

Antibodies were purified using 1ml HiTrap protein G fast flow columns (GE Healthcare) following manufacturers instructions.

Briefly samples were diluted in binding buffer and particulates were removed by filtration using a 0.45µm filter prior to loading. Samples and buffers were added using a 10ml syringe at a flow rate of 1ml/min taking care to prevent introducing air into the column.

After washing with 10ml of binding buffer to remove the ethanol-based storage buffer the sample was passed through the column, which was then rinsed with a minimum of 5ml until the absorbance of the eluate at 280nm had fallen to that of diluted binding buffer. The flow through was retained in the event that not all antibody binds to the column. Bound antibody was eluted using 8ml elution buffer collected sequentially in 2ml fractions that were neutralised immediately by addition of 100µl of 1M Tris-HCl, pH 9.0. Protein Concentrations were determined by absorbance at 280nm using a nanodrop 1000 spectrophotometer (Thermo scientific).

2.7.2 Generation of Fab fragments

Fab fragments were generated using Pierce ® Fab Preparation kit following manufacturer's instructions.

1mg/ml purified IgG was incubated with immobilised papain for 4 hours at 37°C. After digestion, fragments were retrieved by centrifugation at 5000 x g for 1 min and the resin was washed with PBS. Fab fragments were isolated via protein-A chromatography and their purity was assessed by SDS-PAGE.

2.7.3 Purification of patient-derived Nfasc155 specific autoantibodies

Purification and characterisation of Nfasc specific antibodies was performed in collaboration with Dr. A. Arthur (University of Glasgow).

Recombinant rat NF155 (rrNfasc) (500μg) (R&D Systems) was bound to an activated NHS column (GE Healthcare) according to the manufacturer's instructions. Plasmapheresis samples were diluted with PBS, filtered and brought to pH 7 before were run over rNfasc immunosorpbent columns at 4°C. After extensive washing with PBS, bound antibody was eluted with 0.1M glycine pH 2.7 and immediately neutralized using 1M Tris pH 9. The presence of anti-rNfasc antibodies and their isotype was confirmed by ELISA, whilst antibody concentrations were determined using a Nanodrop spectrophotometer (Thermo Scientific).

2.7.3.1 Isotype usage of the Nfasc specific repertoire

Purified human anti-Nfasc155 antibodies were diluted 1:500 in PBS and specific binding rat Nfasc155 detected using anti-human IgG1 (Zymed), IgG2 (Zymed), IgG3 (Zymed), IgG4 (Zymed) or IgM (Dako) reagent conjugated to alkaline phosphatase.

2.8 Using myelinating cultures to detect complement dependent antibody mediated injury

2.8.1 Preparation of fresh rat serum

To obtain fresh sera, SD rats were exsanguinated by cardiac puncture with a 10ml syringe and 21G needle. The blood was allowed to clot on ice and once clotted the serum was isolated by centrifugation at 2000 g for 10 min at 4°C. Sera was stored at -80°C in small aliquots to avoid freeze thawing. To heat inactivate the complement, sera was incubated at 56°C in a water bath for 30 min.

2.8.2 Antibody treatment of myelinating cultures

2.8.2.1 Complement dependent assay

To investigate complement dependent antibody mediated injury. Cultures were treated after 28 days *in vitro* to allow for axonal establishment and robust myelination. Purified antibodies were used at a concentrations indicated in the text. Fresh rat serum was added as a source of complement at a final concentration of 1% and cells were incubated at 37°C/ CO_2 until fixation. In each case cells were labelled by ICC and axonal density and extent of myelination quantified as described previously.

To elucidate whether antibody mediated effects were dose dependant myelinating cultures were treated with varying concentrations of antibody (10µg/ml, 1µg/ml, 100ng/ml, 50ng/ml and 10ng/ml), 1% FRS was used as a source of complement and cells were incubated overnight (~16hrs) at 37°C/7% CO_2 until fixation and ICC.

To determine the time scale in which injury occurs; myelinating cultures were treated with 10µg/ml antibody and taken at specific time intervals (as denoted in the text), 1% FRS was used as a source of complement and cells kept incubated at 37°C/7% CO_2 until fixation and ICC.

2.8.2.2 Complement independent assay

To investigate the pathogenic potential of autoantibodies in the absence of exogenous complement, myelinating cultures were treated at concentrations indicated in the text from 12 DIV, 18 DIV or 24 DIV. Antibodies were reapplied with each media change until 28 DIV.

2.9 Clinical Studies: Identification of pathogenic autoantibodies in MS patients.

2.9.1 Patient samples

Samples were collected from patients at the Southern General Hospital (Glasgow, UK), University of Heidelberg (Heidelberg, Germany) and the University Clinic Grosshardern (Munich, Germany). Clinically definite MS was defined using the Poser or McDonald criteria (Poser et al., 1983). 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. Inclusion criterion for use in the study was positive for an antibody response to Nfasc155 as tested by ELISA.

2.9.2 Treatment of myelinating cultures with patient-derived autoantibodies

After 28 DIV immunopurified Nfasc-specific immunoglobulins were added to the cultures at the concentrations indicated in the text in the presence or absence of fresh rat sera as a source of complement (final concentration 1%). Additional control cultures were treated with antibody alone or in combination with heat inactivated serum as additional controls.

Table 2.3 Clinical data of patient cohort selected for study

Plasma samples were obtained from patients undergoing therapeutic plasma exchange (PEx). The patients were broadly divided into two groups, those diagnosed with multiple sclerosis (MS) and those diagnosed with other neurological diseases OND. Plasma samples were screened by ELISA for Nfasc autoantibody responses and the patients which tested positive were used in this study.

Abbreviations: RRMS: relapsing remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, SPN: Sensory motor polyneuropathy, CIDP: chronic idiopathic polyneuropathy, GBS: Guillian-Barre syndrome, ISAN: Idiopathic sensory ataxic neuropathy, MG: Myasthenia Gravis.

Patient ID	Gender	Age	Diagnosis	Time from onset at TPX
MS1	F	26	MS (Marburg)	8 years
MS2	F	42	RRMS	25 years
MS3	M	45	RRMS	0 years
MS4	F	49	RRMS	7 years
MS5	M	29	SPMS	5 years
MS6	F	49	RRMS	11 years
MS7	F	31	RRMS	7 months
MS8	F	51	RRMS	N/A
MS9	F	43	RRMS	N/A
MS10	M	46	RRMS	N/A
MS11	F	42	RRMS	N/A
MS12	F	42	RRMS	N/A
MS13	F	40	RRMS	N/A
MS14	M	33	RRMS	N/A
MS15	F	53	RRMS	N/A
MS16	F	58	RRMS	N/A
MS17	F	57	PPMS	N/A
MS18	F	56	PPMS	N/A
MS19	M	47	PPMS	N/A
MS20	F	28	PPMS	N/A
OND1	M	38	SPN	9 years
OND2	F	49	GBS	<1 month
OND3	F	55	GBS	<1 month
OND4	M	24	GBS	<1 month
OND5	M	62	CIDP	14 years
OND6	F	55	CIDP	20 years
OND7	M	79	CIDP	4 years
OND8	M	71	ISAN	<1 month
OND9	F	62	MG	5 years
OND10	F	63	CIDP	8 years
HC1	F	45	N/A	N/A
HC2	F	31	N/A	N/A
HC3	M	46	N/A	N/A
HC4	F	33	N/A	N/A
HC5	F	27	N/A	N/A
HC6	F	28	N/A	N/A
HC7	F	32	N/A	N/A
HC8	F	50	N/A	N/A
HC9	F	31	N/A	N/A
HC10	M	80	N/A	N/A
HC11	F	24	N/A	N/A
HC12	M	57	N/A	N/A
HC13	F	37	N/A	N/A

2.10 Statistics

In order to determine whether immunocytochemical data obtained for axonal density and myelination followed a normal distribution, raw data was assessed using the Lilliefors Test for Normality (Lilliefors, 1967) using software provided by the European mirror service (Professor Hossein Arsham, University of Baltimore, http://www.mirrorservice.org/sites/home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Normality.htm). Using this test we determined that our data "likely follows a normal distribution".

Due to the distribution of the data, values from treatments and controls were compared using a parametric method namely the students T-test. When directly comparing raw data values, probability values were calculated using the paired students T-test (two tailed) (Microsoft Excel). In majority of cases, raw data was standardised and expressed as a percentage of control values. In this case the paired T-test was not appropriate; therefore p values were calculated using the one-sample heteroscedastic T-test (Microsoft Excel).

In each instance statistical significance was denoted with * when $p<0.05$ and ** when $p<0.001$. For antibody mediated effects on axons and myelin/glia, p values obtained were comparable between analyses of raw or standardised data [Table 2.4].

All data was plotted as the mean of three independent experiments performed three times in triplicate +/- standard error of the mean, unless stated otherwise.

Table 2.4: Calculation of probality values using the students T-test- comparison of raw and standardised data

Data values were taken from table 4.2. Myelinating cultures (28 DIV) were treated overnight in the presence of complement with 10µg/ml of Z2 (anti-MOG, demyelinating), A12/18.1 (pan-Nfasc, axopathic and demyelinating) or an IgG2a control (no effect to axons or glia). P values from raw data values for axonal density and myelination were calculated using a paired two-tailed T-test. In some cases data was standardised and expressed as a percentage of control values. In these examples p values were calculated using a one-sample heteroscedastic T-test. In each case antibody mediated effects on axons and glia were highly significant and p values were comparable regardless of the data set analysed or statistical test used. Values shown are the average of three independent experiments performed in triplicate ± S.E.M.

		Raw data/ paired t-test			**Standardised data/ one tailed t-test**		
Antibody	**Specificity**	**Axonal density (SMI-31+/ total pixel #)**	**Myelinated axons (%)**	**P value (vs untreated)**	**Axonal loss (% control)**	**Myelin loss (%control)**	**P value (vs untreated)**
Z2	MOG	70.4 ± 3.3	0.03 ± 0.02	$6.9\ e^{-10}$	2 ± 2.2	**99 ± 0.9**	$6.4\ e^{-14}$
A12/18.1	Nfasc155/ Nfasc186	**35.6 ± 3.2**	**0 ± 0**	$1.9\ e^{-18}$ *(axons)* $3.1\ e^{-9}$ *(myelin)*	**40 ± 3.2**	**100 ± 0.0**	$2.3\ e^{-32}$ *(axons)* $1.4\ e^{-10}$ *(myelin)*
IgG2a isotype control	N/A	71.8 ± 3.1	3.6 ± 1.0	0.36	0 ± 1.4	2.7 ± 1.1	0.29

3 Characterisation of *in vitro* myelinating cultures

3.1 Introduction

The purpose of this chapter is to describe the *in vitro* myelinating cultures used in this study and demonstrate their validity as a representative model of myelination. This characterisation is a prerequisite if this *in vitro* system is to be used as a tool to detect pathogenic antibody responses. If myelinating cultures are to provide a valid screening strategy it is crucial they replicate the structural and molecular organisation of the CNS *in vivo*. The advantage of using such a co-culture system over homogenous cell populations such as pure oligodendrocyte cultures is that although oligodendrocytes are capable of extending myelin like membranous sheets *in vitro,* in the absence of neurons they cannot assemble myelin sheathes. However in the myelinating culture model it is possible to obtain myelin sheathes with similar properties to that of compact CNS myelin *in vivo* including formation of higher order structures such as paranodal loops and nodes of Ranvier (Thompson et al., 2006, Sorenson et al., 2008).

If this myelinating culture model was to provide a routine screen for demyelinating autoantibodies it was important to determine the reproducibility of the system by investigating the variation between cultures from the same preparation and between myelinating cultures from different preparations. In order to generate a reliable model it must be proven to be robust, reproducible and statistically sound.

Characterisation was also necessary to determine appropriate time points to assay antibody dependent effects. To do this was important to initially determine at which stages *in vitro* are the antigens of interest expressed.

The aims of this chapter are:

- To analyse the cytoarchitecture of the myelinating cultures and determine similarities between this model and the *in vivo* CNS.

- To determine when various antigens are expressed and their localisation *in vitro*.

- To determine the reproducibility of the system and it suitability as a potential screening strategy in which to detect pathogenic autoantibody responses.

3.2 Results

To investigate *in vitro* development of myelinating cultures, they were characterised by immunofluorescence microscopy at key stages namely 9, 12, 18, 24 and 30 DIV.

3.2.1 Neurite extension and axonal ensheathment

Neurons derived from the embryonic rat spinal cord consist of a heterogeneous population of neuronal cell types and neural progenitors (Nornes et al., 1974).

We can measure neuronal development and axonal extension that occurs throughout the culture period by measuring increases in phosphorylated neurofilament expression by immunocytochemistry using the monoclonal SMI-31 [Figure 3.1A]. After 9 DIV the astrocyte monolayer is covered by clusters of neurons that have already begun to extend neurites which by 12 DIV cover approximately 50% of the surface of the coverslip. Axonal density begins to plateau at approximately 65-70% between 18 DIV to 24 DIV [Figure 3.1B].

Oligodendrocyte maturation and the onset of myelination were followed by measuring MBP immunoreactivity [Figure 3.1A]. MBP^+ cells are not present at earlier culture stages but a small number begin to appear 12 DIV. After 12 days in culture insulin is omitted from the culture media in order to promote oligodendrocyte progenitor differentiation maturation rather than proliferation (Thompson et al., 2006). After insulin withdrawal there is an increase in the number of MBP expressing cells but by day 18 little axonal ensheathment. By 24 DIV we observe substantial axonal ensheathment and myelination by MBP^+ oligodendrocytes. Once myelination has begun the process is relatively rapid as the number of MBP^+ myelin sheathes effectively triples over the final 6 day period [Figure 3.1C]. MBP^+ sheathes also expressed PLP and quantification of PLP staining gave a similar result. PLP was subsequently used for all future quantification.

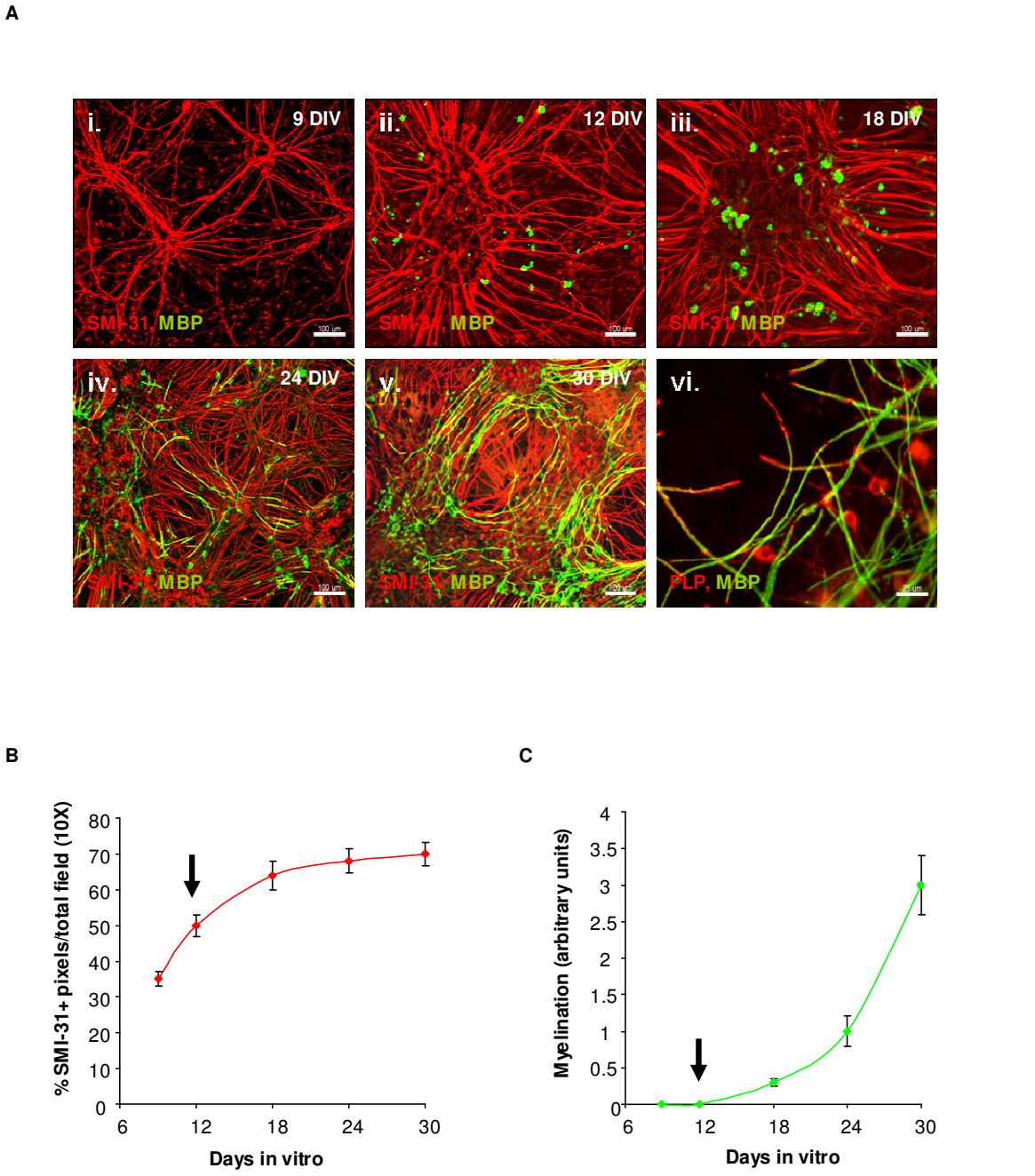


Figure 3.1: Axonal density and myelination increase over time.
A: Immunochemistry of myelinating cultures over a 30 day culture period. Neurites were visualised using SMI-31 (phosphorylated neurofilament: red) and myelin/ oligodendrocytes were stained for myelin basic protein (MBP: green) (10X magnification). Cultures were stained at 9 **(i),** 12 **(ii),** 18 **(iii),** 24 **(iv)** and 30 DIV **(v)**. Myelin sheathes also expressed PLP in addition to MBP **(vi)** (PLP: red, MBP: green) (40X magnification) **B:** Axonal density was calculated by measuring the number of SMI-31+ pixels per field of view (10X magnification). For each time point a minimum of 30 images were analysed. Neurofilament staining was visible at nine DIV where a small number of axons are developing. By 12 DIV a dense network of neurites is formed. Axonal density increases to its maximum by 18 DIV. Values plotted are quantification of immunocytochemical data from three independent experiments performed in triplicate (mean ± S.E.M). **C:** Myelination was quantified by calculating the percentage of myelinated axons in each image. For each time point a minimum of 30 images were analysed. Myelination usually occurs between days 20-22 and by 24 DIV there is a substantial amount of axonal ensheathment. Once myelination has begun the process is relatively rapid as the number of myelin sheathes effectively triples over the final 6 day period. Values plotted are quantification of immunocytochemical data from three independent experiments performed in triplicate (mean ± S.E.M).

3.2.2 Differentiation of the oligodendrocyte lineage

Myelination is a tightly regulated process in which oligodendrocyte precursor cells (OPCs) differentiate into oligodendrocytes which extend processes and can ensheath appropriate axons. OPCs differentiate into mature myelinating oligodendrocytes through a defined path with various stages. Each cell subtype expresses a set of characteristic surface markers that can be used to determine their state of differentiation. Immature OPCs were detected using NG2 (chondroitin sulphate proteoglycan) (Dawson et al., 2000; Stallcup., 1981), late OPCs/ pre- oligodendrocytes were labelled with O4 monoclonal antibody (Bansal et al., 1992, Sommer and Schachner, 1981), which recognises sulphatide. PLP staining was used to visualise oligodendrocytes; however it must be noted that the anti-PLP antibody used in this study also binds to DM20, an isoform of PLP, expressed by pre-OLG populations prior to PLP expression (Yamamura et al., 1991). Mature oligodendrocytes were defined as expressing MOG on the cell surface (Scolding et al., 1989) [Figure 3.2A]. In the early stages of development large numbers of oligodendrocyte precursor cells (OPCs) can be detected by immunocytochemistry [Figure 3.2B]. Between 9 and 12 days in culture, the majority of oligodendrocyte lineage cells present are $NG2^+$, but after insulin is withdrawn from the culture medium (arrow) the number of $NG2^+$ cells rapidly declines. By the end of the culture period only a small proportion of NG2 expressing cells remain. Cells labelled by the O4 antibody include a subset of OPCs (which co-express NG2), pre-oligodendrocytes and oligodendrocytes; therefore there are a high proportion of $O4^+$ cells at each timepoint culture however the cell types recognised will shift from OPCs through pre-oligodendrocyte towards a mature myelinating oligodendrocyte phenotype as the culture progresses. Mature oligodendrocytes are not present within the first 12 days in culture [Figure 3.2B]; however after insulin withdrawal (arrow) there is an increase in the number of MOG expressing cells correlating with the onset of myelination. The number of PLP/DM20 expressing cells follows a similar trend although in greater number due to the number of pre-oligodendrocytes and immature oligodendrocytes labelled. All oligodendrocytes expressing MOG also express PLP and MBP.

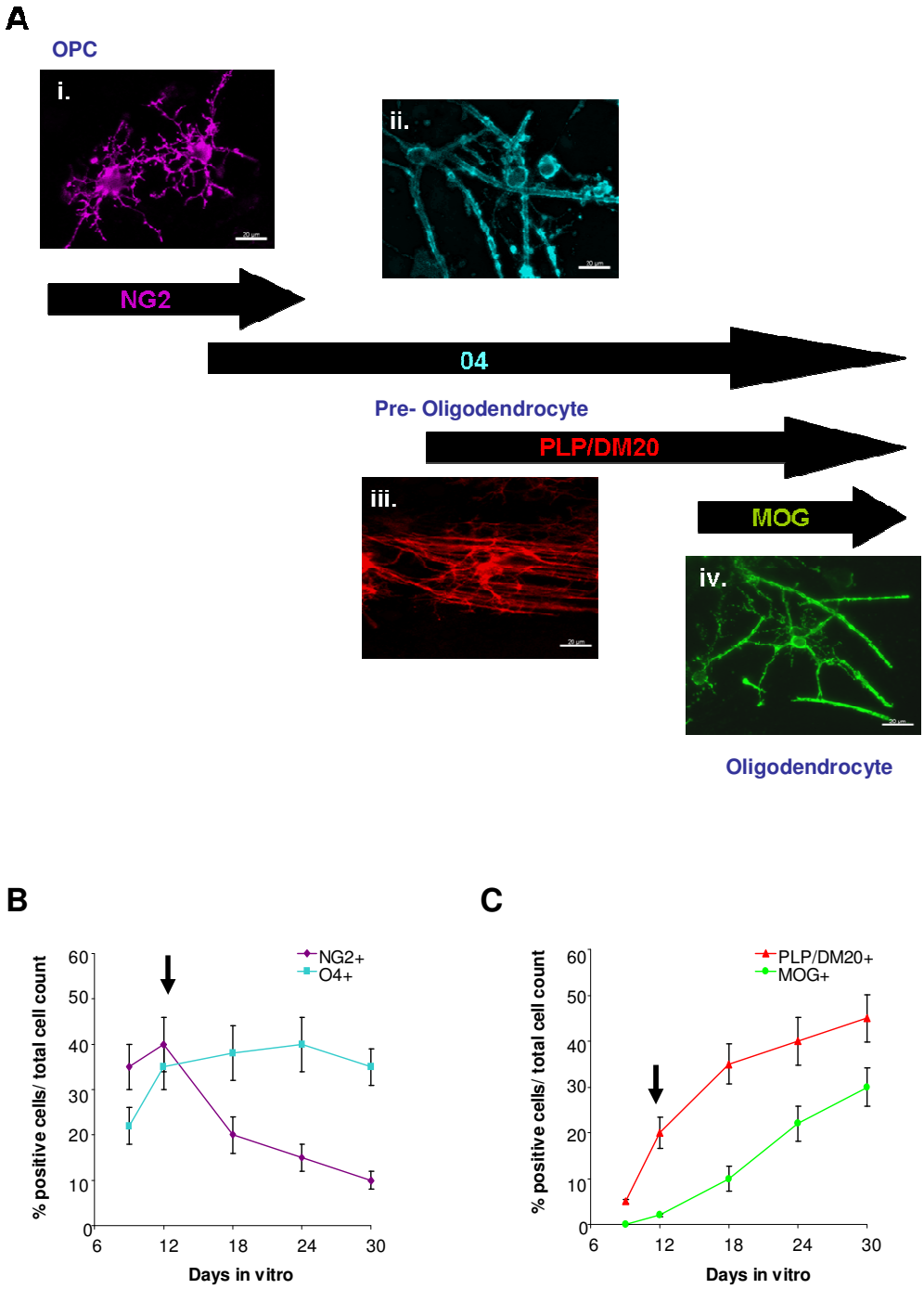


Figure 3.2: Differentiation of the oligodendrocyte lineage within the myelinating cell culture system.
A: OPCs express NG2 chondroitin sulphate proteoglycan **(i)** and as they mature into pre-oligodendrocytes they begin to express sulphatide (04) **(ii).** All oligodendrocytes express PLP **(iii)** (although it must be noted that PLP antibodies also bind to DM20 expressed by pre-OLG populations). MOG is a marker of terminal oligodendrocyte differentiation and commitment to a myelinating phenotype **(iv).** **B:** $NG2^+$ positive cells (purple line) are most abundant in the earlier culture stages reaching a maximum at ~12 DIV and decreasing at later stages. Cells expressing O4 (blue line) arise also in the earlier stages and continue to increase in number as the culture progresses. Arrow denotes day of insulin withdrawal from the media. **C:** PLP^+ cells (red line) appear in large numbers the intermediate and late stages of development coinciding with the onset of myelination. MOG expressing (green line) cells are most abundant in the final stages correlating with the terminal maturation of oligodendrocytes and enhanced myelination. All values plotted are the mean of three independent experiments performed in triplicate (± S.E.M).

3.2.3 Microglial development

In order to determine whether there were a population of resident microglia within the *in vitro* culture system, cultures were analysed by immunocytochemistry at several stages of growth using two microglia markers; CD11b/c and MHC-II.

At the earlier stages of development there are a number of CD11b/c^{+} cells with a round morphology. However as the cultures mature CD11b/c and MHC-II expressing microglia increase in number and they adopt a more classical branched morphology and demonstrate a larger amoeboid shape [Figure 3.3]. The phagocytic potential of these microglia cells will be demonstrated in the next chapter.

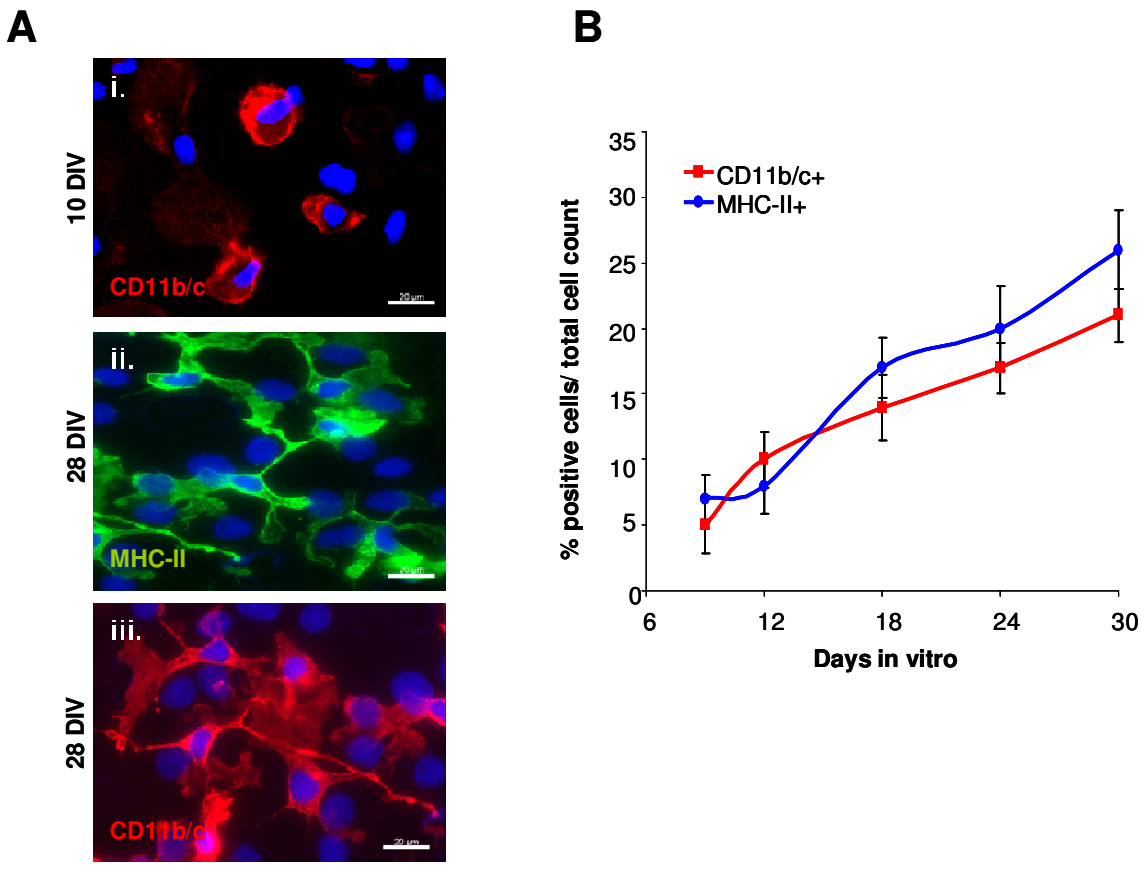


Figure 3.3: Microglia are present in every stage of culture development.
A: In early stages of culture (<12 DIV) the microglia present have a rounded morphology **(i)** (CD11b/c: red) as the cells mature they adopt a more classical branched morphology expressing both MHC-II **(ii)** (green) and CD11b/c **(iii)** (red) (63X magnification). **B:** Quantification of microglia numbers shows a steady increase throughout culture development. Values plotted as the mean of from three independent experiments performed in triplicate (± S.E.M).

3.2.4 Development of axo-glial junctions

3.2.4.1 Neurofascin *expression* in vitro

The neurofascins play key roles in axo-glial interactions and node of Ranvier formation (Zonta et al., 2008). Neurofascin exists as two major isoforms; Nfasc155 expressed by glia and Nfasc186 expressed by neurons and axons.

Live immunocytochemistry using a rabbit polyclonal antibody specific for Nfasc155 detects Nfasc155 expression on the surface of oligodendrocytes at the cell body on along their processes [Figure 3.4A]. Fixation with Bouin's fixative and permeablisation reveals Nfasc155 staining at paranodal domains and cytoplasmic staining within oligodendrocytes. In contrast a rabbit polyclonal specific for Nfasc186 only clearly stained nodes of Ranvier with no binding to oligodendrocytes or myelin. Staining live with A12/18.1, a pan specific monoclonal, revealed not only oligodendrocyte and nodal staining, but also on the surface of a subset of neurons [Figure 3.4B].

To investigate changes in Nfasc gene expression RT-PCR was performed on cDNA samples generated from RNA extracted from myelinating cultures at 9, 12, 18, 24 and 30 DIV. Nfasc155 expression [Figure 3.4C] follows a similar time course as oligodendrocyte maturation. Fold change in gene expression was calculated by the comparative Ct method (Livak et al., 2001) with gene expression values for 9 DIV used as the base line. There is no change in Nfasc gene expression between days 9 and 12. However from day 12 there is a rise in Nfasc155 gene expression increasing steadily as the culture progresses reaching a 3 fold increase after 30 DIV. In contrast Nfasc186 expression increases by 2 fold from day 9 to day 12 there is no change in Nfasc186 expression between day 12 and 18. From the cells present at these timepoints (mainly OPCs and no myelin formation) this increase in Nfasc186 is due to neuronal Nfasc186 expression. Nfasc186 gene expression increases sharply once myelination has begun (post day 18) due to increased Nfasc186 protein expression by the axon at the AIS and at the node of Ranvier.

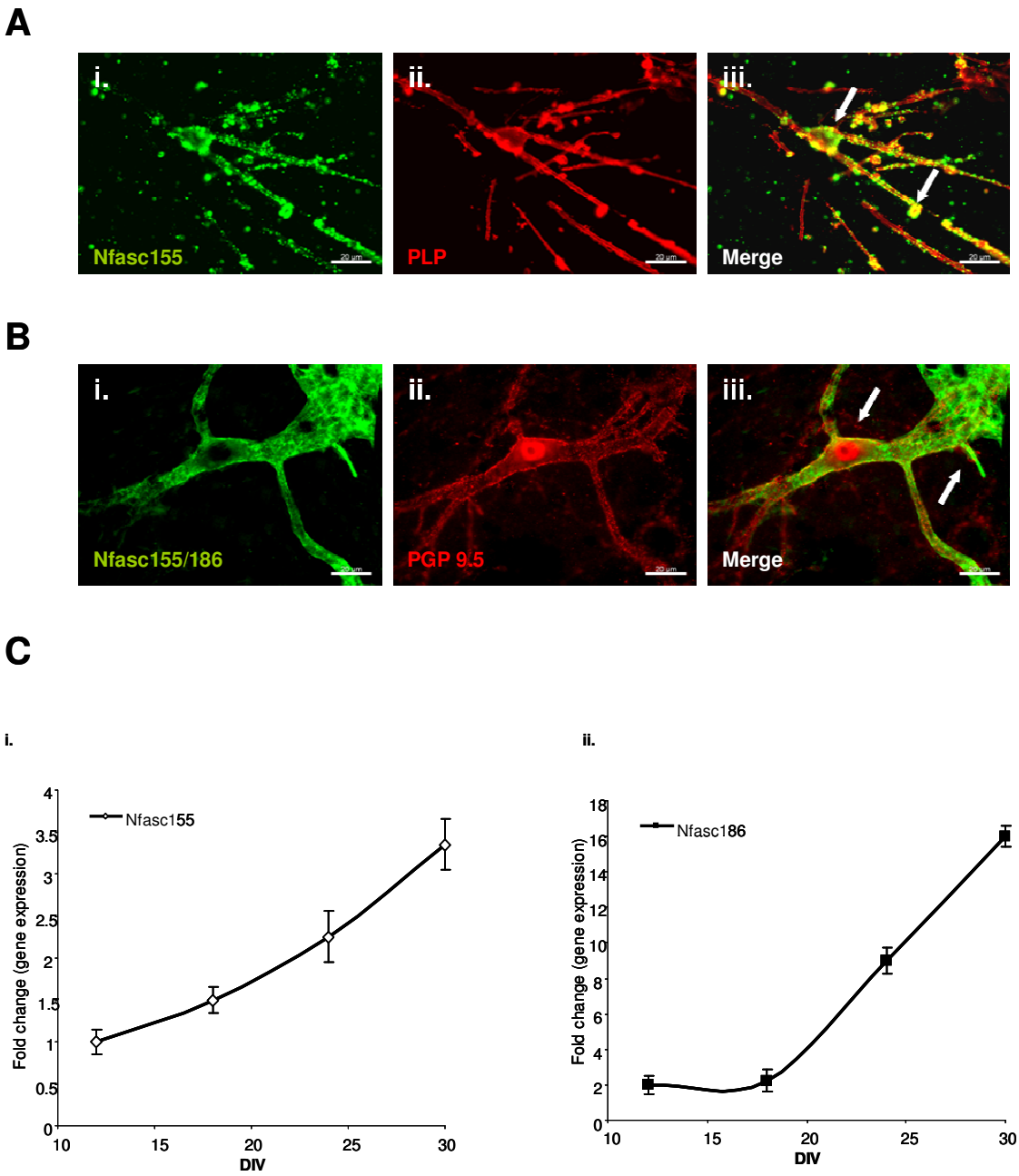


Figure 3.4 Neurofascin expression in vitro
A: Live staining for Nfasc155 (green) demonstrates surface expression by oligodendrocytes at the cell body and processes (PLP: red) with intense staining at the tip of the cell process (40X magnification). **B:** Live staining for Nfasc155/186 using a pan-specific monoclonal (A12/18.1) revealed Nfasc186 expression on a subset of PGP9.5^{+} neurons at the soma (red), axon initial segment and at the node of Ranvier (63X magnification). **C:** RNA was purified from myelinating cultures at 9, 12, 18, 24 and 30 DIV and cDNA created. Subsequent RT-PCR analysis of gene expression revealed that mRNA levels for both Nfasc155 and Nfasc186 continually increase over time in culture from 6 DIV. Nfasc155 expression increases from 12 DIV and increases gradually as myelination proceeds **(i).** Nfasc186 expression increases from day 6 as the neuronal population expands and after 18 DIV there is a large increase in Nfasc186 expression coinciding with the onset of myelination **(ii).** Fold change values calculated in comparison to day 9 gene expression (±S.D). ß-actin was used as housekeeping gene.

3.2.4.2 Molecular organisation of the node of Ranvier

In order to assess whether myelin sheathes formed in the cultures reproduced the characteristics to *in vivo* compact myelin and whether myelin internodes were bridged by typical nodes of Ranvier an immunocytochemical approach was adopted. Myelinating oligodendrocytes *in vitro* assemble paranodal loops expressing both Caspr and Nfasc155. These paranodal domain flank nodal regions where Nfasc186, ankyrin G and voltage gated sodium channels (NaV) are clustered. Myelinating cultures are capable of forming nodes of Ranvier between myelinated segments that are "typical" of those described *in vivo* with respect to their molecular composition [Figure 3.5]. In however in this culture system the formation of "heminodes" is much more common. At the heminode there is clustering of Nfasc155 and Caspr at the end of PLP^+ myelin sheathes but no detectable axonal expression of Nfasc186 or NaV [Figure 3.5B].

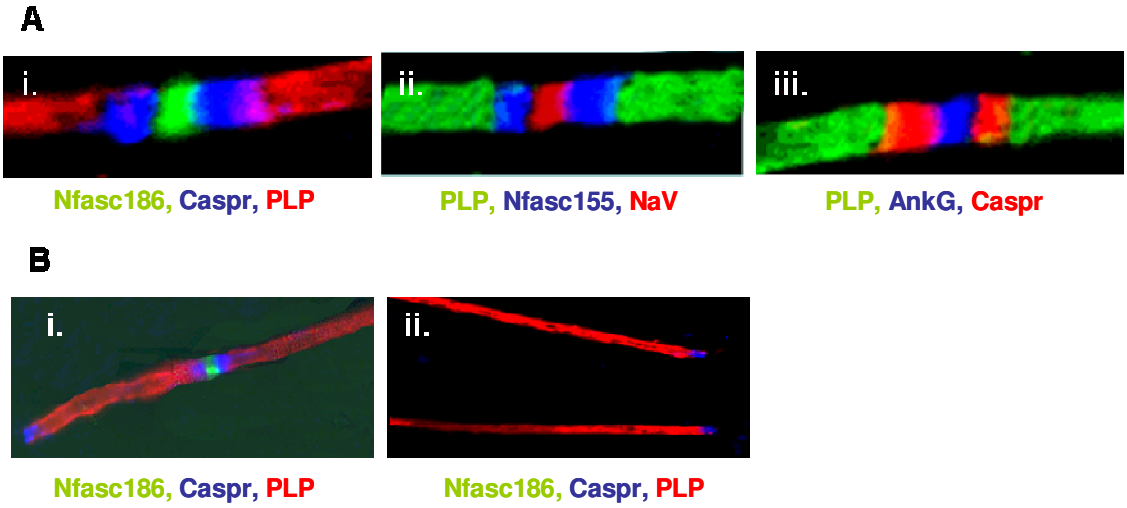


Figure 3.5: Nodes of Ranvier formed in vitro are representative of those formed in vivo.
A: Node of Ranvier between two myelin internodes (PLP, red) visualised by Nfasc186 (green) staining bounded by paranodal Caspr staining (blue) (100x magnification) **(i).** Node of Ranvier between two myelin internodes (PLP, green) visualised by staining of the voltage gated sodium channel (red) bounded by Nfasc155 at the paranode (blue) (100x magnification) **(ii).** Node of Ranvier between two myelin internodes (PLP, green) visualised by staining for ankyrinG (blue) bounded by Caspr at the paranode (red) (100x magnification) **(iii). B:** Staining showing a node of ranvier and a "hemi" node. Heminodes consist of paranodal Caspr **(i)** and Nfasc155 **(ii)** but no expression of voltage gated sodium channel or Nfasc186.

3.3 Discussion

In vitro myelinating cultures provide a model which reproduces the cellular composition and molecular organisation observed *in vivo* CNS. However it must be noted that this dissociated cell system is a developmental model of myelination and does not reproduce the 3D cytoarchitecture as seen in myelinated tracts *in vivo*. Nevertheless, individual sheathes form paranodal loops and heminodes as defined by Nfasc155 and Caspr immunoreactivity and between myelin internodes nodes of Ranvier are formed where Nfasc186, NaV and AnkG are clustered. Furthermore this system provides a cell based assay in which to determine the effects of a molecule of interest on myelination and axonal survival.

The advantage of using this rat-derived system over the mouse equivalent is the number of coverslips (individual cultures) one can generate from a typical preparation. One litter of P1 neonatal rats and one pregnant E15.5 SD rat (~12-15 embryos) will generate approximately 140 coverslips/ individual cultures. In contrast one pregnant mouse E13.5 (~6-8 embryos) will only generate only a third of this number. Using mouse-derived cultures could be used to confirm the specificity of the target such as using MOG knockout mice against anti-MOG antibody responses. However for the myelinating culture system to be used routinely as a model to detect pathogenic autoantibody responses it must be statistically reproducible both between individual coverslips within a single preparation but also between coverslips from different preparations. Within a batch of cultures the amount of myelination and the approximate axonal density remain relatively reproducible between coverslips [Table 3.1].

There is little variation in axonal density between either coverslips of the same batch or between preparations (C_V<10%). Myelination is much more variable (C_V>10%). Myelinating cultures were checked by immunocytochemistry and microscopy the day before use and cultures with <2% myelinated axons were discarded.

Table 3.1: Variations between preparations of myelinating cultures at 28 DIV
Values for axonal density and the percentage of myelinated axons were calculated from six independent batches of myelinating cultures generated over a six week period. Values plotted as the mean from three coverslips per preparation ± standard deviation (SD).

	Batch 1 (mean ± SD)	Batch 2 (mean ± SD)	Batch 3 (mean ± SD)	Batch 4 (mean ± SD)	Batch 5 (mean ± SD)	Batch 6 (mean ± SD)
Axonal Density (% SMI-31+/ total field)	71.9 ± 4.2	70.2 ± 3.7	68.6 ± 3.5	67.9 ± 4.1	69.1 ± 2.8	60.2 ± 2.2
Coefficient of Variance (Cv) (%)	5.8	5.3	5.1	6.0	4.0	3.7

	Batch 1 (mean ± SD)	Batch 2 (mean ± SD)	Batch 3 (mean ± SD)	Batch 4 (mean ± SD)	Batch 5 (mean ± SD)	Batch 6 (mean ± SD)
% Myelinated axons	15.1 ± 5.6	9.28 ± 3.1	8.49 ± 2.3	7.11 ± 2.9	4.48 ± 2.1	3.45 ± 1.5
Coefficient of Variance (Cv) (%)	37.1	33.4	27.1	40.8	46.9	43.5

The major advantage of this system is that images can be taken from coverslips randomly and subsequently analysed in a semi-automated fashion to give quantitative data. However it must be noted that quantifying the number of myelinated axons by highlighting individual myelin sheathes and discriminating between myelin and oligodendrocyte processes can be subjective depending on the individual performing the analysis. When quantifying cell numbers it is difficult to obtain and accurate figure when many cells are grouped closely together. One possible way to overcome this is to take Z stacks and quantify only those cells in that plane of focus.

I considered other culture models in addition to the dissociated cell culture models established here in Glasgow. One such model was the organotypic slice culture model. Organotypic slice cultures (OSCs) typically consist of neonatal murine CNS explants (such as the hippocampus or cerebellum), which are maintained in culture for several weeks (Stoppini et al., 1991). Such systems have been used for a wide range of applications from measuring the effects of long term addition of a particular substance (review: Peña. 2010) such as investigating the effects of neuroactive drugs (Drexler et al., 2010), growth factors (review: McAllister et al., 1999) and studies regarding antibody demyelination and subsequent remyelination (Harrer et al., 2009).

The advantage of such a system is that the 3D architecture seen *in vivo* is conserved in culture [Figure 3.6]. OSCs are therefore ideally suited to electrophysiological studies and have been used to expand our understanding of action potential propagation and synaptic transmission (Maletic-Savatic et al., 1999, Shi et al., 1999). The use of such as system has become an increasingly powerful tool with the incorporation of various transgenic mouse strains from auto fluorescent cells for enhanced imaging (review: Hechler et al., 2006) or the use of knockouts to investigate the mechanistics of a molecule of interest. I considered using OSCs as the model screening strategy for my thesis and spent two weeks in the laboratory of Professor N. Goebels (University of Zurich, Switzerland) learning the methodology. I discovered that the system was not without its drawbacks. It would require many animals and be very labour intensive to produce enough slices each week for a routine high throughput screening protocol and the slices require more maintenance in comparison to dissociated cell systems. The slices are also more sensitive to environmental changes and may spontaneously die in culture due to small media pH changes or hypoxia.

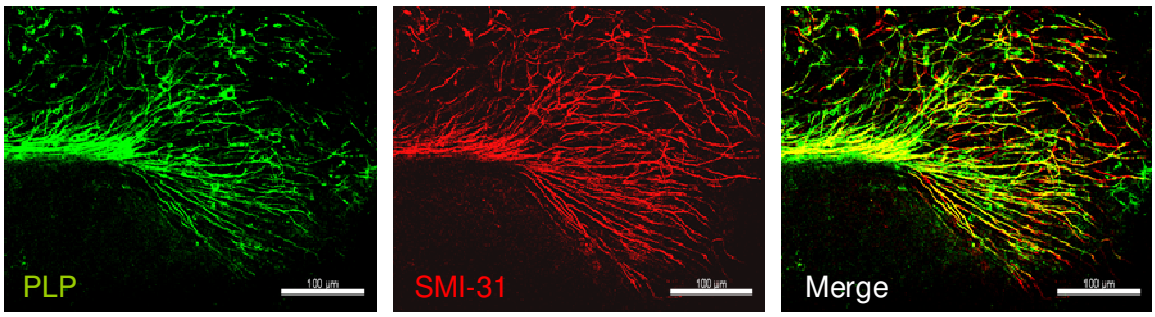


Figure 3.6: 3D architecture of white matter tracts in cerebellar slice cultures.
3D architecture of myelinated tracts in OSCs created from GPF-PLP transgenic mice (PLP; green, neurofilament; red) (20X magnification). OSCs were created and images taken whilst visiting the lab of Prof. N Goebels in collaboration with Dr. M Harrer (Zurich).

Characterising the rat dissociated myelinating cultures allowed me to determine the best time point in which to investigate antibody mediated injury *in vitro*. I decided for future experiments to add antibody at day 28. This was based on the cellular and biological composition of the cultures at this time point. Axonal density is well established with a substantial amount of oligodendrocyte maturation and myelin formation with nodes of Ranvier [Figure 3.7]. Usually the cells begin to decline after 30-35 days in culture and therefore it is crucial to use the cultures whilst they remained "healthy".

Through understanding which cell populations are present at this later time point and which molecules are expressed by these cells together with what is already known about autoantibody mediated pathogenesis in MS. We can begin to compile a list of candidate autoantigens in which to investigate using our system.

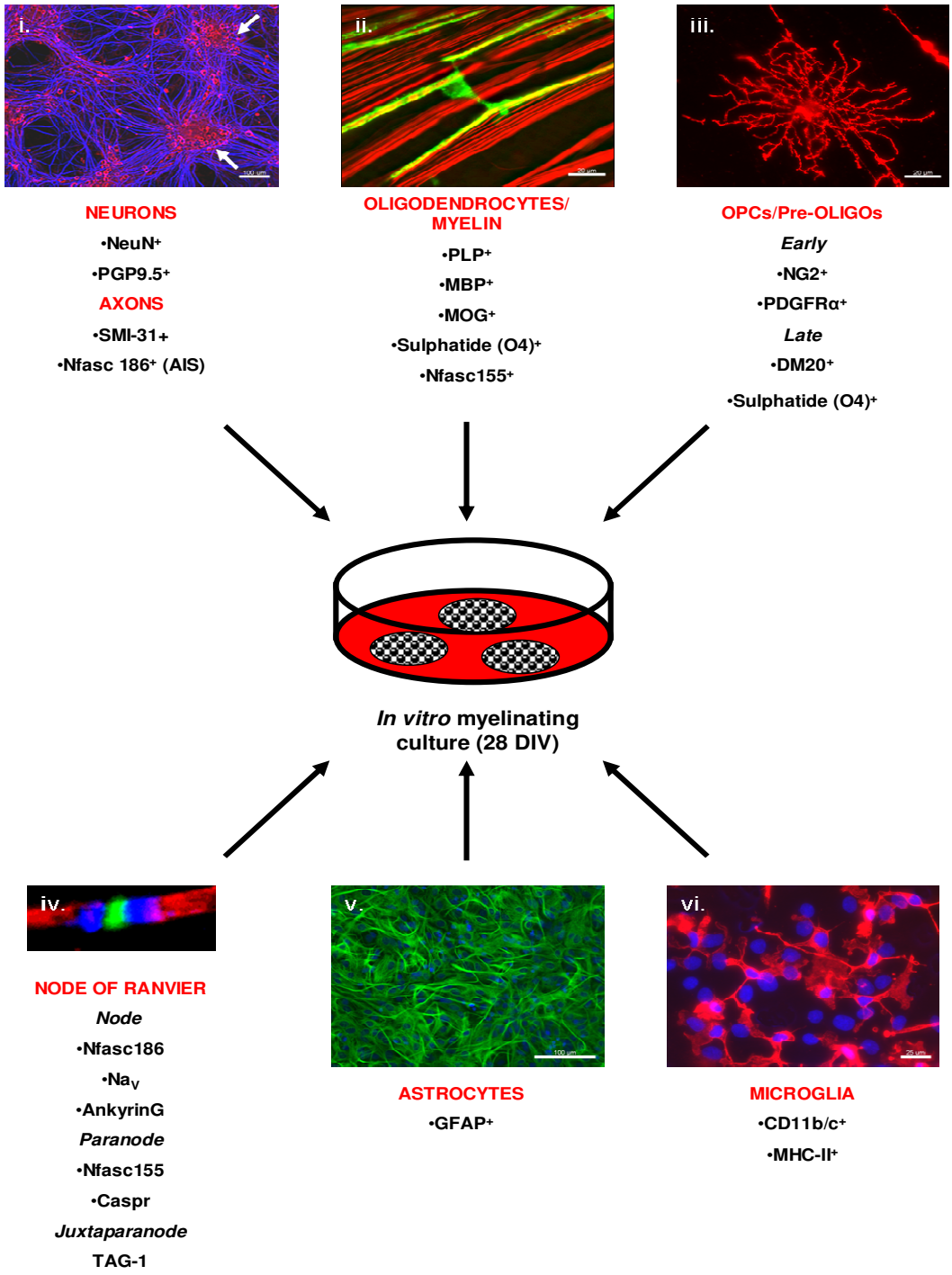


Figure 3.7: Summary of mature myelinating cultures after 28 DIV.
Myelinating cultures consist of a many different cell types after 28 days in culture. There is a well established populations of neurons (red, PGP9.5) extending many neurites (SMI-31, blue, 10X magnification) **(i).** These axons eventually become myelinated by endogenous oligodendrocytes expressing wide variety of typical markers (PLP, green; SMI-31 red) (63X magnification) **(ii).** These oligodendrocytes arise from a pool of oliogodendrocyte precursor cells, which are still present in small numbers even in mature cultures (O4, red, 63X magnification) **(iii).** Myelinated internodes formed within the system form heminodes and are also bridged by characteristic nodes of Ranvier **(iv).** Within these myelinated cultures it must be noted that there is a substantial populations of astrocytes (GFAP, green, 10X magnification **(v)** and microglia (CD11b/c, red, 40X magnification) **(vi).**

4 Validation of myelinating cultures as a model to detect pathogenic autoantibody responses.

4.1 Introduction

In the previous chapter myelinating cultures were described as an *in vitro* model with cellular and molecular composition of the CNS *in vivo* which reproduces the structural organisation of myelin internodes and nodes of Ranvier. The purpose of this chapter is to build on this and to describe the experiments undertaken to demonstrate that this model can be adapted to reproduce antibody mediated CNS injury. From the previous chapter and what is known about antibody mediated effects in animal models of MS we can compile a list of candidate autoantigens to investigate in order to validate our model as a tool to screen patients for the presence of axopathic and/or demyelinating antibody responses [Table 4.1].

Table 4.1: Summary of candidate MS autoantigens and their localisation *in vitro*
Summary of key autoantigens implicated in MS autoantibody responses. Localisation of these antibodies within myelinating cultures was determined using well characterised monoclonal antibodies. Antibody binding to myelinating cultures was determined either live (without fixation or permeablisation) (L) or post fixation with 4% PFA and permeablisation with 0.5% Triton X-100 (P) [+: strong staining, ++: enhanced staining, --: no staining]

Antigen	References	Localisation	Binding (L)	Binding (P)
MOG	Linington et al., (1988) Storch et al., (1998) Von Budingen et al., (2001)	myelin sheathes oligodendrocytes	+	++
MBP	Cruz et al., (1987) Olsson et al., (1990) Warren et al., (1995)	myelin sheathes oligodendrocytes	--	++
PLP	Johnson et al., (1986) Seil et al., (1984) Sun et al., (1991) Warren et al., (1994a) Warren et al., (1994b)	myelin sheathes oligodendrocytes	--	++
Sulphatide	Kanter et al., (2006)	pre-oligodendrocytes oligodendrocytes myelin sheathes	++	--
Nfasc155	Charles et al., (2002) Mathey et al., (2007)	*Boiuns fixative:* paranode *Live:* oligodendrocyte processes	++	++
Nfasc186	Charles et al., (2002) Mathey et al., (2007)	*Using A12/18.1:* Live: node of Ranvier, AIS and neuronal soma *Using a Nfasc186 specific rabbit IgG:* node of Ranvier and AIS	++	--
TAG-1	Derfuss et al., (2009)	*Bouins fixative:* Juxtaparnode	--	+

From what is already known about antibody mediated pathogenesis in MS and EAE we decided to investigate complement dependent antibody mediated injury (although complement independent effects will be discussed in a later chapter). This was for a number of reasons:

1. In antibody mediated demyelinating EAE (ADEAE) CNS injury is complement dependent. In animals with ADEAE there is extensive deposition of C9 in white matter tracts (Linington et al., 1989). However it has also been reported that antibody mediated injury *in vivo* is associated with an antibody dependent cellular cytotoxicity mechanism, where antibody binding makes the target vulnerable to attack from other immune cells such as macropahges (Brosnan et al., 1981). Therefore we will focus on acute antibody mediated effects.

2. There is a correlation of deposition of complement activation products and immunoglobulins in MS lesions (Lucchinetti et al., 2000; Prineas et al., 1981).

3. In paediatric MS and ADEM there is strong evidence implicating a role of pathogenic α-MOG autoantibodies in disease pathogenesis (McLaughlin et al., 2009, Di Pauli et al., 2010).

4. From a technical standpoint using a single time point will reduce the amount of antibody required for screening and therefore will conserve patient samples.

The aim of this chapter is to determine the validity of our *in vitro* system as a model of antibody mediated CNS injury and therefore must fulfil certain criterion:

- Antibody mediated injury *in vitro* must correspond to published data with respect to antigen specificity, accessibility and activity.

- Injury must reproducible and readily detectable at low antibody concentrations as reported in other autoantibody mediated diseases (Myathenia Gravis [α-AChR] is ~10-20nM) (Lindstrom et al., 1976).

4.2 Results

4.2.1 Antibody mediated injury in vitro is complement dependant and antigen specific

To determine the validity of our *in vitro* myelinating culture model as a potential screening strategy to detect pathogenic autoantibody responses we performed a series of experiments using well characterised monoclonal and polyclonal reagents directed against a multitude of CNS antigens some of which have been reported previously and implicated in the pathogenesis of EAE and MS [Table 4.2]. Throughout this initial study, myelinating cultures (28 DIV) were incubated overnight with 10µg/ml antibody in the presence of 1% fresh rat serum (FRS) as a source of complement. Targets expressed at the outer lamellae of the myelin sheath such as myelin oligodendrocyte glycoprotein (Z2, 8-18C5) and sulphatide (O4) were readily accessible for antibody binding and complement deposition resulting in a complete loss of PLP^+ myelin sheathes. In contrast targeting a cytosolic myelin antigen such as myelin proteolipid protein (PLP) or myelin basic protein (MBP) resulted in no loss of myelin due to the inability of the antibody to bind to its target.

Using live immunochemistry we can detect Nfasc155 staining on the surface of oligodendrocytes and their processes. Nfasc155 expressed at paranodal domains is not accessible to antibody binding without permeablisation. Targeting Nfasc155 with a Nfasc155 specific anti-sera mediates complement dependent loss of all PLP^+ myelin sheathes. In the previous chapter we demonstrated that Nfasc186 is expressed by the axon at the node of Ranvier and axon initial segment. Targeting myelinating cultures with a Nfasc186 specific anti-sera mediated a selective loss of myelinated axons (~10% of total axons) and subsequent loss of myelin sheathes. Live immunochemistry using A12/18.1 (pan-Nfasc specific mAb) revealed in addition to the Nfasc155 and Nfasc186 staining observed with the specific anti-seras, staining on the cell body of a subset of neurons. Treatment of myelinating cultures with A12/18.1 resulted in a dramatic loss of ~40% of all axons (both myelinated and unmyelinated) and complete demyelination.

TAG-1/contactin-2 is a recently identified autoantigen highlighted for its potential to cause T cell mediated cortical pathology *in vivo*. In our system TAG-1 is buried deep within the juxtaparanodal region and is completely inaccessible to antibody binding without fixation and permeablisation. Therefore it was not surprising that incubation of myelinating cultures with two TAG-1 specific monoclonal Abs (4D7, 3.1C6) did not mediate complement dependent injury to axons nor glia. The pathological effects of these antibodies *in vitro* were complement-dependent and antigen-specific, as demonstrated using heat inactivated sera and appropriate isotype or species specific control antibodies.

Table 4.2: Using myelinating cultures as a model of autoantibody mediated injury

Using our in vitro myelinating cultures we are able to detect selective antigen specific effects using a series of monoclonal reagents. Myelinating cultures were treated overnight in each case with 10µg/ml antibody in the presence of 1% fresh rat sera as a source of complement. Targeting an extracellular myelin antigen, such a MOG or sulphatide in the presence of complement results in complete and selective demyelination. In contrast targeting an cytosolic antigen such as PLP or MBP unable to mediate complement dependent demyelination *in vitro*. Antibody mediated effects were complement dependent and antigen specific. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. P values obtained using the suitable statistical test were comparable between raw data or values standardised to the control (* $p<0.05$, ** $p<0.01$ T-test).

			Raw data		Standardised to control	
Ab specificity	**Species**	**Isotype**	**Axon density (% SMI-31+/ total field)**	**Myelination (arbitrary units)**	**% Axonal loss**	**% Myelin loss**
MOG (Z2)	mouse	IgG2a	69.4 ± 3.3	**0.03 ± 0.02****	2 ± 2.2	**99 ± 0.9****
MOG (8-18C5)	mouse	IgG1	70 ± 4.1	**0.06 ± 0.03****	1.2 ± 3.2	**98 ± 1.2****
Nfasc155	rabbit	IgG	68 ± 2.2	**0.144 ± 0.09****	3 ± 0.6	**96 ± 3.3****
Sulphatide (O4)	mouse	IgM	69.4 ± 3.2	**0 ± 0.06****	1.5 ± 1.8	**100 ± 2.9****
PLP	rabbit	IgG	71 ± 3.2	3.7 ± 2.2	0 ± 2.0	0 ± 2.5
MBP	mouse	IgG2a	71.2 ± 3.4	3.64 ± 1.5	0 ± 3.6	0 ± 3.8
TAG-1 (4D7)	mouse	IgM	69.2 ± 3.8	3.62 ± 1.2	1.4 ± 2.6	0 ± 2.9
TAG-1 (3.1C12)	mouse	IgG1	68.6 ± 2.2	3.52 ± 0.9	2.3 ±1.2	2 ± 1.3
Nfasc186	rabbit	IgG	**60 ± 3.0***	**0.09 ± 0.03****	**15 ± 3.6***	**97 ± 2.4****
Nfasc 155/186 (A12/18.1)	mouse	IgG2a	**35.6 ± 3.2****	**0 ± 0****	**40 ± 3.2****	**100 ± 0.0****
Myeloma Protein (MOPC1)	mouse	IgG1	71.5 ± 2.4	3.65 ± 1.2	0 ± 1.8	0 ± 2.4
Myeloma Protein (UPC10)	mouse	IgG2a	69 ± 3.1	3.61 ± 1.0	1 ± 3.0	0 ± 2.2

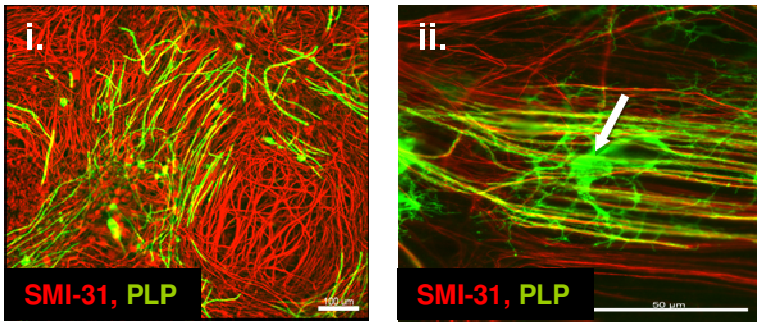
4.2.2 MOG: the classic target for autoantibody mediated injury

In EAE it has been demonstrated that MOG specific antibodies drive antibody mediated demyelination and exacerbate disease (Linington et al., 1988). In paediatric MS and ADEM there is strong evidence suggesting that α-MOG autoantibodies have a key role in disease pathogenesis (McLaughlin et al., 2009, Di Pauli et al., 2011). However in classical adult onset MS the issue remains controversial.

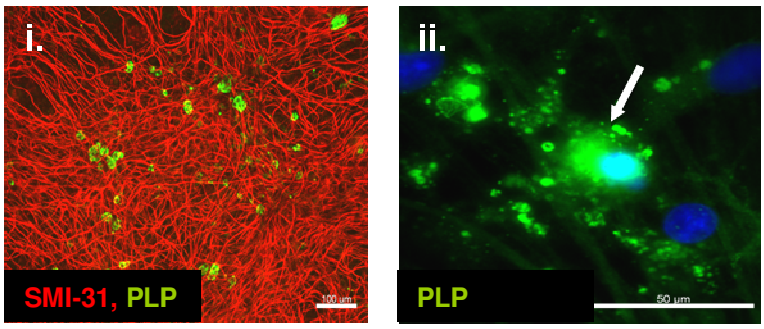
The accessibility of MOG on the outer lamellae of the myelin sheath makes it an obvious candidate for antibody mediated injury. To model anti-MOG mediated injury *in vitro* there are many MOG specific monoclonals available most of which recognise the extracellular Ig-like domain (Breithaupt et al., 2008). For this thesis I used the monoclonal antibody Z2; a well defined anti-MOG monoclonal antibody generated by Piddlesden et al, (1993). In this study the authors extensively characterised this antibody both *in vitro* and *in vivo*. Z2 is mouse IgG2a and therefore is an excellent complement-fixing antibody. This makes Z2 an ideally candidate mAb to use in this initial proof of principle study [Figure 4.1].

Overnight treatment with 10μg/ml Z2 in the presence 1% FRS as a source of complement results in a selective and complete loss of PLP^{+} myelin sheathes and total loss of MOG immunoreactivity. Injury is restricted to the oligodendrocyte and myelin sheathes as there is no detectable effect on axonal survival [Figure 4.1A]. Antibody treatment in the absence of 1% rat serum as a source of complement mediated no detectable injury when compared to untreated controls and at higher magnification individual oligodendrocytes can be seen extending several processes and maintaining multiple myelin internodes [Figure 4.1B]. This antibody mediated demyelination was a complement dependent phenomenon as no pathogenic activity was detected in cultures treated with either antibody alone, 1% FRS alone or after heat inactivation (56°C for 30 min) of the complement source. [Figure 4.1C].

A



B



C

Condition	% Axonal loss	% Myelin loss	% OLG loss
Z2 (10µg/ml)	0 ± 0.1	5 ± 2.3	7 ± 4.6
Z2 (10µg/ml) + fresh rat serum (1%)	2 ± 2.2	**100 ± 0.9****	**95 ± 3.5****
Z2 (10µg/ml) + heat inactive rat serum (1%)	2 ± 1.5	3 ± 1.8	6 ± 5.2
fresh rat serum (1%) (FRS)	1.5 ± 0.5	1.8± 1.3	5 ± 3.7

Figure 4.1: MOG: The classic target for autoantibody mediated injury
A: Overnight antibody treatment 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 **(i).** High magnification images (100x) show the lysis of oligodendrocytes after treatment with an MOG specific monoclonal antibody in the presence of complement (PLP: green, DAPI: blue) **(ii). B:** In contrast, treatment of myelinating cultures with 10µg/ml Z2 (MOG-specific monoclonal antibody) in the absence of 1% FRS does not induce axonal injury as visualised by intact SMI-31 (phosphorylated neurofilament) staining (red) or demyelination visualised with PLP (green) (10x magnification) **(i).** High magnification (63X) images show oligodendrocytes are unaffected after overnight treatment with Z2 in the absence of a source of complement **(ii). C:** Pathology observed was both antibody mediated and complement dependant further confirmed by various control conditions such as heat inactivation of complement and addition of normal rat sera in the absence of antibody. Values shown are an average of three independent experiments performed in triplicate ± S.E.M (* $p<0.05$, ** $p<0.01$; T-test).

4.2.2.1 Time course of complement dependent demyelination mediated by MOG specific antibodies

To visualise MAC pore formation it was not possible to use Z2 (both Z2 and α-MAC antibody are mIgG2a). Therefore for this experiment I used 8-18C5, another α-MOG monoclonal that is of the IgG1 isotype (Linington et al., 1988). Most importantly 8-18C5 is capable of fixing complement and mediating demyelination in our myelinating culture system (described above).

Two hours after addition of 10μg/ml 8-18C5 and 1% FRS, MAC pore formation can be seen on the surface of PLP^+ oligodendrocytes and myelin sheathes [Figure 4.2A]. At this time point oligodendrocytes and myelin sheathes remain intact. However MAC pore formation on the oligodendrocyte/myelin surface is accompanied by a disruption in MOG distribution on the outer surface of the myelin sheath which can be detected as early as 2 hours after antibody addition [Figure 4.2B].

To plot the time course of α-MOG antibody mediated injury myelinating cultures were treated with 10μg/ml Z2 with 1% FRS. Cultures were fixed at key intervals and stained for PLP, SMI-31 and MOG.

Analysis of PLP staining demonstrates that despite injury to the outer surface, the myelin sheath remains stable and attached to the axon until 3.5 hours after addition. The first signs of PLP loss are detected after 3 hours and demyelination is complete within 4 hours. Demyelination does not have a detrimental effect on axonal survival as detectable by measuring SMI-31 immunoreactivity [Figure 4.3A]. Complete destruction of compact myelin is preceded by injury to the outer surface of the myelin sheath. To monitor the fate of bound antibody at the myelin surface; cultures were washed, fixed (4% PFA) and incubated with the appropriate fluorochrome conjugated secondary antibody. Within 30 minutes of addition we can detect Z2 bound to the surface of intact myelin sheathes and oligodendrocytes. Within one hour of addition we can detect a loss of Z2 immunochemistry (~10%) and as injury progresses the amount of detectable Z2 immunoreactivity sharply declines. After 3 hours Z2 binding can be no longer detected on oligodendrocytes, myelin or myelin debris. This loss of Z2 bound at the myelin surface precedes a loss of MOG^+ myelin

sheathes detected using 8-18C5. Loss of all MOG reactivity (8-18C5 and Z2) was observed after 4 hours [Figure 4.3B]. In this series of experiments a rabbit pAb against neurofilament was used to label axons as 8-18C5 and SMI-31 are both mIgG1.

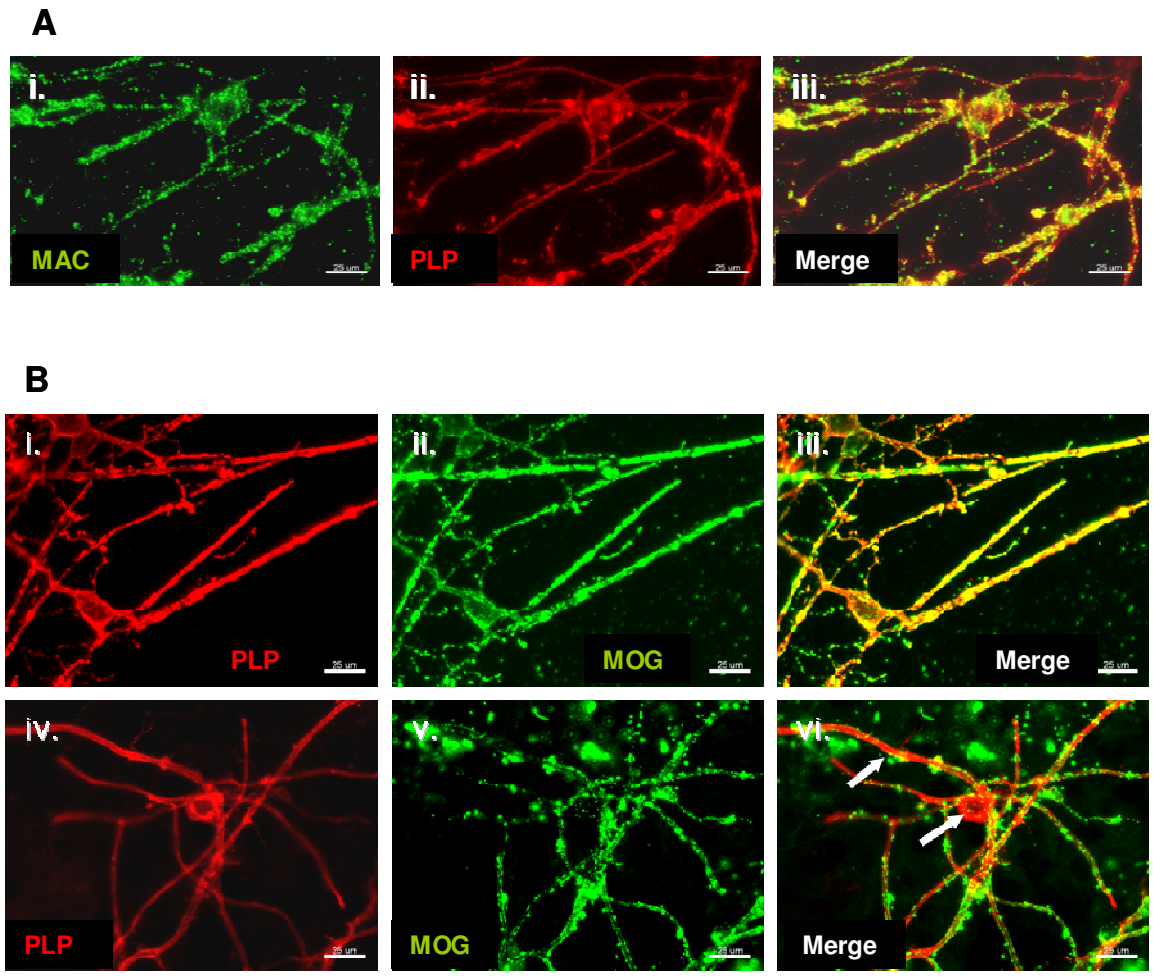


Figure 4.2: MOG specific antibody mediated complement dependent myelin injury occurs within to hours via MAC deposition on the surface of oligodendrocytes and myelin
A: Myelinating cultures were treated with 10µg/ml 8-18C5 in the presence of complement. Two hours after antibody addition MAC (poly-C9) (green) **(i)** can be detected on the surface of PLP^+ oligodendrocytes and myelin (red) **(ii-iii). B:** Treatment of myelinating cultures with 10µg/ml 8-18C5 in the absence of complement does not induce demyelination. Three hours after antibody addition; high magnification images (63X) of myelin show intact sheathes expressing both cytosolic PLP (red) **(i)** and MOG (Z2) on the outer myelin lamellae (green) **(ii) (iii;** merged image**).** In contrast three hours after treatment with 10µg/ml 8-18C5 with 1% FRS as a source of complement shows that although the myelin appears intact with respect to PLP staining (red) **(iv)** there is major disruption of MOG localisation at the myelin surface (green) and the oligodendrocyte cell body **(v) (vi;** merged image**).**

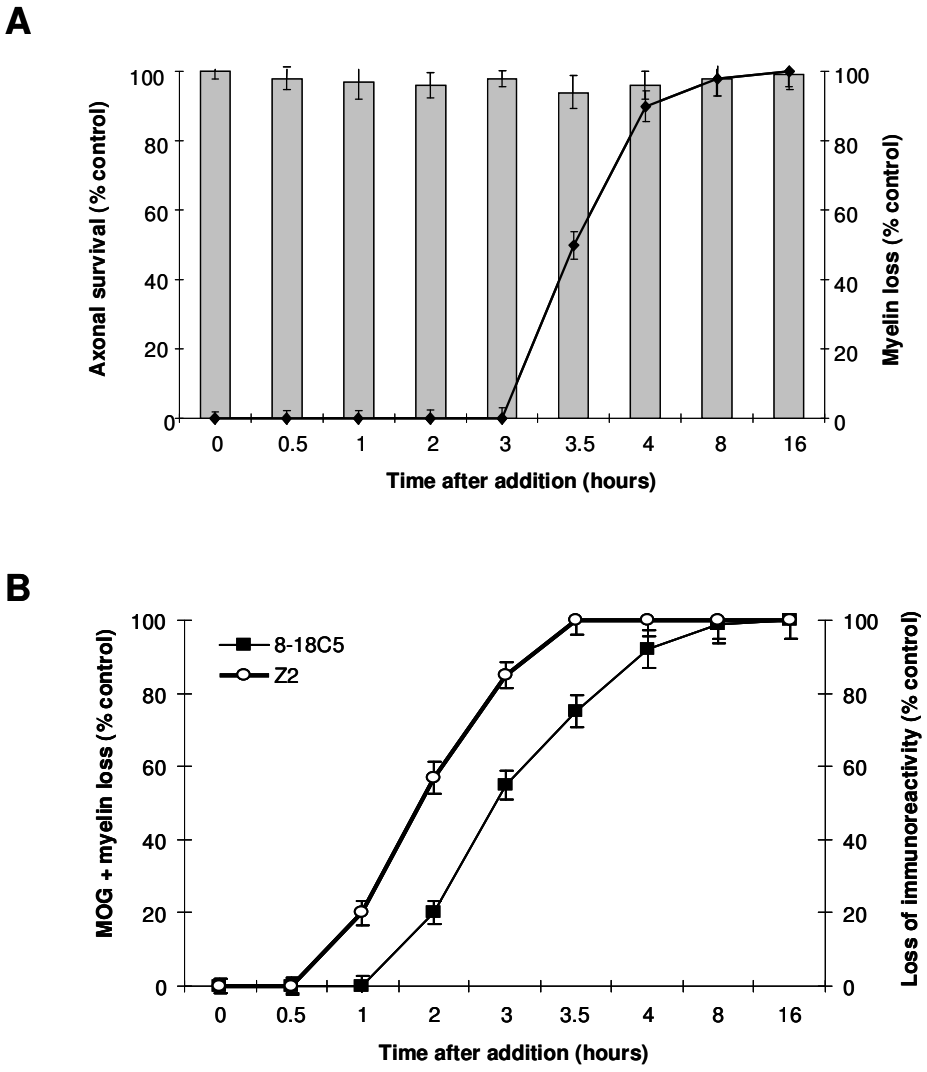


Figure 4.3: Time course of anti-MOG antibody mediated complement dependent injury.
A: Demyelination occurs rapidly after antibody addition. Analysis of PLP staining reveals that myelin sheathes remain relatively stable and attached to the axon until after ~4 hours *in vitro* after which the myelin is completely degraded (line). Demyelination does not have a detrimental effect on axonal survival as detected by SMI-31 immunoreactivity (bars). **B:** Complete destruction of PLP^+ myelin sheathes is preceded by a disruption of MOG staining at the myelin surface. Significant loss of MOG^+ immunoreactivity can be detected as early as 2 hours after antibody addition as detected by using 8-18C5 mAb. Loss of bound Z2 from the myelin surface is detectable after one hour after antibody addition. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

4.2.2.2 Fate of myelin debris

After complement mediated demyelination by the α-MOG response, PLP^{+} myelin debris was found to be internalised by CD11b/c expressing microglia 4.5 hours after addition. This was not seen in untreated cultures or in cultures treated with and isotype control antibody [Figure 4.4].

There reports suggesting that antibody mediated injury may occur by ADCC, where antibody binding to the target leads to destruction by immune cells. However at no point during antibody treatment and subsequent injury; were microglia observed making direct contact with the damaged myelin sheath. Suggesting that microglia do not have an active role in driving demyelination *in vitro* but rather serve to clear myelin debris and phagocytose opsonised material.

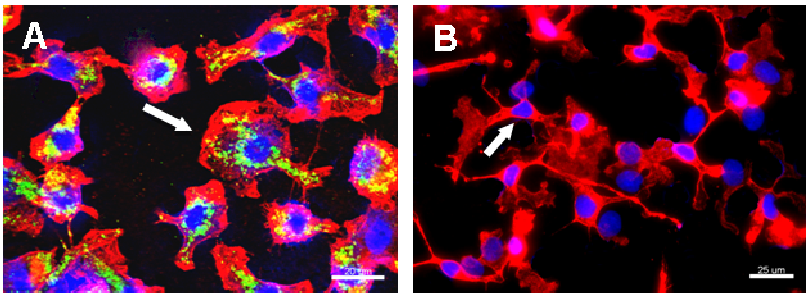


Figure 4.4: Uptake of myelin debris by microglia
After α-MOG mediated demyelination myelin debris is internalised by resident microglial population **(A)**. This was not observed in non-demyelinated cultures **(B)** (PLP; green, CD11b/c; red, DAPI; blue) (63X magnification).

4.2.2.3 Summary of MOG specific antibody mediated complement dependent myelin injury

In summary α-MOG mediated complement dependent injury occurs rapidly; the first signs of complement of activation and myelin disruption are detectable one hour after addition. Substantial MAC deposition on the myelin surface can be detected at approximately two hours after antibody addition, leading to a reduction in MOG expression at the myelin surface. Despite damage to outer lamellae the myelin sheathes (PLP^+) remain stable until 3.5 hours where the myelin rapidly degrades and demyelination is complete within 4 hours. Residual PLP reactivity is associated with myelin debris, which is internalised by microglia [Figure 4.5].

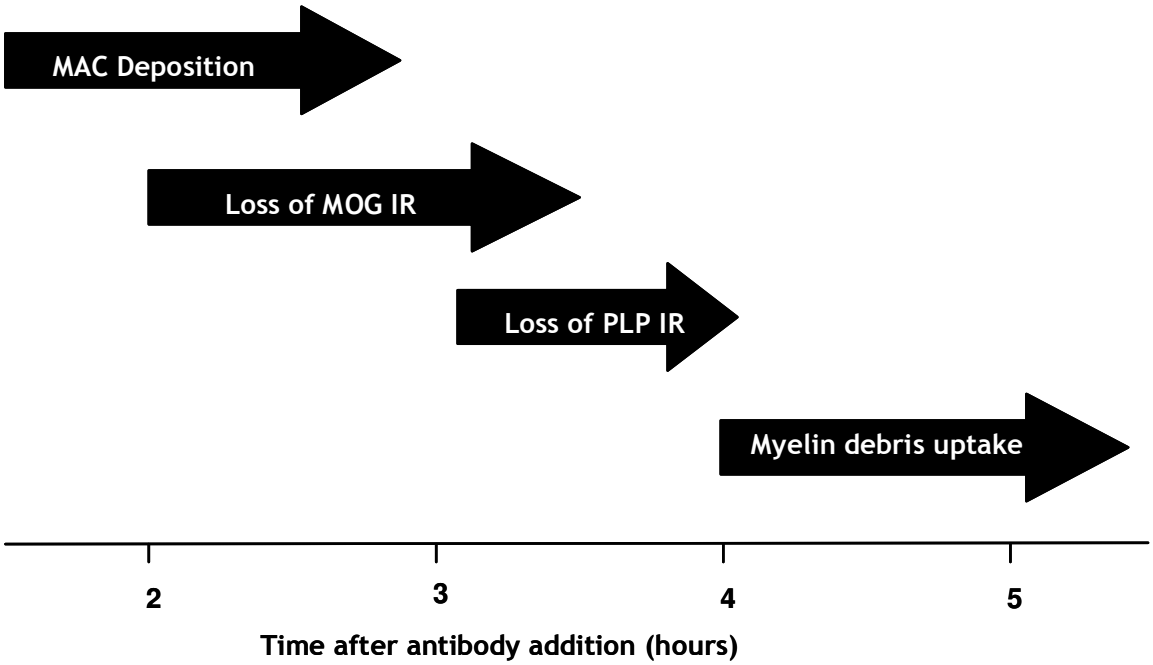
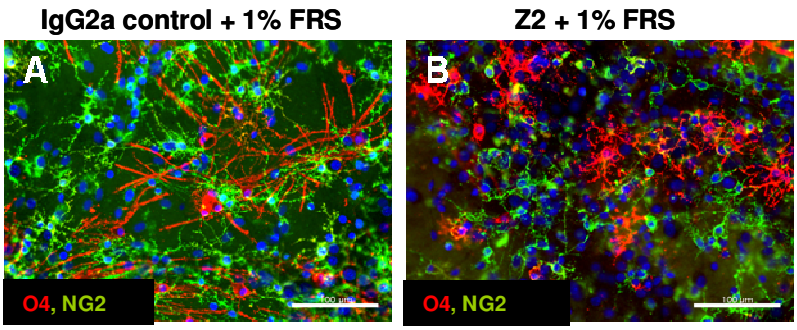


Figure 4.5: Summary of the time course of anti-MOG antibody mediated injury.
MAC formation can be detected one hour after antibody addition reaching a peak at approximately 2 hours after addition. At this point there is a significant loss of MOG immunoreactivity (IR) at the surface of the myelin sheath. Destruction of compact myelin occurs much later (3 hours after addition). From three hours after addition internalised PLP^+ myelin debris can be detected in microglia.

4.2.2.4 Complement mediated demyelination by a MOG specific antibody has a limited effect on oligodendrocyte progenitor cells

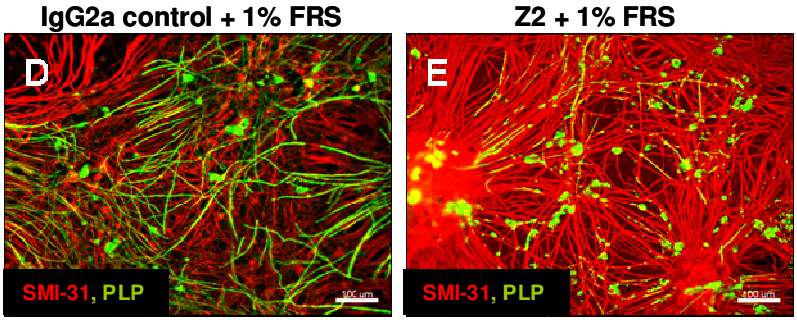
To investigate the long term consequences of Z2 mediated demyelination I determined changes in OPC populations post treatment. Immediately after demyelination cultures were stained for NG2, a marker for OPCs and O4 for late OPC pre-oligodendrocytes. In cultures treated with an isotype control there is a population of NG2 expressing cells and a mixed population of $O4^+$ cells including OPCs, pre-oligodendrocytes and mature oligodendrocytes with their associated myelin sheathes [Figure 4.6A]. After Z2 mediated demyelination there is a complete loss of $O4^+$ myelin sheathes accompanied by a loss of mature oligodendrocytes expressing both sulphatide and MOG [Figure 4.6B]. Quantification of cell numbers revealed the number of $NG2^+$ OPCs is unchanged after demyelination; there is a small reduction of $O4^+$ cells which can be attributed to the loss of $O4^+/MOG^+$ mature oligodendrocytes [Figure 4.6C].

To determine whether these remaining OPCs still retained their ability to differentiate and myelinate; cultures were demyelinated using Z2 in the presence of complement and after demyelination cells were washed extensively and placed back into culture with fresh media. After 5 days in culture there was a considerable amount of myelin formed in the non-demyelinated cultures [Figure 4.6D]. In demyelinated cultures there is a partial reconstitution of the mature PLP^+ oligodendrocyte pool and some myelination has taken place [Figure 4.6E]. Analysis of immunocytochemical data reveals that after 5 days in culture the level of myelination is almost the level it was before demyelination [Figure 4.6F]. This demonstrates that OPCs are spared after demyelination retain their functionality. It also supports the previous observation that demyelination has no effect on axonal integrity as many axons remain permissible to myelination.



C

Cells/field of view (20X)	% NG2+ cells (green)	% O4+ cells (red)
Z2 (+ 1% FRS)	47 ± 4.2	31 ± 14
IgG2a (+1% FRS)	50 ± 1.8	48 ± 5



F

	Myelination (arbitrary units)
Before Demyelination (24DIV)	1 ± 0.24
Z2 (+ 1% FRS) (30 DIV)	0.65 ± 0.16
IgG2a (+1% FRS) (30 DIV)	2.8 ± 0.62

Figure 4.6: Complement mediated demyelination by anti-MOG antibody has a limited effect on oligodendrocyte progenitors
A: At 28 DIV there is a residual population of $NG2^+$ and $O4^+$ OPCs. It must be noted that mature oligodendrocytes and myelin are also labelled by the O4 antibody (20X magnification). **B:** At 28 DIV cultures are demyelinated using 10µg/ml Z2 in the presence of complement resulting in a complete loss of $O4^+$ myelin sheathes and a loss of mature oligodendrocytes expressing both MOG and sulphatide (NG2; green, O4; red) (20X magnification). **C:** Comparison of cultures demyelinated using Z2 and those treated with an isotype control reveal that the numbers of $NG2^+$ OPCs are unchanged in response to demyelination there is a modest reduction in the number of $O4^+$ cells. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **D:** At 30 DIV there was considerable myelin formed in the non-demyelinated cultures (SMI-31; red, MOG green) (10X magnification). **E:** Five days after demyelination there was a reconstitution the mature oligodendrocyte population with a modest amount of axonal ensheathment (SMI-31; red, MOG green) (10X magnification). **F:** Analysis of myelination after antibody treatment demonstrates that OPCs remain functional and capable of differentiating into a myelinating phenotype. This also implies that there are no major axonal changes in response to demyelination as the remaining axons remain permissible to myelination. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

4.2.2.5 Myelinating cultures provide a highly sensitive screening strategy in which to detect pathogenic MOG specific antibody responses

In order to test the sensitivity of our system to detect pathogenic activity at low antibody concentrations a dose response study was performed. Z2 was diluted across a concentration range from 10µg/ml to 10ng/ml.

To determine the binding affinity of Z2 to myelinating cultures at sub-optimum concentrations used for immunochemistry a titration curve was plotted. Cultures were stained with 10µg/ml, 1µg/ml, 100ng/ml, 50ng/ml or 10ng/ml. Cultures were imaged using immunofluorescence microscopy (10X magnification); images were capture using the same exposure thresholds and imaging parameters defined using 10µg/ml cultures. Analysis was performed using image J software using the same settings for each concentration (again thresholds were set using highest concentration). At 10µg/ml approximately 45% of the total pixel number is $Z2^+$; as the concentration is lowered the number of $Z2^+$ pixels detected per field of view drops rapidly. At concentration ranges below 100ng/ml it was not possible to visualise significant Z2 binding via immunocytochemistry [Figure 4.7].

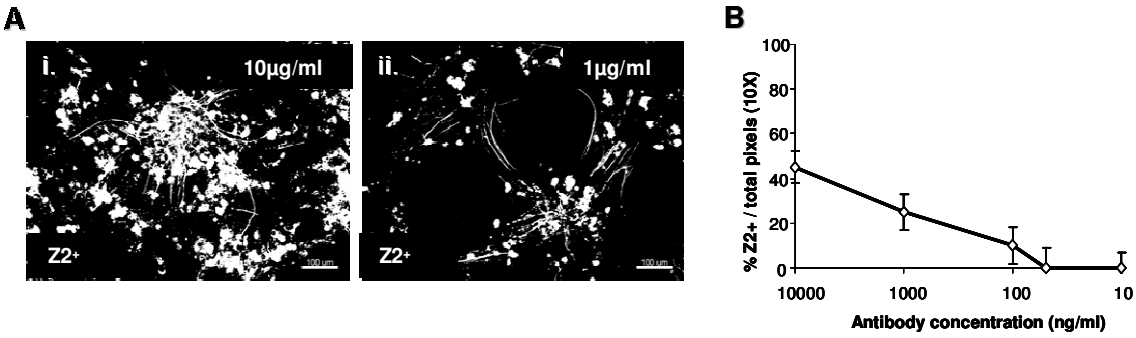


Figure 4.7: Detection of Z2 binding *in vitro* by immunofluorescence is concentration dependent.
A: Staining of myelinating cultures (28 DIV) with 10µg/ml Z2 gives a strong staining of oligodendrocytes and myelin sheathes (i). In contrast immunochemistry using 1µg/ml Z2 gives a much weaker staining (ii). Black and white images were created using imageJ under the same exposure parameters and can be used to quantify the number of $Z2^+$ pixels per field of view (10X magnification). **B:** Titration of Z2 over a concentration range 10µg/ml to 10ng/ml demonstrates that at antibody concentrations below 100ng/ml it was not possible to detect Z2 binding by immunofluorescence microscopy. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

Fortunately our model is far more sensitive as it was possible to detect significant demyelinating activity at antibody concentrations below the detection threshold for immunofluorescence. Significant demyelinating activity can be detected at antibody concentrations ≥50ng/ml [Figure 4.8].

This gives our system a great advantage over other cell based assay system which relies solely on immunofluorescence as their mode of detection (either by FACS or microscopy). In this paradigm addition of 50ng/ml Z2 would give a "negative" result however using our system we can still detect a pathogenic antibody response.

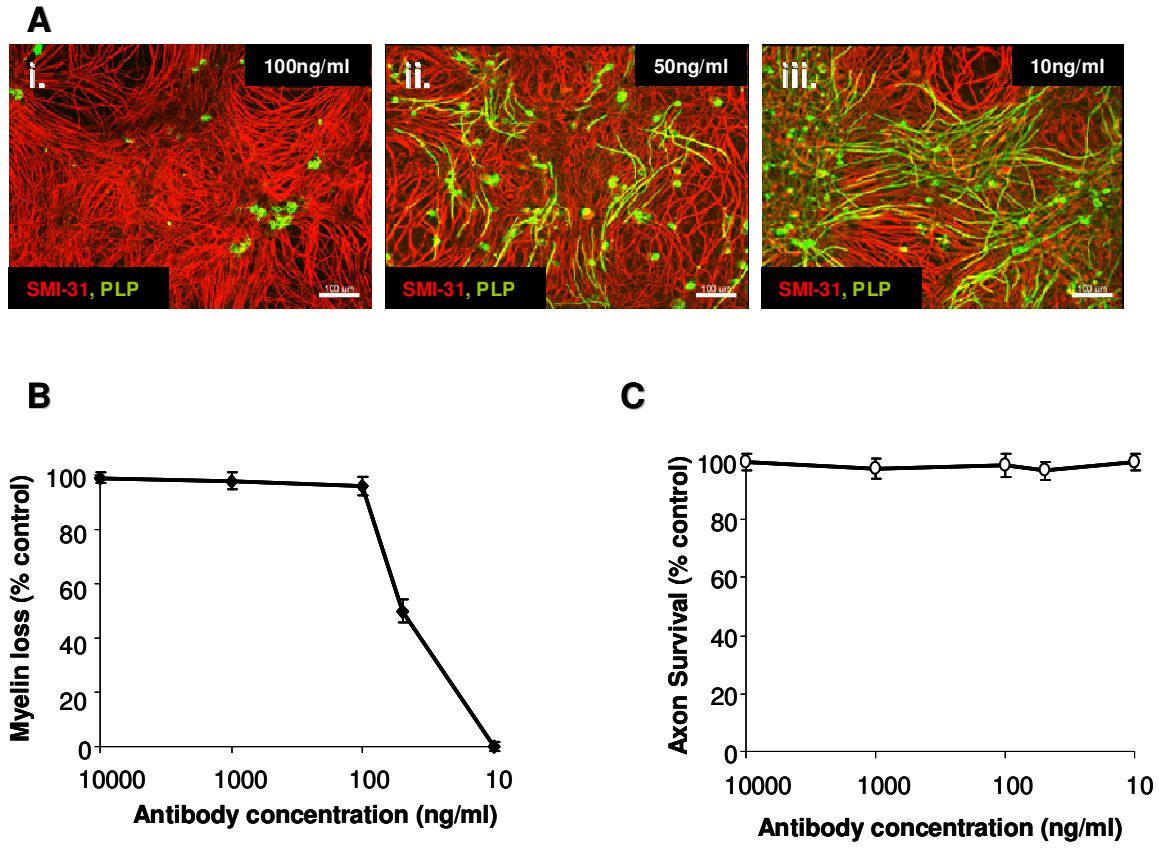


Figure 4.8: Myelinating cultures provide a highly sensitive screening strategy in which to detect pathogenic α-MOG antibody responses
A: Treatment of myelinating cultures with 100ng/ml Z2 in the presence of complement mediates complete demyelination similar as that observed with treatment with antibody concentrations 100 fold higher (SMI-31: red, PLP: green) **(i).** In contrast treatment of myelinating cultures with 50ng/ml Z2 in the presence of complement mediates significant demyelination with a loss of ~50% total myelin sheathes (SMI-31: red, PLP: green) **(ii).** Lowering antibody concentration to 10ng/ml abolishes all demyelinating activity when compared to controls (SMI-31: red, PLP: green) **(iii). B:** Dose response studies show that α-MOG mediated demyelination is detectable to antibody concentrations >50ng/ml. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **C:** Axonal density was unaltered and equivalent across treatments when compared to controls. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

In samples of unknown antigen specificity one way to determine antibody binding sites at low antibody concentrations would be to visualise MAC deposition on the target. To investigate this we treated myelinating cultures with 100ng/ml 8-18C5 and stained for MAC formation 2 hours after antibody addition. At this low concentration we can only detect a small amount of antibody binding by immunochemistry but treatment ultimately results in complete demyelination [Figure 4.9]. Unfortunately at this low concentration we were unable to detect MAC formation as seen with higher antibody concentrations. This suggests that analysis of MAC deposition may not be a useful method of determining sites of antibody binding in samples with low antibody concentrations.

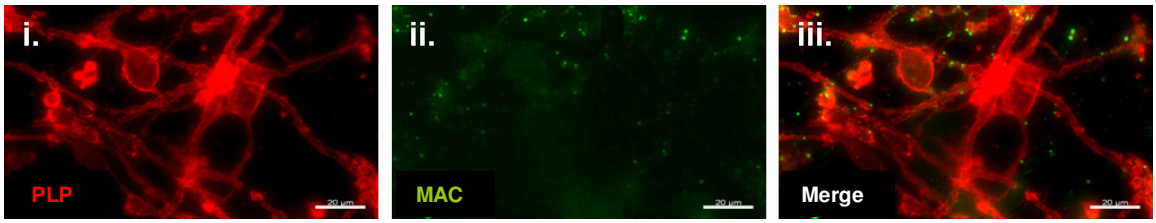


Figure 4.9: Detection of MAC formation is dependent on antibody concentration
In contrast, when myelinating cultures were treated with 100ng/ml 8-18C5 in the presence of complement, MAC (green) was not detected at the surface of oligodendrocytes or myelin (PLP; red) after 2 hours, although treatment ultimately leads to complete demyelination after 4 hours (63X magnification).

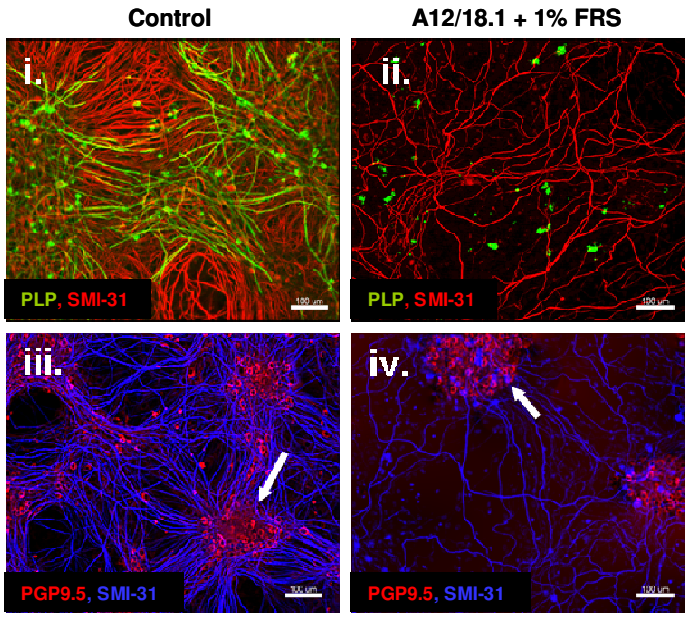
4.2.3 Nfasc: One molecule; two distinct pathologies?

4.2.3.1 Nfasc specific antibodies can mediate complement dependent demyelination and axonal injury

Recently a number of axoglial proteins have been implicated as potential MS autoantigens, one of which is Nfasc (Mathey et al, 2007). In this study the authors used the monoclonal antibody A12/18.1, which has a high affinity for the extracellular domain of Nfasc186 and Nfasc155, the target moiety is shared between the isoforms however the exact epitope is unknown. The authors also demonstrated the ability of this mAb to fix complement making it an excellent model Nfasc specific antibody to use in this proof of concept experiment. To draw parallels between the MOG specific and Nfasc specific antibody responses we characterised the α-Nfasc response using a similar approach as described above for α-MOG responses.

Overnight treatment of myelinating cultures with A12/18.1 in the presence complement mediates complete demyelination and loss of mature MOG^+ oligodendrocytes. Antibody treatment also mediates a loss of both myelinated and demyelinated axons (~40% loss of total axons) accompanied by a loss of $PGP9.5^+$ neurons [Figure 4.10A]. Antibody mediated CNS injury was a complement dependent phenomenon as no pathogenic activity was detected in cultures treated with either antibody alone or after heat inactivation of the complement source. Demyelination was also antigen specific as the use of an isotype control antibody or 1% FRS in the absence of antibody was insufficient to induce injury [Figure 4.10B].

A



B

Condition	% Axonal loss	% Myelin loss	% OLG loss	% Neuronal loss
A12/18.1 (10µg/ml)	0 ± 0.1	5 ± 2.3	7 ± 4.6	2 ± 1.2
A12/18.1 (10µg/ml) + normal rat serum (1%)	**40 ± 3.2****	**100 ± 0.8****	**97 ± 4.6****	**44 ± 5.2****
A12/18.1 (10µg/ml) + heat inactive rat serum (1%)	2 ± 1.5	3 ± 1.8	6 ± 5.2	0 ± 0.8
normal rat serum (1%)	1.5 ± 0.5	1.8± 1.3	5 ± 3.7	2.9 ± 1.8

Figure 4.10: Nfasc; an axo-glial antigen providing a potential link between antibody mediated demyelination and axonal injury
A: Myelinating cultures 28 days *in vitro* consist of a dense network of axons, some of which are myelinated by oligodendrocytes **(i).** Overnight treatment of myelinating cultures with 10µg/ml A12/18.1 a pan-Nfasc specific monoclonal in the presence of 1% FRS as a source of complement induces complete demyelination accompanied by a significant loss of both myelinated and unmyelinated axons (SMI-31; red, MOG; green) **(ii).** Myelinating cultures 28 days *in vitro* have an abundant neuronal populations (PGP9.5; red), which extend axons (SMI-31; blue) **(iii).** Axonal loss induced by A12/18.1 treatment is accompanied by a loss of neurons (PGP9.5; red, SMI-31; blue) **(iv).** **B:** Pathology observed was both antibody mediated and complement dependant further confirmed by various control conditions such as heat inactivation of complement and addition of normal rat sera in the absence of antibody. Values shown are an average of three independent experiments performed in triplicate ± S.E.M (* $p<0.05$, ** $p<0.01$; T-test).

4.2.3.2 Time course of A12/18.1 mediated complement dependent injury

Similar to that described above for the MOG specific response; to determine whether demyelination mediated by α-Nfasc antibody was due to acute injury caused by the activation of the complement cascade it was important to determine the time scale in which antibody mediated complement dependent injury occurs. To this end myelinating cultures were treated with 10µg/ml of A12/18.1 in the presence of 1% FRS as a source of complement. Cultures were analysed at 30 minute intervals until 4 hours after antibody addition.

Using A12/18.1, a pan specific monoclonal antibody recognising both Nfasc155 and Nfasc186, induces a loss of both myelinated and unmyelinated axons accompanied with complete demyelination in a complement dependant manner. Demyelination precedes the axonal loss as there is a significant loss of myelin (~50%) at 3.5 hours after addition [Figure 4.11A] prior to a detectable significant reduction in axonal density [Figure 4.11B]. However after 4 hours both axonal injury and demyelination is complete.

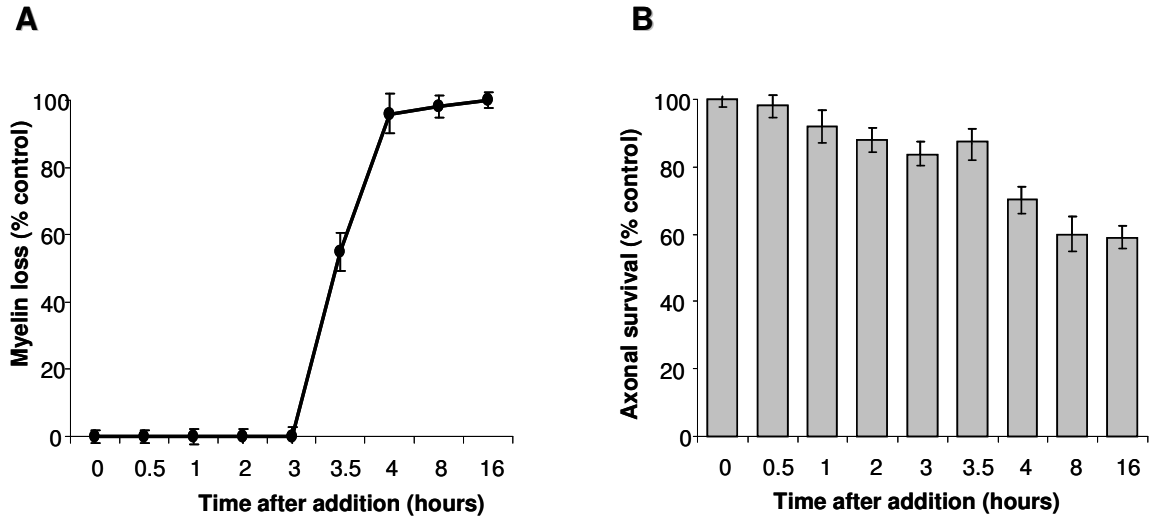


Figure 4.11: Time course of α-Nfasc mediated injury
A: Treatment of *in vitro* myelinating cultures with 10µg/ml A12/18.1 (a pan Nfasc specific monoclonal antibody) in the presence of 1% fresh rat sera induced complete loss of PLP^+ myelin sheathes within 4 hours of antibody addition. Values plotted are an average of three independent experiments performed in triplicate ± S.E.M. **B:** Demyelination was accompanied by a significant axonal loss as detected by a loss on $SMI\text{-}31^+$ immunoreactivity. This injury occurs in a similar time course as demyelination as significant decreases in both axonal density and myelination are detectable after 3.5hrs and are complete after 4 hours. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

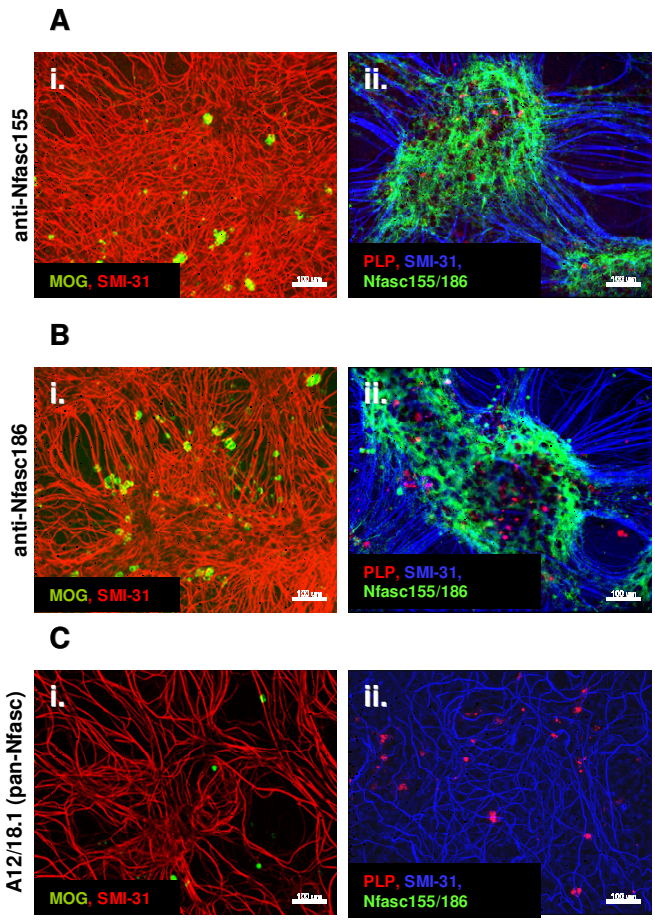
4.2.3.3 Targeting Nfasc reveals isoform specific effects on axons and glia

As treatment of myelinating cultures with an antibody recognising both Nfasc isoforms is capable of mediating a dual pathology effecting neurons/ axons and oligodendrocytes we can postulate that antibodies recognising Nfasc may be capable of mediating two distinct pathologies based on which isoform is targeted. In order to dissect the pathology seen using the pan specific monoclonal into is axonal/ neuronal and glial components we used two polyclonal antibodies one specific for the extracellular domain of Nfasc155 and the other specific for Nfasc186.

In our system Nfasc155 is expressed exclusively by the oligodendrocyte processes and at the paranodal domain. Treatment of myelinating cultures overnight with a Nfasc155 specific polyclonal antibody in the presence of complement were completely demyelinated with a significant reduction in the number of mature OLG^+ oligodendrocytes. Demyelination had no detrimental effect on axonal integrity. Nfasc186 immunochemistry is unchanged after demyelination with a Nfasc155 specific reagent when compared to controls [Figure 4.12A].

Using a Nfasc186 specific polyclonal antibody expression within our system can be detected on the axon at the axon initial segment (AIS) and at the Node of Ranvier. Targeting myelinating cultures with a Nfasc186 specific polyclonal antibody in the presence of complement mediates loss of only myelinated axons (~15%) with subsequent loss of myelin. Antibody treatment has a minor effect on the number of mature MOG^+ oligodendrocytes (~10%) but this was just statistically significant ($p=0.48$, T-Test). Neuronal Nfasc immunochemistry was present on the majority of neurons after axonal loss and subsequent myelin loss using a Nfasc186 specific reagent [Figure 4.12B].

In contrast immunocytochemistry using A12/18.1 also reveals that in addition to the staining at the node and the AIS, Nfasc 186 staining on the neuronal soma. Treatment with A12/18.1 mediates major injury and subsequent loss of neurons, axons and oligodendrocytes. This injury is associated with a total loss of Nfasc immunoreactivity [Figure 4.12C].



D

Condition	Binding *in vitro*	% Axonal loss (±SD)	% Myelin loss (±SD)	% OLG loss (±SD)
α-Nfasc155 pAb	Oligodendrocytes and myelin (paranode)	3 ± 0.6	96 ± 3.3**	80 ± 4.4**
α-Nfasc186 pAb	Node of Ranvier and AIS	15 ± 3.6*	97 ± 2.4**	9 ± 3.1*
A12/18.1 mAb	Node of Ranvier, AIS and neuronal soma	40 ± 3.2**	100 ± 0.8**	90 ± 3.5**

Figure 4.12: Targeting Nfasc reveals isoform specific effects on axons and glia.
A: Targeting myelinating cultures with a polyclonal antibody specific for Nfasc155; the glial isoform expressed at the oligodendrocyte cell surface and at the paranode. Treatment overnight of myelinating cultures (28 DIV) with 10µg/ml Nfasc155 specific antibody induces selective demyelination and loss of oligodendrocytes with no loss of axons (SMI-31; red, MOG; green) (10X magnification) **(i).** Nfasc186 reactivity is retained after α-Nfasc155 meditated demyelination (Nfasc; green, PLP; red; SMI-31; blue) (10X magnification) **(ii). B:** In contrast treatment using 10µg/ml Nfasc186 specific antibody mediated complement dependant loss of myelinated axons and subsequent myelin loss accompanied by a small decrease in the number of mature MOG^+ oligodendrocytes (SMI-31; red, MOG; green) (10X magnification) **(i).** Nfasc reactivity on the neuron is retained after treatment with Nfasc186 specific antibody (Nfasc; green, PLP; red; SMI-31; blue) (10X magnification) **(ii). C:** Targeting both Nfasc155 and Nfasc186 simultaneously induces complete demyelination accompanied by a significant loss of both myelinated and unmyelinated axons (SMI-31; red, MOG; green) (10X magnification) **(i).** This major loss of both axons and glia is associated with a complete loss of Nfasc (Nfasc155 and Nfasc186) immunoreactivity (Nfasc; green, PLP; red; SMI-31; blue) (10X magnification) **(ii). D:** Analysis of immunochemical data confirmed initial observations. Values are plotted as a percentage of equivalent control cultures. Antibody mediated effects were complement dependent phenomenon as the use of heat inactivated complement completely abolishes activity. Values shown are an average of three independent experiments performed in triplicate ± S.E.M (* $p<0.05$, ** $p<0.01$; T-test).

4.2.3.4 CNS injury by anti-Nfasc155 and anti-Nfasc186 antibodies is initiated by MAC deposition at the site of antibody binding

To determine where MAC is deposited after treatment with either a Nfasc155 or a Nfasc186 specific reagent cells were fixed and stained for MAC two hours after addition of 10 µg/ml α-Nfasc155 or α-Nfasc186 with 1% FRS as a source of complement.

Two hours after addition of α-Nfasc155 and 1% FRS, MAC formation was visible on the surface of PLP^+ oligodendrocytes and myelin sheathes [Figure 4.13A]. In cultures treated with Nfasc186 after two hours I could detect MAC deposition at the node of Ranvier [Figure 4.13B]. Unfortunately the isotype of the antibody used to visualise MAC (mouse IgG2a) is the same as A12/18.1 so could not be used in these experiments. As the Nfasc186 polyclonal was a gift (Prof P. Brophy, University of Edinburgh) I had only a small quantity and this unfortunately limited the number experiments I could perform.

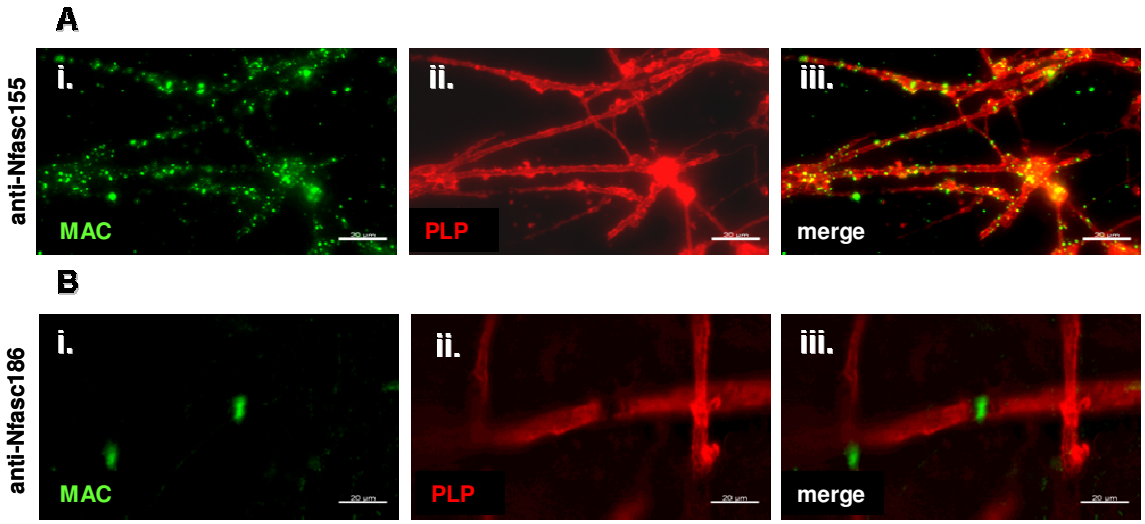


Figure 4.13: α-Nfasc 155/α-Nfasc186 antibody mediated complement dependent CNS injury occurs via MAC deposition on the surface of oligodendrocytes/and at the node of Ranvier respectively.
A: Myelinating cultures were treated with 10µg/ml Nfasc155 in the presence of complement. Two hours after antibody addition MAC (poly-C9) (green) (i) can be detected on the surface of PLP^+ oligodendrocytes and myelin (red) (ii-iii). **B:** Myelinating cultures were treated with 10µg/ml Nfasc186 in the presence of complement. Two hours after antibody addition MAC (poly-C9) (green) (i) can be detected between PLP^+ myelin internodes (red) at the node of Ranvier (ii-iii) (63X magnification).

4.2.3.5 Time course of anti-Nfasc mediated complement dependent injury

Similar to that described above for the α-MOG response; to determine whether demyelination mediated by α-Nfasc antibody was due to acute injury caused by the activation of the complement cascade it was important to determine the time scale in which antibody mediated complement dependent injury occurs. To this end myelinating cultures were treated with 10µg/ml of α-Nfasc155 polyclonal antibody in the presence of 1% FRS as a source of complement. Cultures were analysed at 30 minute intervals until 4 hours after antibody addition.

Nfasc155 mediated injury occurs rapidly with loss of PLP^+ myelin sheathes initially detectable after 3.5 hours [Figure 4.14A]. Demyelination is complete within 4 hours after antibody addition. Nfasc155 mediated demyelination is has no pathogenic effect on axonal survival [Figure 4.14B].

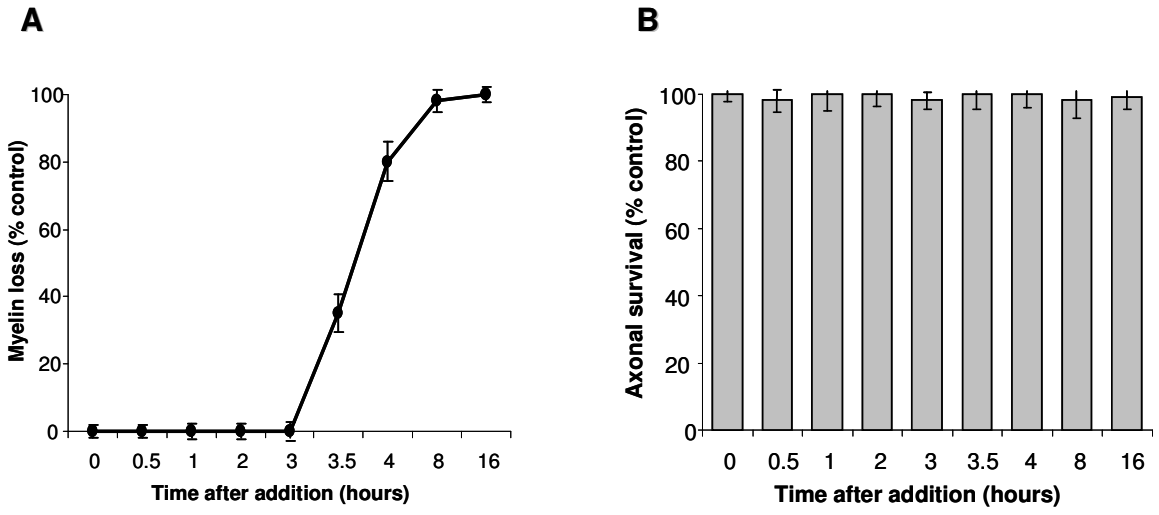


Figure 4.14: Time course of α-Nfasc 155 mediated demyelination
A: Targeting myelinating cultures with 10µg/ml of an α-Nfasc155 polyclonal antibody induces complement mediated demyelination. Loss of PLP^+ myelin sheathes is initially detectable 3.5 hours after antibody addition and demyelination is complete within 4 hours. Values plotted are an average of three independent experiments performed in triplicate ± S.E.M. **B:** Nfasc155 mediated demyelination has no effect on axon integrity as detectable by changes SMI-31 immunoreactivity. Axonal density remains consistent throughout the treatment period. Values plotted are an average of three independent experiments performed in triplicate ± S.E.M.

4.2.3.6 Nfasc specific antibody responses are detectable at ng/ml antibody concentrations

To determine whether our myelination culture system would be a feasible screening strategy for detecting pathogenic antibody responses we set up a dose response to determine the sensitivity of the system. Nfasc specific antibodies were diluted in series from 10μg/ml to 10ng/ml and added to myelinating cultures in the presence of complement.

Using an α-Nfasc155 specific polyclonal antibody we can detect pathogenic antibody responses at antibody concentrations ≥100ng/ml. A small amount of demyelination can be detected in cultures treated with 50ng/ml however this was just statistically significant (p=0.48, T-test) [Figure 4.15A]. Axonal density as measured by SMI-31 immunoreactivity was unaltered and equivalent across treatments when compared to controls [Figure 4.15B].

Using A12/18.1, a pan-specific monoclonal, we can detect demyelinating antibody responses down to antibody concentrations ≥50ng/ml [Figure 4.15C]. However the axo-pathic component could only be detected at antibody concentrations ≥100ng/ml [Figure 4.15D].

These results are similar as that described for the α-MOG response i.e. at concentration ranges below 1μg/ml it is not possible to visualise antibody binding via immunocytochemistry however pathogenic activity can be detected at much lower antibody concentrations.

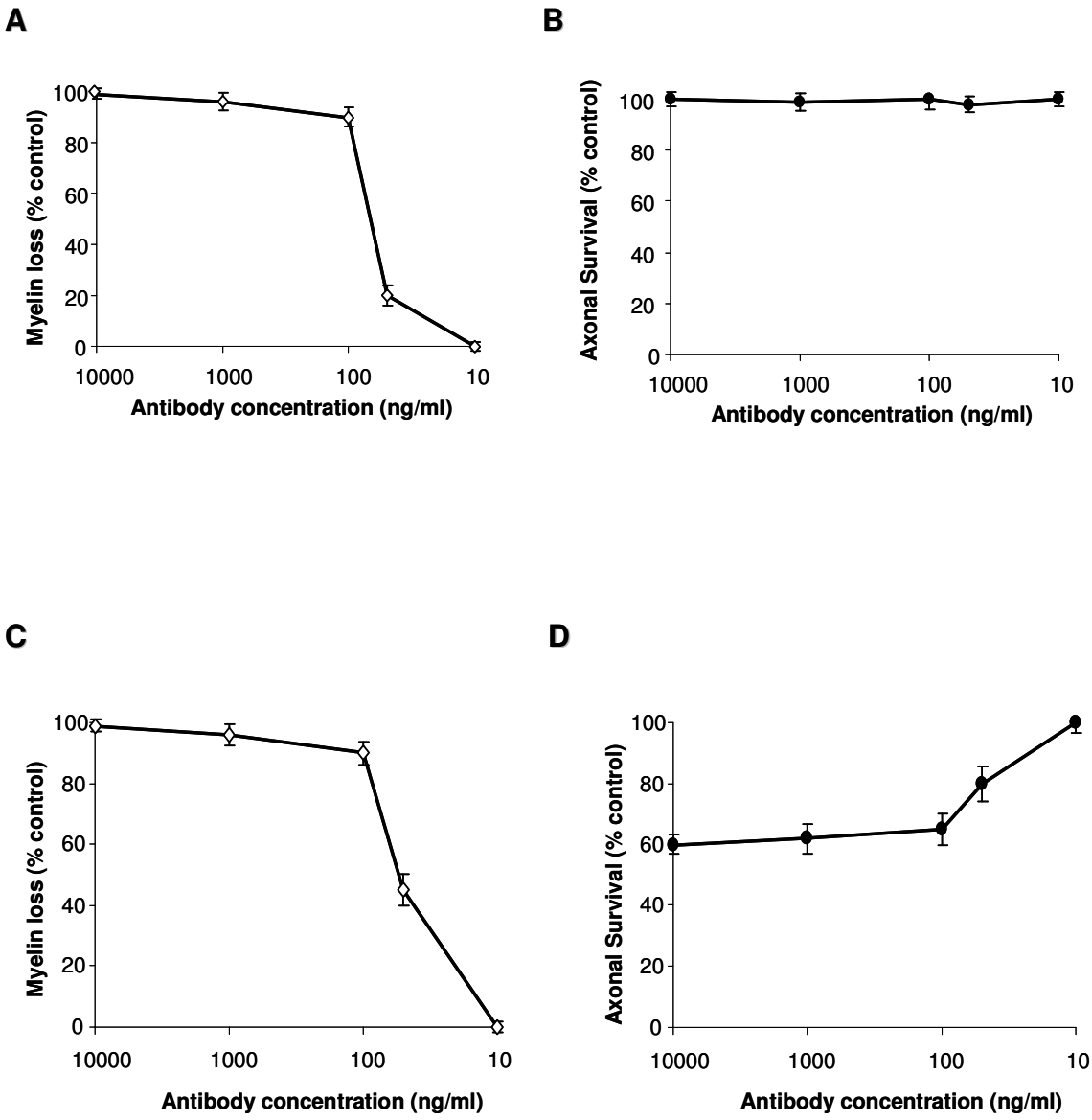


Figure 4.15: Myelinating cultures provide a highly sensitive screening strategy in which to detect pathogenic α-Nfasc antibody responses

A: Dose response studies show that α-Nfasc155 mediated demyelination is detectable to antibody concentrations >50ng/ml. Complete demyelination was observed at antibody concentrations >100ng/ml. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **B:** Axonal density was unaltered and equivalent across treatments when compared to controls. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **C:** Dose response studies show that A12/18.1 mediated demyelination is detectable to antibody concentrations >50ng/ml. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **D:** A12/18.1 mediated complement dependent axonal injury is detectable at antibody concentrations >100ng/ml. Axonal loss detected at cultures treated with 50ng/ml A12/18.1 and below were not statistically significant ($p>0.05$, T-test). Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

4.2.4 Complement mediated antibody driven CNS injury occurs by Fc activation of the classical pathway

To determine whether antibody mediated complement dependent CNS injury is through activation of the classical complement cascade via the Fc region I generated Fab fragments. Fab fragments were produced by papain digestion of whole IgG (Z2 and A12/18.1) followed by protein A chromatography to separate Fabs from Fc domain. Complete digestion and Fab purity was confirmed by SDS-PAGE [Figure 4.16A]. Fabs purified from Z2 still retained there binding capacity as detected by immunocytochemical staining on both myelin and oligodendrocytes [Figure 4.16B]. Similarly Fab purified from A12/18.1 retained binding as seen with the whole antibody with binding at the node of Ranvier, neuronal soma and oligodendrocyte [Figure 4.16C]. No binding was detected in any of the purified Fc region preparations.

When myelinating cultures are treated with 3.3µg/ml Z2 Fab fragments in the presence of complement no demyelination is detected. Similarly treatment with purified Z2 Fc region had no effect on myelin or mature oligodendrocytes [Figure 4.16D].

In the case of A12/18.1, which can mediate complement dependent axonal injury and demyelination, treatment of myelinating cultures with 3.3µg/ml A12/18.1 Fab does not induce axonal loss or demyelination. Similarly treatment with purified A12/18.1 Fc domain mediated no detectable to axons, neurons or glia [Figure 4.16E].

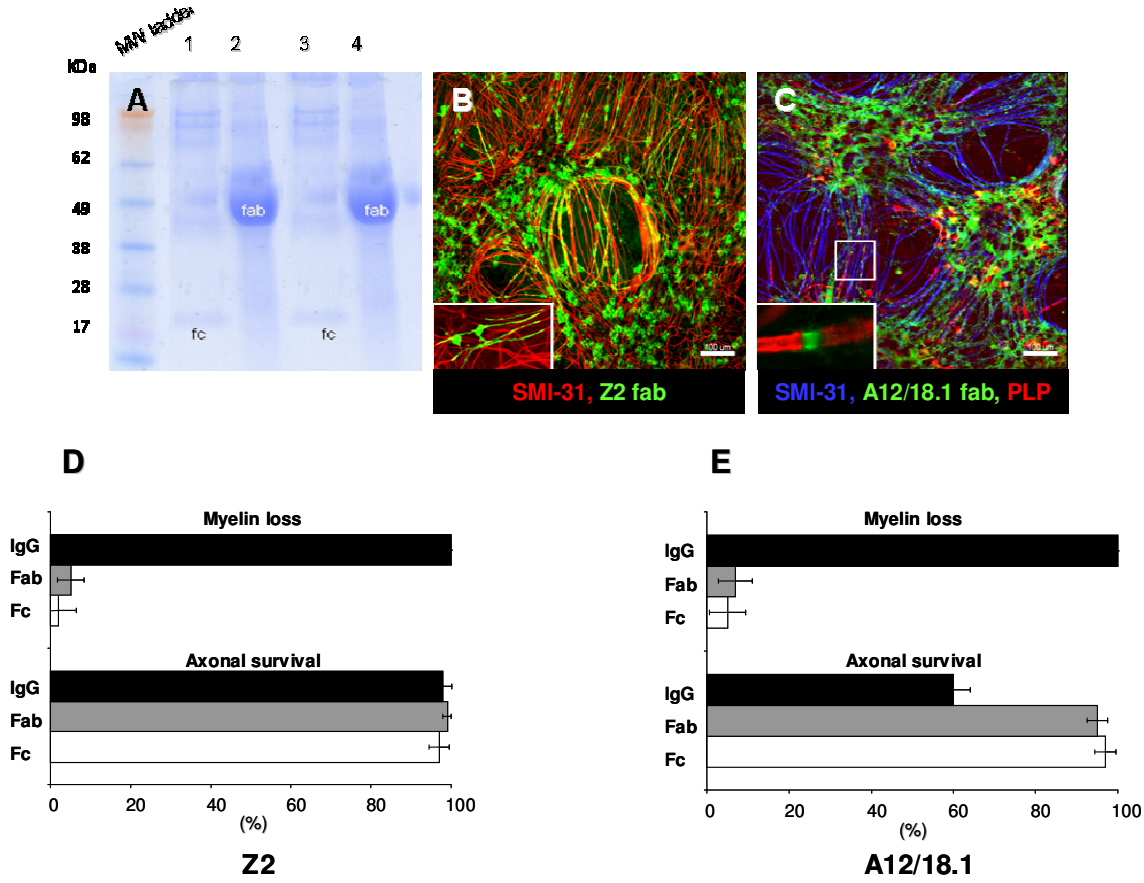


Figure 4.16: Complement mediated antibody driven CNS injury is Fc dependent
A: SDS-PAGE analysis of IgG after papain digestion and protein A chromatography showing clear separation of Fab fragments from the Fc region. Lanes: 1: Z2 protein A column eluate containing Fc regions and undigested IgG, 2: Z2 protein A flow through containing Fab fragments and Ig light chain, 3: A12/18.1 protein A column eluate containing Fc regions and undigested IgG, 4: A12/18.1 protein A flow through containing Fab fragments and light chain. **B:** Purified Z2 Fab fragments retain their binding activity. Z2 Fab fragments bind to MOG on the surface of the myelin sheath and to oligodendrocytes (inset) (SMI-31; red, Z2 Fab; green) (10X magnification). **C:** Purified A12/18.1 Fab fragments retain their binding activity. A12/18.1 Fab fragments bind to myelinating cultures on neurons, oligodendrocyte and by the axon at the AIS and the node of Ranvier (SMI-31; blue, PLP; red, A12/18.1 Fab; green) (10X magnification). **D:** Overnight treatment of myelinating cultures with 10µg/ml Z2 (whole IgG) in the presence of 1% FRS as a source of complement mediates complete demyelination (black bar). However addition of an equivalent concentration of either purified Z2 Fab fragments (grey bar) or Fc domain (white bar) in the presence of 1% FRS as a source of complement was insufficient to mediate demyelination. Values shown are an average of three independent experiments performed in triplicate ± S.E.M. **E:** Overnight treatment of myelinating cultures with 10µg/ml A12/18.1 (whole IgG) in the presence of 1% FRS as a source of complement mediates complete demyelination and axonal injury (black bars). However addition of an equivalent concentration of either purified Z2 Fab fragments (grey bars) or Fc domain white bars) in the presence of 1% FRS as a source of complement was insufficient to mediate demyelination or axonal injury. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

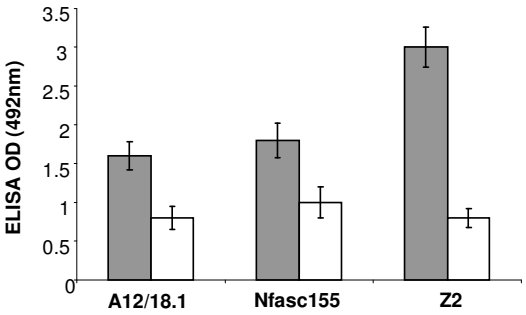
4.2.5 Adsorption of IgG using purified compact myelin diminishes its pathogenic potential

Using the myelinating culture model we have identified a number of pathogenic antibody responses that can induce complement mediated demyelination which in some cases may be accompanied by significant axonal injury. To determine whether this effect is truly antigen specific I tried to remove the pathogenic antibody component using purified rat myelin. Whilst one alternative to this approach would be to use recombinant antigens, in the case of Nfasc155 this would be extremely expensive and Nfasc186 is commercially unavailable. A recombinant antigen may lack the correct three dimensional structure or post-translational modifications.

Incubation with 1µg/ml IgG (A12/18.1, α-Nfasc155, Z2) with 2mg/ml total myelin protein greatly reduced binding to rat myelin as detectable by ELISA when compared to antibody binding prior to adsorption [Figure 4.17A]. This diminished binding was accompanied in a reduction in demyelinating activity when 1µg/ml residual unadsorbed antibody was added to myelinating cultures in the presence of complement. In the case of A12/18.1 where demyelination is accompanied by a significant axonal loss, this axopathic activity was also significantly reduced [Figure 4.17B].

This technique is extremely useful when characterising patient samples with unknown specificities. Myelin adsorption may provide confirmation of antibody specificity especially in cases where antibody binding may not be visible using fluorescence microscopy. Our model also allows us to determine the effect of myelin adsorption on the pathogenic activity and therefore we can determine whether antibodies generated against myelin *are* pathogenic.

A



B

	IgG Pre-adsorption	
	Axonal loss (± SD)	**Myelin loss (± SD)**
A12/18.1	40 ± 3.2**	100 ± 0.8**
α-Nfasc155	3 ± 0.6	96 ± 3.3**
Z2	2 ± 2.2	100 ± 0.9**
	IgG Post-adsorption	
	Axonal loss (± SD)	**Myelin loss (± SD)**
A12/18.1	28 ± 4.9**	46 ± 6.6**
α-Nfasc155	4 ± 1.2	51 ± 4.4**
Z2	3 ± 1.6	39 ± 5.7**

Figure 4.17: Adsorption of IgG using purified compact myelin diminishes its pathogenic potential

A: : Adsorption of 1µg/ml IgG (Z2, α-Nfasc155, A12/18.1) with 2mg/ml rat myelin resulted in reduced binding to rat myelin as detected by ELISA. Data plotted as mean OD (± S.E.M). All reductions in IgG binding were statically significant ($p<0.05$, T-test). **B:** Myelin adsorption of IgG resulted in a marked reduction in the pathogenic activity when compared to samples prior to adsorption. Myelin adsorption lowered the amount of complement dependent demyelination detected in myelinating cultures in comparison to unabsorbed IgG. In the one case of A12/18.1 where axonal injury was also detected this was also significantly reduced after myelin adsorption. Values shown are an average of three independent experiments performed in triplicate ± S.E.M.

4.3 Discussion

In this chapter it has been demonstrated that *in vitro* myelinating cultures can be used as a model of antibody mediated injury, which is:

- Antigen specific and dependent on antigen accessibility *in vitro.*

- Complement dependent through activation by Fc region and subsequent MAC deposition at sites of antibody binding.

This model of antibody mediated injury is extremely effective. The results seen are usually "all or nothing" i.e. antibody treatment mediates either complete demyelination or none. This makes the data easy to interpret but provides no information on the variation or reproducibility of detecting antibody mediated injury using this model. However using data obtained from the Z2 dose response curve, addition of 100ng/ml mediates an almost complete loss of myelin there is very little inter and intra variation (coefficient of variance= 6.6%). In contrast using 50ng/ml Z2, which mediates ~50% demyelination the variability is much greater (32.2%). Antibody mediated axonal injury is much less variable. Using the A12/18.1 dose response data, where antibody treatment mediates significant axonal injury the mean coefficient of variance from three independent experiments is much lower (5.1% (100ng/ml); 3.1 % (50ng/ml)) [Table 4.3]. From this we can conclude that antibody mediated injury in this system is fairly reproducible with respect to both inter and intra variability and therefore provides a relatively robust assay for antibody screening.

Table 4.3: Variability of antibody mediated injury.
Values were taken from a series of experiments performed three times. Values shown are an average from three coverslips ± standard deviation. Coefficient of variance was calculated as standard deviation/ mean and expressed as a percentage.

A

50ng/ml Z2			
	Exp 1	**Exp 2**	**Exp 3**
Myelin loss (% control)	56.7 ± 26.1	50.5 ± 14.0	42.4 ± 9.7
Coefficient of variance (%)	46.1	27.6	22.9

B

10µg/ml A12/18.1			
	Exp 1	**Exp 2**	**Exp 3**
Axonal survival (% control)	62.6 ± 3.0	54.1 ± 3.8	59.8 ± 1.6
Coefficient of variance (%)	4.8	6.2	2.7

A major advantage of this system over other antibody detection strategies is that it is extremely sensitive and can detect pathogenic antibody activity at antibody concentration in the ng range. Pathogenic activity can be detected using this system at antibody levels much lower than the detection thresholds of immunofluorescence. For our model to be used as a valid screening strategy to detect pathogenic autoantibody responses in clinical samples it must be sensitive enough to detect low antibody titres. In has been reported in the literature that patients with myasthenia gravis have α-AChR antibody titres ranging from 10-20nM (Lindstrom et al., 1976). In our model we can detect pathogenic antibody responses down to picomolar levels; this is extremely promising support for the viability of our model.

Antibody mediated injury is highly specific and injury is confined to only cells expressing the antigen. For example treatment using Z2 or α-Nfasc155 induces complement mediated demyelination and a significant loss of mature oligodendrocytes with a notable sparing of axons. However neurofilament staining is not the most sensitive technique to monitor subtle changes in axonal physiology. Minor axonal changes in response to antibody treatment may also be

transient. Further investigation is required to determine the effect of antibody mediated demyelination on the axon.

It has been documented that rat serum can activate complement cascade and can mediate lysis of oligodendrocytes in culture in the absence of antibody (Scolding et al., 1989; Wren and Noble, 1989). Rat oligodendrocytes lack expression of CD59, a complement inhibitor that blocks MAC formation (Wing et al., 1992; Piddlesden and Morgan, 1993). However in our *in vitro* system addition of fresh rat serum in the absence of antibody has no pathogenic effect on oligodendrocytes or OPCs. One explanation is that within the myelinating cultures there are sufficient levels of endogenous complement inhibitors preventing this autolytic effect. Astrocytes and neurons are known sources of complement inhibitors (Rogers et al., 1996; Gasque et al., 2000) and are present in myelinating cultures in abundance. Co-culturing oligodendrocytes in close proximity to large numbers of neurons and astrocytes is sufficient to protect them from auto-lysis by complement.

It has been reported that in the presence of complement activated microglia can mediate oligodendrocyte cell death and uptake of debris, which is antibody independent (Zajicek et al., 1992). In cultures treated with either rat serum alone or in the presence of an isotype/CNS irrelevant antibody there was no detectable injury to oligodendrocytes. This could be due to the activation state of microglia within the cultures. The majority of microglia in untreated cultures are deemed “inactive” as they lack ED1/CD68 staining (Graeber et al., 1990).

The pathology observed using our model is antibody driven and complement mediated phenomenon. Therefore one can postulate that in the presence of low concentrations of fresh rat serum there is a must be a delicate balance between complement activation and inhibition and addition of a CNS specific antibody is sufficient to overcome this endogenous complement inhibition.

Nfasc186 localisation within our myelinating cultures is also inconsistent with what is reported *in vivo*. Using A12/18.1, a pan-Nfasc specific monoclonal antibody, which binds to the extracellular domain of Nfasc155 and Nfasc186, reactivity is detected on the axon at the node of Ranvier and the AIS. In addition the antibody also labels Nfasc expressed on the surface of a subset of

neurons *in vitro*. This neuronal staining was not detected using a Nfasc186 specific polyclonal antibody where Nfasc186 immunoreactivity was seen only at the AIS and the node of Ranvier. Titration of these antibodies suggests that this neuronal Nfasc reactivity is not a staining artefact. As myelinating cultures are a developmental system it is possible that this neuronal Nfasc expression is due to the recognition of an alternative Nfasc isoform which is not expressed in the adult CNS. There are reports in the literature to support this and suggest that during development CNS neurons express an alternative intermediate isoform of Nfasc within the cell body prior to myelination (Burkarth et al., 2007). This was identified as Nfasc166, a lower molecular weight isoform that lacks the fifth fibronectin-like region and precedes neuronal Nfasc186 expression (Pruss et al., 2006). Nfasc186 is not expressed by adult neurons apart from at the AIS and node of Ranvier however Nfasc186 is readily available for antibody binding at these sites (Mathey et al., 2007).

There is also a discrepancy between Nfasc155 expression *in vitro* and reported expression *in vivo*. Nfasc155 in our system is expressed on the surface of the oligodendrocyte at the cell body and along the cell process with staining at the paranodal domain. *In vivo* neurofascin expression by oligodendrocytes is transient and once axonal contact is made gene expression falls rapidly (Collinson et al., 1998). Once myelination is complete expression Nfasc155 is sequestered to the paranodal loops (Tait et al., 2000; Charles et al., 2002) and is inaccessible to antibody binding. Lack of binding of our Nfasc155 specific antibody to fully myelinated *in vivo* structures was demonstrated using the OSC system described in the previous chapter [Figure 4.18]. However that is not to say that Nfasc155 specific antibody responses are clinically irrelevant as Nfasc155 may be accessible in damaged myelin and therefore may exacerbate demyelination. Nfasc155 may also become accessible to antibody binding during remyelination and therefore could disrupt lesion repair.

Despite these caveats; *in vitro* myelinating cultures provide an unbiased, extremely sensitive and statically reproducible model of antibody mediated CNS injury. This model can be used as a tool to detect pathogenic autoantibody responses in patient samples of undefined specificity. This will be demonstrated in the next chapter.

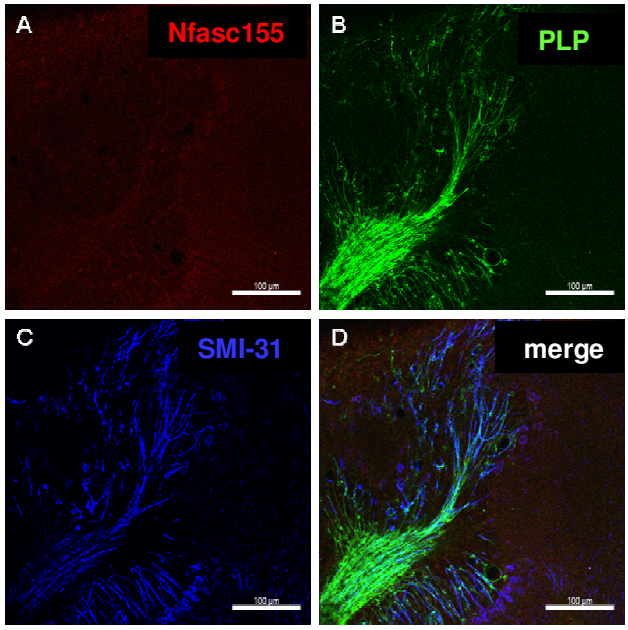


Figure 4.18: Nfasc155 is sequestered to the paranode in intact myelinated tracts *in vivo* and is inaccessible for antibody binding
Nfasc155 (red) is not accessible to antibody binding in intact myelinated fibres *in vivo* (PLP; green, neurofilament; blue) (20X magnification). OSCs were created and images taken whilst visiting the lab of Prof. N Goebels in collaboration with Dr. M Harrer (Zurich).

5 Identification of pathogenic antibody responses in Multiple Sclerosis

5.1 Introduction

Previously we demonstrated that the myelinating cultures can provide an *in vitro* system which reproduces the molecular and structural organisation of the CNS *in vivo* and have comprehensively demonstrated that this model provides a bioassay which can be used to quantify complement dependent antibody mediated injury. The previous chapters were necessary prerequisites building up to the main goal of this thesis, to determine whether or not MS is associated with a pathogenic autoantibody response.

We are certainly not the first to attempt to address this question using a tissue culture based approach. Studies describing the *in vitro* pathogenic properties of MS sera date back to the 1960s (Bornstein and Appel, 1965). These early studies indicated that a significant number of MS cases were associated with a serological factor capable of mediating demyelination *in vitro* (reviewed in Seil, 1977 and Caspary, 1977). However whether this factor was an anti-myelin autoantibody response was controversial. The use of *in vitro* myelinating cultures to model demyelination was largely abandoned from the late-1980s. Our understanding of EAE pathogenesis lead to the long held assumption that MS was likely to be purely a T cell mediated disease, however the pathogenesis of MS has been revealed to be far more complex. Circumstantial evidence has steadily accumulated implicating a role for autoantibodies. Within the past 5 years there have been reports supporting a strong association between α-MOG antibodies and paediatric MS/ADEM (McLaughlin et al., 2009, Di Pauli et al., 2010). However despite immense effort, current opinion as to whether adult onset MS is associated pathogenic autoantibody responses remains divided. This scepticism mainly stems from the lack of formal evidence that autoantibody associated with adult-onset MS actually pathogenic.

The main aim of this chapter is to address this question and use the myelinating cultures as a bioassay to:

- Determine whether the total IgG fraction purified from adult MS patient serum can mediate complement dependent demyelination and/or axonal injury *in vitro*.

- Investigate the potential specificity of any pathogenic responses with particular focus on Nfasc.

5.2 Results

5.2.1 Detection of pathogenic antibody responses from patients with MS

To determine whether the "*in vitro* demyelinating factor" previously reported in MS sera was due to an anti-myelin autoantibody response, total IgG was purified from MS patients (n=20), patients with other neurological diseases (OND; n=10) and healthy control donors (HC; n=13) by protein G chromatography.

5.2.1.1 MS patient derived IgG mediates complement dependent demyelination and axonal injury

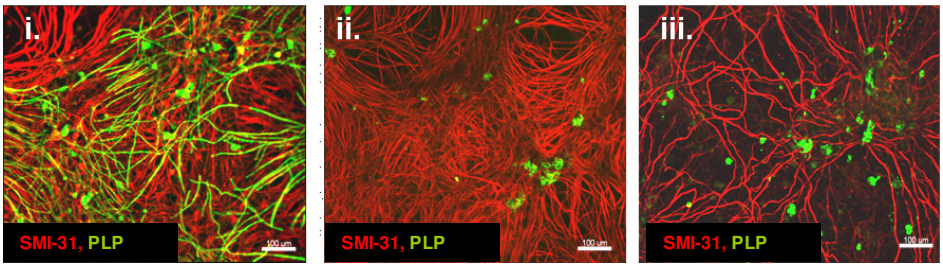
Treating myelinating cultures with total IgG preparations from individual donors revealed a striking heterogeneity in their ability to mediate axonal loss and/or demyelination [Figure 5.1].

At 100 µg/ml IgG preparations from 50% of MS cases mediated complete demyelination reproducing the effects observed by targeting an oligodendrocyte/myelin specific antigen such as MOG or Nfasc155 [Figure 5.1A (ii)]. It must be noted that in two cases (MS5 and MS14) demyelination was also accompanied by varying degrees of axonal loss ranging from 22% to 34% of the total number of axons. This pathology is similar to that observed after treatment with a pan-Nfasc specific monoclonal (A12/18.1) [Figure 5.1A (iii)]. Within the MS group there is a trend suggesting that the pathogenic antibody responses observed are associated more with MS patients suffering for non-steroid responsive relapses requiring plasmapheresis in comparison to patients with more the classical form of RRMS or PPMS ($P<0.05$, Fisher's exact test) [Figure 5.1B]. These pathogenic effects were disease specific as no pathogenic effects were detected within the OND group or from samples taken from healthy controls [Figure 5.1C]. However the remaining 10 MS samples tested did not mediate a pathogenic effect or either axons or glia, however it is possible that not all antibodies are pathogenic as pathogenicity in this assay is dictated by several factors such as antigen localisation or the relative ability of the antibody to fix rat complement. To address the possibility that the concentration of

pathogenic antibodies was below the bioassays detection threshold in some donors, the assay was repeated at an IgG concentration of 1 mg/ml. This failed to identify any additional donors with a pathogenic (axopathic/demyelinating) serum IgG response [Table 5.1].

In all cases control experiments demonstrated that these effects were an antibody-mediated complement-dependent phenomenon. Heat activation of the sera used as a source of complement abolished all antibody mediated effects and no loss of myelin or axons was observed after heat inactivation of the serum used as the source of complement.

A



B

		+ active complement		- inactivated complement	
Patient ID	Diagnosis	% Axonal loss	% Myelin loss	% Axonal loss	% Myelin loss
MS1	Marburg MS	2 ± 2.9	**95 ± 3.5****	0 ± 2.4	5.2 ± 5.5
MS2	RRMS	3 ± 2.6	**96 ± 4.0****	2 ± 3.4	4.9 ± 5.0
MS3	RRMS	4 ± 3.0	**97 ± 2.2****	2 ± 1.9	3.2 ± 2.7
MS4	RRMS	2 ± 2.1	**98 ± 1.3****	4 ± 2.9	0 ± 3.3
MS5	SPMS	**34 ± 5.7****	**92 ± 2.2****	1 ± 3.5	2 ± 2.4
MS6	RRMS	2 ± 3.0	0 ± 0.5	0 ± 3.7	0 ± 1.0
MS7	RRMS	3 ± 3.7	**94 ± 2.0****	3 ± 3.7	4.9 ± 2.4
MS8	RRMS	0 ± 2.2	0 ± 4.6	0 ± 2.4	0 ± 2.6
MS9	RRMS	0 ± 2.0	0 ± 3.7	0 ± 2.8	0 ± 1.9
MS10	RRMS	0 ± 2.3	0 ± 4.0	0 ± 2.9	0 ± 4.0
MS11	RRMS	0 ± 2.1	**95 ± 2.0****	0 ± 3.4	0 ± 2.0
MS12	RRMS	0 ± 2.5	**93 ± 1.7****	0 ± 4.8	0 ± 1.7
MS13	RRMS	3 ± 2.4	1.5 ± 4.1	3 ± 2.6	0 ± 1.1
MS14	RRMS	**22 ± 3.5****	**96 ± 3.2****	2 ± 3.5	0 ± 3.2
MS15	RRMS	0 ± 2.1	3 ± 4.2	1 ± 2.3	3.4 ± 2.1
MS16	RRMS	0 ± 2.2	**94 ± 2.9****	1.6 ± 1.2	4.0 ± 1.9
MS17	PPMS	0 ± 2.9	1.6 ± 4.0	1.4 ± 2.8	3.6 ± 1.4
MS18	PPMS	0 ± 2.3	0 ± 4.5	2.5 ± 2.7	0 ± 4.5
MS19	PPMS	0 ± 2.0	1.2 ± 4.3	4 ± 2.0	1.4 ± 4.5
MS20	PPMS	1 ± 0.6	4.6 ± 4.0	1.9 ± 2.6	3.2 ± 4.6
OND1	SPN	1.5 ± 2.5	2.4 ± 1.5	0 ± 3.5	0 ± 3.6
OND2	GBS	0.8 ± 1.4	0 ± 3.4	0 ± 3.3	0 ± 4.2
OND3	GBS	0 ± 4.1	3.3 ± 1.2	0 ± 3.2	0 ± 2.2
OND4	GBS	1.5 ± 1.6	0 ± 2.2	0 ± 2.5	0 ± 2.5
OND5	CIDP	2.5 ± 1.5	0 ± 6.0	0 ± 2.8	0 ± 2.6
OND6	CIDP	0 ± 2.4	0 ± 1.3	0 ± 3.9	0 ± 2.9
OND7	CIDP	0 ± 3.0	0 ± 2.4	0 ± 2.0	0 ± 2.8
OND8	ISAN	1.1 ± 2.3	0 ± 4.0	0 ± 3.3	0 ± 4.4
OND9	MG	1.6 ± 3.7	2 ± 5.2	0 ± 4.8	0 ± 2.6
OND10	CIDP	0 ± 5.1	0 ± 3.2	0 ± 2.2	0 ± 3.8
HC1	--	0.2 ± 2.5	1.3 ± 6.7	2.2 ± 2.5	2.3 ± 5.4
HC2	--	1.2 ± 2.4	1.8 ± 5.2	3.4 ± 2.4	2.3 ± 3.1
HC3	--	0.5 ± 2.6	1.1 ± 5.0	1.5 ± 2.6	3.1 ± 2.0
HC4	--	0 ± 3.1	1.2 ± 4.3	1.3 ± 2.1	3.2 ± 3.2
HC5	--	0.5 ± 4.4	1.8 ± 6.9	2 ± 2.6	2.8 ± 4.4
HC6	--	0.5 ± 5.9	1 ± 3.8	2.2 ± 3.9	3 ± 3.3
HC7	--	0 ± 2.2	0 ± 4.1	0 ± 3.1	0 ± 2.1
HC8	--	0 ± 2.1	0.6 ± 4.4	0 ± 3.0	0 ± 3.4
HC9	--	0.3 ± 3.0	0.7 ± 4.3	1.3 ± 1.0	0 ± 5.3
HC10	--	0.4 ± 2.9	0.4 ± 3.8	0 ± 2.6	0 ± 4.1
HC11	--	0 ± 2.7	0 ± 4.2	0 ± 1.8	0 ± 3.5
HC12	--	0 ± 2.6	1.1 ± 4.1	0 ± 3.2	0 ± 4.2
HC13	--	0.6 ± 4.0	0 ± 5.1	0 ± 3.0	0 ± 1.5

Figure 5.1: MS patient derived IgG mediates complement dependent demyelination and axonal injury at 100µg/ml.
A: A representative image of a myelinating culture after an overnight treatment with 100µg/ml IgG purified from patient MS6 **(i),** MS1 **(ii)** or MS5 **(iii)**; resulting in a striking heterogeneity between patients [SMI-31: red, PLP: green, 10X magnification]. **B:** Summary of pathogenic potential of the IgG fraction from the 20 MS patient samples, 10 OND samples and 13 healthy controls. Half of the MS samples tested mediated complete demyelination. In two patients this was also accompanied by a loss of axons ranging from 22% to a loss of 34% of the total number of axons. No significant axopathic or demyelinating activity was detected in OND or healthy controls. Antibody mediated effects were complement dependent phenomenon as heat inactivation of the FRS abolished all pathogenic activity. Values shown are from one representative data set performed three times in triplicate ± standard deviation (*p<0.05, **p<0.01; T-test).

Table 5.1: 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).

A

Patient ID	% Axonal loss	% Myelin loss
MS1	1 ± 1.9	**100 ± 4.5****
MS2	4 ± 2.9	**100 ± 3.0****
MS3	0 ± 1.0	**100 ± 2.4****
MS4	0 ± 2.8	**100 ± 2.2****
MS5	**30 ± 6.4****	**100 ± 1.0****
MS6	2 ± 3.0	0 ± 1.2
MS7	3 ± 3.7	**100 ± 1.0****
MS8	0 ± 2.2	0 ± 3.9
MS9	0 ± 2.0	0 ± 2.6
MS10	0 ± 2.3	2 ± 4.4
MS11	0 ± 2.1	**100 ± 1.5****
MS12	0 ± 2.5	**100 ± 1.3****
MS13	3 ± 2.4	3 ± 2.1
MS14	**18 ± 1.3***	**100 ± 1.6****
MS15	0 ± 2.1	3 ± 2.8
MS16	0 ± 2.2	**100 ± 1.2****
MS17	0 ± 2.9	2.6 ± 2.2
MS18	1 ± 2.3	1 ± 4.0
MS19	2 ± 1.8	2 ± 3.3
MS20	2 ± 1.6	5 ± 3.1

B

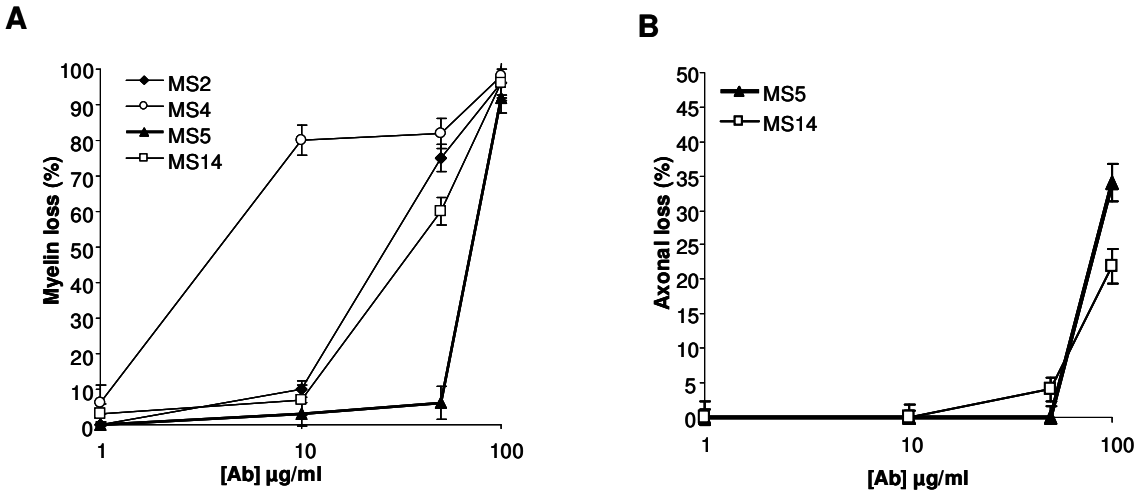
Patient ID	% Axonal loss	% Myelin loss
OND1	0 ± 1.6	1.5 ± 2.3
OND2	2 ± 1.8	1 ± 1.2
OND3	1.2 ± 2.6	1 ± 1.8
OND4	0 ± 1.0	0 ± 1.6
OND5	0 ± 2.2	0 ± 2.4
OND6	1 ± 2.5	0 ± 3.2
OND7	4.4 ± 1.2	0 ± 1.0
OND8	1 ± 1.4	2 ± 1.2
OND9	0 ± 2.2	0 ± 1.2
OND10	0 ± 1.9	0 ± 1.2

5.2.1.2 Dose response studies using patient IgG

To determine the sensitivity of our bioassay dose response studies were performed using IgG from four patients; two with a purely demyelinating response at 100µg/ml (MS2 and MS4) and two with demyelination accompanied by some degree of axonal injury (MS5 and MS14). IgG was added to myelinating cultures (28 DIV) at 100µg/ml (667pM), 50µg/ml (333pM), 10µg/ml (67pM) and 1µg/ml (6.7pM) with 1% FRS as a source of complement [Figure 5.2].

For MS2 significant demyelination was detected at IgG concentrations ≥50µg/ml. In contrast pathogenic responses using IgG purified from patient MS4 were seen at concentrations as low as 10µg/ml. In sample MS5 where demyelination was accompanied by axonal loss, reducing the IgG concentration to 50µg/ml resulted in a complete loss of all pathogenic activity. IgG from MS14 had extensive axopathic activity at antibody concentrations ≥50µg/ml whereas demyelinating activity was still observed at ≥10µg/ml.

Figure 5.2: Dose dependence of patient derived autoantibody mediated CNS injury.
IgG from four MS patients (MS2, MS4, MS5, and MS14) known to contain demyelinating and axopathic activity was added to myelinating cultures at 100, 50, 10 and 1µg/ml in the presence of 1% FRS as a source of complement (mean ± SEM, n = 3) (*p<0.05, **p<0.001, T-test). **A**: Demyelinating activity in all cases was significantly reduced at 50µg/ml and was no longer detected at 1µg/ml. **B:** Axopathic activity present in samples MS5 and MS14 was not detected at IgG concentrations below 100µg/ml (mean ± SEM, n = 3) (*p<0.05, **p<0.001, T-test).



5.2.1.3 Pathogenic IgG components purified from MS patients bind selectively to myelin

Using our *in vitro* system we have identified a demyelinating IgG response, therefore it was important to determine whether this activity can be attributed to the presence of myelin specific antibodies.

Live immunocytochemistry of myelinating cultures using 100µg/ml human IgG revealed a weak staining mainly restricted to the surface of the myelin sheath with minor staining on the oligodendrocyte cell body [Figure 5.3A]. There was no detectable staining on neuronal/axonal structures. However high background levels seen using IgG on live cells may obscure relatively weak immunoreactivity. Antibody binding was only observed in those samples with demyelinating activity i.e. (MS1-5 and MS7) and was not detected in non-pathogenic samples (MS6; OND1-4; HC1-4) [Figure 5.3B].

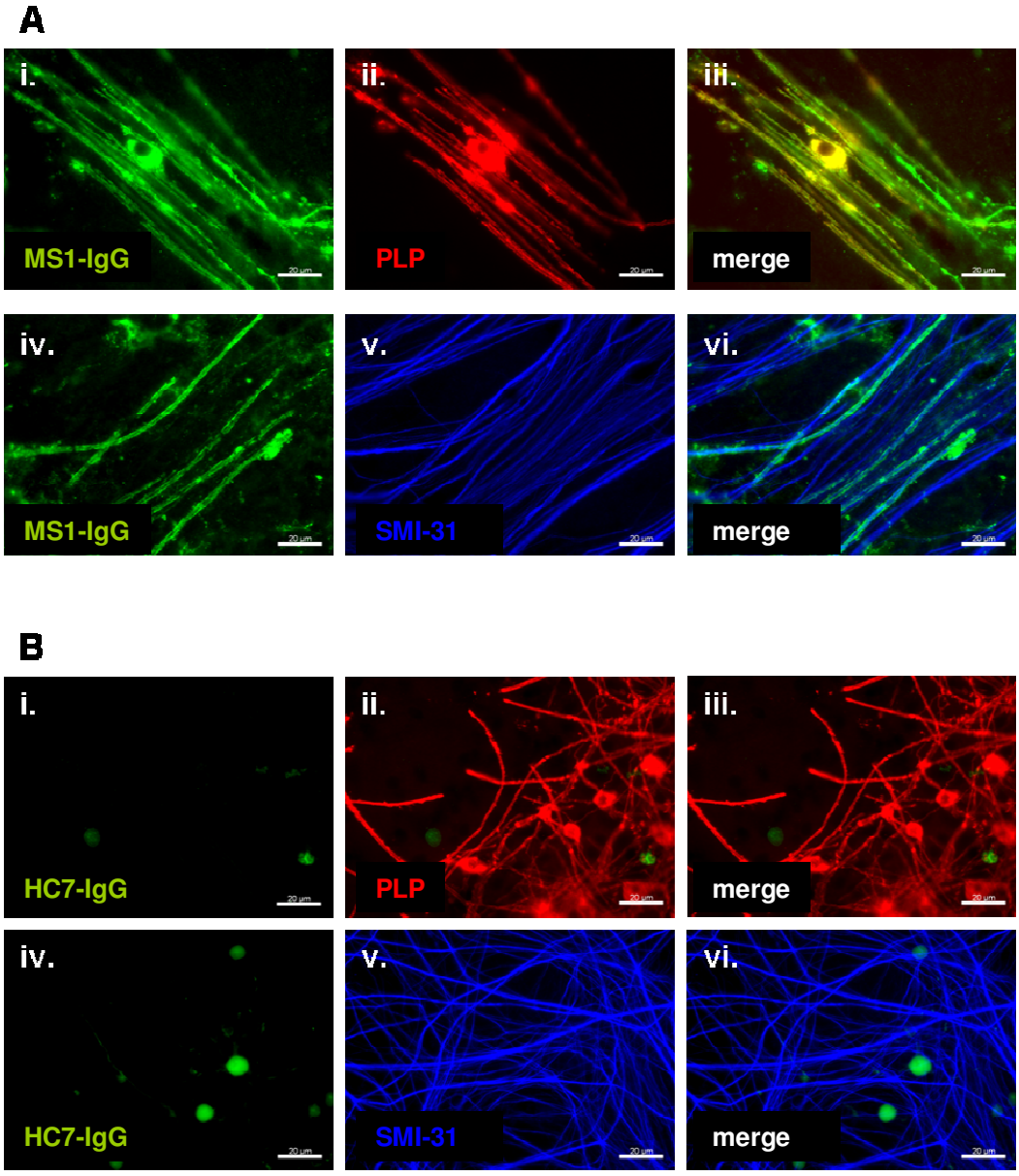
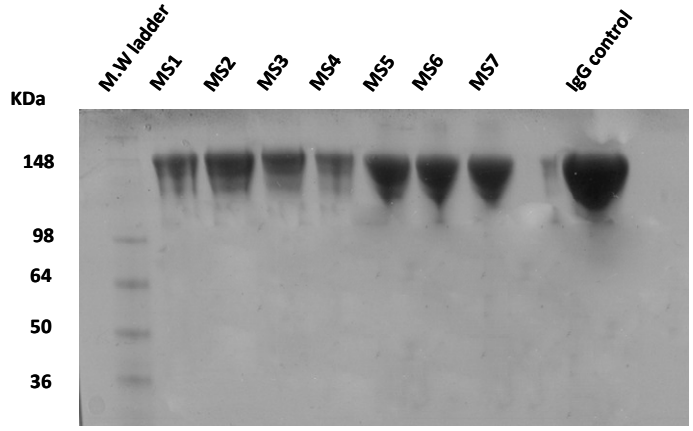


Figure 5.3: IgG purified from MS patient plasma binds selectivity to myelin
A: Live immunocytochemistry using 100µg/ml IgG purified from MS patients (green) revealed binding to PLP positive oligodendrocytes (red) **(i-iii).** Binding is strongest at the myelin sheath/ cell process there is a weak staining on the oligodendrocyte cell body. These structures stained with human IgG align with SMI-31^{+} axons (blue) indicating that demyelinating IgGs are capable of binding to a component of the myelin sheath. Images shown are from IgG purified from patient MS1 but identical staining patterns were observed using other MS patient IgG preparations. **B:** Live immunochemistry using 100µg/ml IgG purified from donor HC7 demonstrated no binding to oligodendrocytes **(i-iii)** or axons **(iv-vi).** A similar lack of immunoreactivity was observed with all other non-pathogenic IgG samples.

5.2.1.4 Adsorption of patient derived IgG using purified compact myelin diminishes its pathogenic potential

In order to determine whether these demyelinating responses were truly due to autoantibody responses directed against components of the myelin sheath we attempted to adsorb the pathogenic IgG component using highly purified rat CNS myelin and used our myelinating culture system to detect a potential reduction in pathogenic activity. Incubation of MS patient IgG (100µg/ml) with 2mg/ml total myelin protein resulted in a decrease in total IgG in the samples as seen by SDS-PAGE [Figure 5.4A] and a significant reduction in detectable binding by ELISA to rat myelin and human MOG [Figure 5.4B]. This diminished binding was accompanied in a reduction in demyelinating activity of the IgG fraction in three of the six patients tested demyelinating activity was completely removed by myelin adsorption (MS1, MS3, MS5). In the remaining samples pathogenicity was greatly reduced ranging from a 64% decrease (MS7) to 88% decrease in demyelinating potential (MS2) when compared to samples pre adsorption [Figure 5.3C]. The retention of some residual demyelinating activity may be a result of incomplete adsorption of pathogenic component or an indication that an element of this demyelinating response does not recognise a component of compact rat myelin. Another possibly is that the epitope is unavailable or altered between purified myelin and the myelin generated *in vitro*.

A



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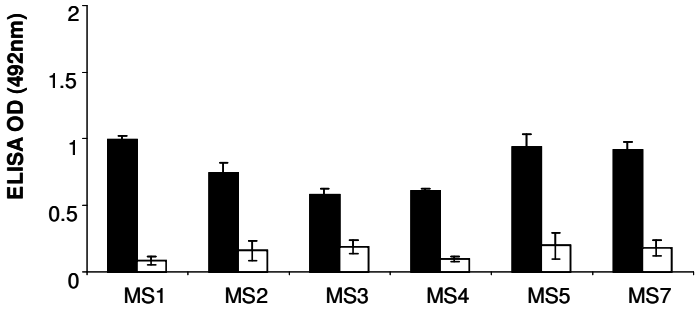


C

	IgG pre myelin absorption		IgG post myelin absorption	
Patient ID	**mean axonal loss (±SD)**	**myelin loss (±SD)**	**axonal loss (±SD)**	**myelin loss (±SD)**
MS1	2 ± 2.9	95 ± 3.5**	1.5 ± 2.5	**10 ± 2.2**
MS2	3 ± 2.6	**96 ± 4.0****	0.8 ± 1.4	**21 ± 3.2***
MS3	4 ± 3.0	97 ± 2.2**	0 ± 4.1	**6 ± 3.8**
MS4	2 ± 2.1	**98 ± 1.3****	1.5 ± 1.6	**25 ± 4.2***
MS5	34 ± 5.7**	**92 ± 2.4****	15 ± 1.3*	**8± 4.2**
MS6	2 ± 3.0	**0 ± 0**	0 ± 2.4	**3 ± 3.8**
MS7	3 ± 3.7	94 ± 2.0**	0 ± 3.0	**33 ± 3.8****

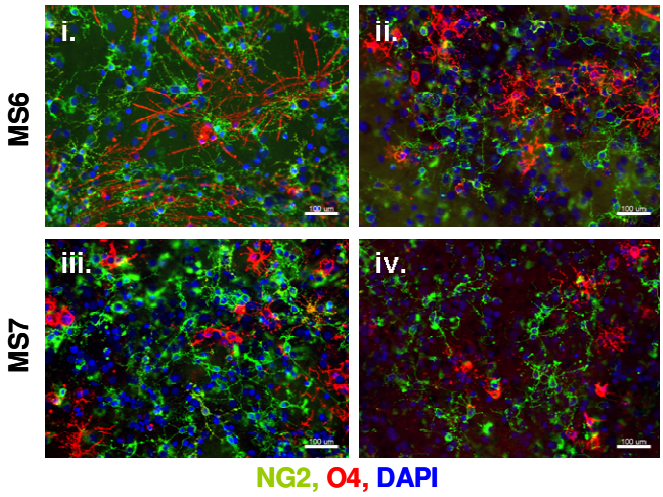
Figure 5.4: Adsorption of patient derived IgG using purified compact myelin diminishes its pathogenic potential

A: SDS-PAGE of IgG preparations post adsorption revealed a reduction in the total IgG present in the sample when compared to an non-adsorbed IgG control. SDS-PAGE analysis also revealed that the remaining IgG is intact (~150KDa) and has not been subjected to degradation from endogenous proteases. **B:** Adsorption of 100µg/ml IgG purified from MS patients with 2mg/ml rat myelin resulted in reduced binding to rat myelin as detected by ELISA (Black bars non-adsorbed IgG, white bars IgG adsorbed with myelin). Data plotted as mean OD (± standard deviation). All reductions in IgG binding were statically significant (**p<0.01, T-test). **C:** Myelin adsorption of IgG samples purified from MS patients resulted in a marked reduction in the pathogenic activity when compared to samples prior to adsorption. In the majority of cases myelin adsorption was lowered the amount of demyelination detected in myelinating cultures treated with 100µg/ml unbound IgG in the presence of 1% fresh rat sera as a source of complement. In the one case where axonal injury was detected in the IgG preparation prior to absorption (MS5) this was also significantly reduced after myelin adsorption. Values shown are from one representative data set performed three times in triplicate ± standard deviation (*p<0.05, **p<0.01; T-test).

5.2.1.5 Demyelination by human IgG has a limited effect on OPC survival

Using this system we have been able to identify a set of MS patients that possess IgG responses capable of mediating demyelination and destruction of mature MOG^+ and PLP^+ oligodendrocytes. To determine whether these antibodies had a pathogenic effect on oligodendrocyte progenitor cells (OPCs) we determined changes in the $NG2^+$ and $O4^+$ populations post treatment [Figure 5.5]. From this we can determine that antibody treatment with either patient derived IgG or with the anti-MOG monoclonal Z2 had no effect on the number of $NG2^+$ cells when compared to isotype and untreated controls. In 6 of 7 MS patients tested there was a complete loss of $O4^+$ myelin sheathes accompanied by a significant loss of $O4^+$ cells ranging from (24%-40%). A similar effect was observed after demyelination using Z2 which can be attributed to a loss of mature oligodendrocytes and myelin sheathes that express both sulphatide and MOG. Therefore we can predict that the human IgG preparations are targeting epitopes expressed on the mature oligodendrocyte and myelin sheath and not a target expressed only by a subset of OPCs. As when mature oligodendrocytes and OPCs are targeted simultaneously using the O4 monoclonal antibody there is a complete loss of myelin sheathes and 90% reduction in the total number of $O4^+$ cells.

A



B

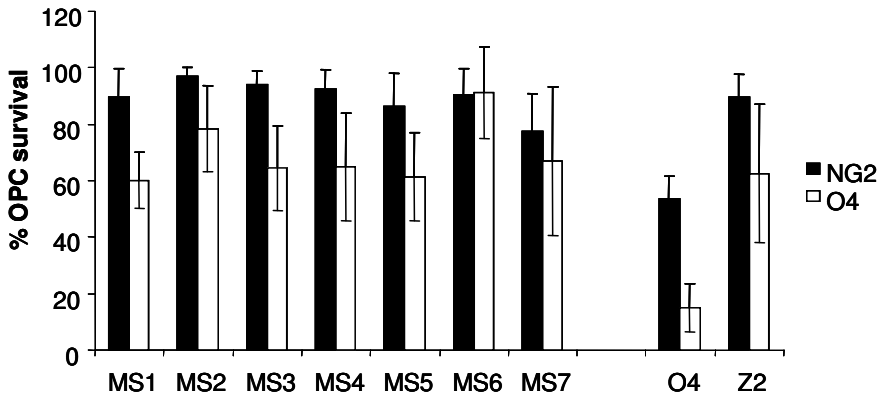


Figure 5.5: Treatment of in vitro myelinating cultures with MS patient derived IgG preparations has limited effect on oligodendrocyte precursor cells (OPCs).
A: Representative image (20X) taken from myelinating cultures treated with IgG from patient MS6 **(i)** showing $O4^+$ OPCs, oligodendrocytes and their associated myelin sheathes (red). Early OPCs are labelled using NG2 (chondroitin sulphate proteoglycans) (green). Representative image (20X) taken from cultures treated with IgG purified from patient MS7 **(ii)** showing a complete loss of $O4^+$ myelin sheathes and a modest reduction in the total number of $O4^+$ cells. There was not significant change in the number of $NG2^+$ OPCs when compared to controls. This reduction is comparable to that seen when cultures are demyelinated with 10µg/ml Z2 (α-MOG mAb) **(iii)** due to the loss of mature oligodendrocytes and myelin sheathes that express both sulphatide and MOG. In contrast when myelinating cultures are demyelinated with 10µg/ml O4 there is an almost complete loss of all O4 immunoreactivity **(iv).** There is also a reduction in the amount of NG2 positive cells, resulting from the loss of $O4^+$ and $NG2^+$ double positive OPCs. **B:** Treatment of myelinating cultures with 100µg/ml MS patient derived IgG in the presence of complement results in a complete loss of O4 (α-sulphatide mAb) positive myelin sheaths and a significant reduction in the $O4^+$ cell population (24-40%) in 6 of the 7 MS patient samples tested. Values shown are from one representative data set performed three times in triplicate ± standard deviation.

5.2.2 Nfasc: a potential specificity for pathogenic autoantibodies?

5.2.2.1 Characterisation of the rrNfasc155 reactive repertoire

Purification and characterisation of patient derived Nfasc155 reactive antibodies was performed by Dr A. Arthur (University of Glasgow).

To define a potential specificity for these observed pathogenic autoantibody responses, we screened our patient cohort for Nfasc155 responses. When screened using ELISA 18 of the 20 MS patients possessed significant antibody titres to recombinant rat Nfasc155 (rrNfasc155), with 7 patients possessing particularly high titres (MS1-7). However this response to rrNfasc155 was not unique to MS as responses were also detected with the OND group.

We isolated the Nfasc155-reactive component of antibody repertoire by immunoaffinity chromatography using recombinant rat Nfasc155. The Nfasc155-reactive immunoglobulin preparations contained varying proportions of IgM and IgG antibodies, IgG levels being significantly highly in samples isolated form patients with MS than those with other neurological diseases ($p<0.05$). This resulted in a significant difference the IgG:IgM ratio between the two groups. Isotype subclass usage was similar in both groups, predominately IgG1 and IgG2 accompanied by low levels of Nfasc-specific IgG3 and IgG4 [Figure 5.6A]. The latter was significantly higher in MS patients ($p<0.05$). All these Nfasc155-specific immunoglobulin preparations recognised both glycosylated and deglycosylated Nfasc155, although recognition of the peptide backbone varied significantly between individual patients [Figure 5.6B].

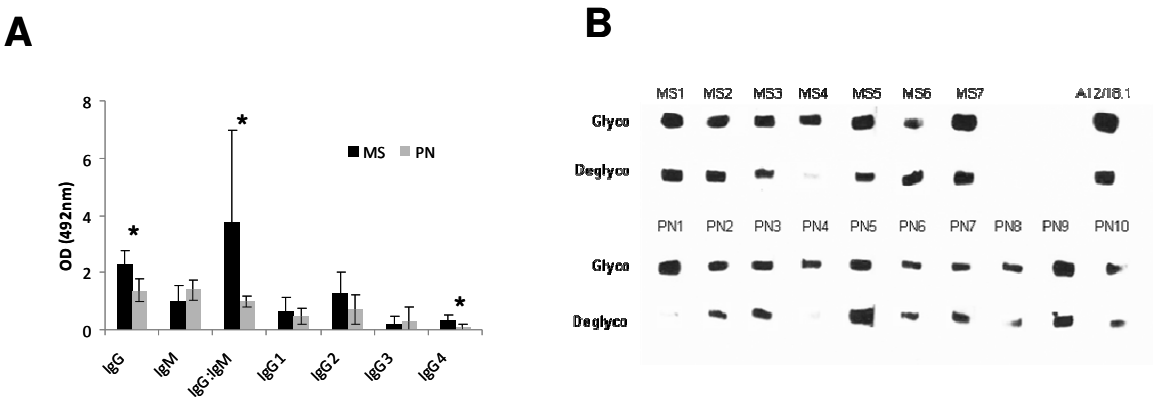


Figure 5.6: Characterisation of the Nfasc specific repertoire
A: Nfasc responses were detected by ELISA in patients with MS (black bars) and patients with ONDs (grey bars). When the isotype usages were quantified they was a significant difference between the two groups in the amount of total IgG present, the IgG to IgM ratio and the amount of IgG4 present all of which were higher in the MS cohort. However there were no significant differences between the two patient groups for the total amount of IgM, IgG1, IgG2 or IgG3. Values shown are an average of three independent experiments performed in triplicate ± standard deviation (* $P<0.05$, T-test). **B:** Western blot was performed to determine binding to Nfasc peptide epitopes rather than glycosylated moieties. Patient derived Nfasc-specific antibodies were blotted against 200ng glycosylated and 500ng deglycosylated rNfasc. With the exception of MS4, antibodies isolated from all the MS patients gave a strong ECL signal against both glycosylated and deglycosylated antigen after 2 minutes exposure. Antibodies from patients with peripheral neuropathies gave a similar signal against glycosylated rNfasc, but the exposure time had to be increased to 20 minutes to demonstrate binding of these samples to the deglycosylated protein.

5.2.2.2 Patient derived rrNfasc155 antibodies mediate demyelination and axonal injury *in vitro*

Treating myelinating cultures with Nfasc155-specific immunoglobulin preparations from individual donors revealed a striking heterogeneity in their ability to mediate axonal loss and/or demyelination. Nfasc155-specific immunoglobulins isolated from all cases of MS mediated complement-dependent demyelination. However in four cases this was also accompanied by variable degrees of axonal loss which ranged from 11% to 39% [Table 5.2A]. This axopathic effect was attributed to cross-reactivity of Nfasc155-specific antibodies with epitopes shared with Nfasc186, as demonstrated previously. Demyelination induced by the Nfasc specific repertoire may not be strictly MS specific as a demyelinating response was also detected in two OND patients (OND1: sensory motor polyneuropathy, OND3: GBS) [Table 5.2B].

Table 5.2: Nfasc specific antibodies purified from MS patients mediate axonal and glial pathology *in vitro*.
A: Patient derived Nfasc155 specific autoantibodies are able to mediate demyelination *in vitro*. In all seven MS cases tested we detected a strong demyelinating antibody response when added to myelinating cultures at 10µg/ml in the presence of 1% rat serum as a source of complement. In four patients (MS1, MS5, MS6, MS7) we could also detect an axonal response resulting in axonal loss ranging from 11% to 39% of the total number of axons. Values shown are an average of three independent experiments performed in triplicate ± standard deviation (* P<0.05, T-test). **B:** Demyelinating responses were more common in the MS cohort but are not disease specific as we are able to detect demyelinating activity in two patients from the OND control group (OND1 and OND3). Axonal pathology however was unique to the MS group. Values shown are from one representative data set performed three times in triplicate ± standard deviation (*p<0.05, **p<0.01; T-test).

A

Patient ID	Diagnosis	Mean Axonal Loss (±SD)	Mean Myelin Loss (±SD)
MS1	Marburg MS	22 ± 4.2**	100 ± 0**
MS2	RRMS	1.4 ± 1.4	100± 0**
MS3	RRMS	1.8± 1.2	99 ± 1.5**
MS4	RRMS	1.5 ± 1.0	98 ± 2.0**
MS5	SPMS	39 ± 4.9**	100 ± 0**
MS6	RRMS	11 ± 5.0*	100 ± 0**
MS7	RRMS	37 ± 4.3**	100± 0**

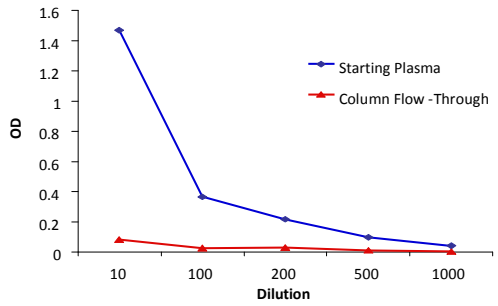
B

Patient ID	Diagnosis	Mean Axonal Loss (±SD)	Mean Myelin Loss (±SD)
OND1	SPN	1.5 ± 2.5	98 ± 1.5**
OND2	GBS	0.8 ± 1.4	0 ± 3.4
OND3	GBS	0 ± 4.1	99 ± 1.2**
OND4	GBS	1.5 ± 1.6	0 ± 2.2
OND5	CIDP	2.5 ± 1.5	0 ± 6.0
OND6	CIDP	0 ± 2.4	0 ± 1.3
OND7	CIDP	0 ± 3.0	0 ± 2.4
OND8	ISAN	1.1 ± 2.3	0 ± 4.0
OND9	MG	1.6 ± 3.7	0 ± 5.2
OND10	CIDP	0 ± 5.1	0 ± 3.2

5.2.2.3 Nfasc155 can provide a dominant target for demyelinating and axopathic autoantibody responses in MS

The use of immunopurified antibodies formally demonstrates that components of the Nfasc155-specific autoantibody repertoire have the potential to mediate axonal injury and/or demyelination *in vivo*. However, this approach provides no indication as to whether this pathogenic response is a dominant component of the autoantibody repertoire. We therefore compared the pathogenic potential *in vitro* of total IgG fractions prepared from patient plasma post-depletion of Nfasc155 reactivity. Using our immunopurification method we are able to virtually remove all Nfasc155 reactivity from the plasma [Figure 5.7A], we subsequently collected this flow through and purified the residual IgG remaining in the plasma after Nfasc immunoabsorption. In one particular case (patient MS5) removal of the Nfasc155-specific repertoire completely abolished the ability of the IgG fraction to mediate any pathogenic effect *in vitro* [Figure 5.7B]. This formally identifies Nfasc as a dominant target for pathogenic autoantibodies in this particular patient. This was not the case for the remaining patients, as depletion of the Nfasc155 reactive component of the antibody repertoire had no significant effect its ability to mediate demyelination *in vitro*.

A



B

	IgG before depletion		Nfasc Depleted IgG	
Patient ID	**% Axonal loss (±SD)**	**% Myelin loss (±SD)**	**% Axonal loss (±SD)**	**% Myelin loss (±SD)**
MS1	2 ± 2.9	95 ± 3.5**	2 ± 2.6	93 ± 4.5**
MS2	3 ± 2.6	96 ± 4.0**	3 ± 2.5	97 ± 3.0**
MS3	4 ± 3.0	97 ± 2.2**	4 ± 2.3	97 ± 2.0**
MS4	2 ± 2.1	98 ± 1.3**	3 ± 2.9	94 ± 1.8**
MS5	**34 ± 5.7***	**92 ± 2.4****	**2 ± 3.0**	**0 ± 1.2**
MS6	2 ± 3.0	0 ± 0	4 ± 3.4	0 ± 0
MS7	3 ± 3.7	94 ± 2.0**	2 ± 1.6	92 ± 2.2**

Figure 5.7: Nfasc155 can provide a major autoantigen for autoantibody responses in MS
A: Using our immunoabsorption protocol using Nfasc155 we are able to remove virtually all Nfasc155 reactivity, as detected by ELISA. We can see that there is a large amount of detectable Nfasc155 reactivity in the starting plasma and after immunodepletion binding to Nfasc155 is diminished (patient MS5). **B:** For patient MS5 we purified the IgG fraction present in the column flow through after the removal of the Nfasc155 specific component. This was then added to myelinating cultures at 100µg/ml in the presence of 1% rat serum as a source of complement. In this particular patient we were able to see that IgG purified from plasma prior to Nfasc155 immunodepletion mediated both a striking demyelinating and axopathic response. However after the removal of the Nfasc155 specific repertoire this activity was abolished, implicating Nfasc155 as the main autoantigen in this particular case. However in the remaining patients the pathogenic activity was completely retained suggesting that Nfasc155 is not the key autoantigen driving these responses. Values shown are from one representative data set performed three times in triplicate ± standard deviation (*$p<0.05$, **$p<0.01$; T-test).

5.3 Discussion

The use of *in vitro* CNS models to detect pathogenic activity in patient serum is not a novel concept. The *in vitro* demyelinating potential of MS sera was first described over 50 years ago (Bornstein and Appel, 1965), an observation which was later confirmed by others (Hughes and Field, 1967; Lumsden, 1971; Bradbury et al., 1985). However whether this *in vitro* demyelinating activity was mediated by a myelin-specific autoantibody response remained controversial (review: Seil, 1977; Caspary, 1977).

Recent studies have identified a plethora of potential autoantigens associated with MS; however most failed to determine whether these responses are actually involved in disease pathogenesis. In order to address this question we established a novel *in vitro* screening strategy which not only detects demyelinating and axopathic autoantibody responses but provides a quantitative readout of their pathogenic potential. Using this model we can identify a subset of MS patients who possess pathogenic autoantibodies capable of mediating axonal injury and/or demyelination. This is the first formal demonstration that adult onset MS is associated with pathogenic autoantibody responses. In contrast to earlier studies we demonstrated that these responses are specific for MS and not present in the OND or healthy controls (Bronstein and Appel., 1965; Hughes and Field 1967).

Unfortunately due to the nature of this study and characterisation of clinical samples, coding of patient and control samples was not possible. In order to prevent introducing any potential bias, future experiments should be performed in a blinded fashion.

A number of studies have attempted to determine if there is any correlation between myelin specific antibody responses and the course of disease but this issue remains controversial (Berger et al., 2003; Kuhle et al., 2007). Within our MS cohort we observed a trend suggesting that pathogenic antibody responses were associated with patients with aggressive onset disease and steroid non-responsive relapses. This suggests the exciting possibility that a correlation may exist between disease severity and the presence of a pathogenic antibody

response. Unfortunately the clinical data was not available in order for us to determine whether plasma exchange was most effective in patients with pathogenic autoantibody reponses than seronegative patients. This pilot study also lacks sufficient patient numbers to draw any solid conclusions regarding this point. Studies of AQP-4 specific antibody titres in NMO demonstrated that there is a marked correlation between disease progression and pathogenic antibody titre (Jarius et al., 2010).

Similar studies could be adopted to investigate many aspects of MS pathogenesis in which our model could be used as a potential readout of pathogenic antibody activity. Further clinical investigations using this model will be discussed in greater detail in the final chapter.

We demonstrated that these pathogenic autoantibodies recognise a component of intact myelin. Antibody binding was only observed in IgG preparations with pathogenic activity unlike previous studies which reported that human IgG can bind non-specifically to myelin/oligodendrocytes *in vitro* (Aarli et al., 1975; Traugott et al., 1979). The anti-myelin reactivity of these pathogenic autoantibodies was further confirmed when antibody adsorption using purified myelin was shown to significantly reduce pathogenic activity. This is in contrast to studies described in the 1970s (Wolfgram and Duquette, 1976). However the precise specificity of this pathogenic response remains unknown. It is probable, given the complexity and immunopathological heterogeneity of MS that multiple targets may be involved, the identity of which may vary between patients. In fact we have data to suggest this is the case as one of our patients developed pathogenic autoantibody responses against Nfasc155 and depletion of this repertoire abolished all pathogenic activity (MS5), however Nfasc155 was not a dominant autogen in the rest of the cohort.

From the data in this chapter we can make a number of assumptions about the antigen:

- It is accessible to bind antibody at the myelin surface.

- It is not expressed by oligodendrocyte precursor cells as antibody treatment spares the majority of $NG2^+$ and $O4^+$ OPCs.

- Its expression is restricted to terminally differentiated oligodendrocytes.

Potential targets that satisfy these criteria are MOG (Brunner et al., 1989; Kroepfl et al., 1996), PLP (Hudson et al., 1989) and opalin (Yoshikawa et al., 2008; Kippert et al., 2008). Nothing is known about the encephalitogenic potential of opalin whilst MOG has been implicated in paediatric MS/ADEM (McLaughlin et al., 2009; DiPauli et al., 2010). Further investigation is required in order to identify the antigen recognised by these pathogenic antibodies and methods by which the candidate autoantigens may be identified will be discussed in a later chapter.

Although our data are highly suggestive that the responses we detect *in vitro* may contribute to disease pathogenesis. This has still to be confirmed *in vivo*. Classically this is achieved by passive transfer in an *in vivo* disease model. In attempting to model antibody mediated effects in CNS disease most studies use cotransfer models in which patient-derived antibody is transferred into animals with EAE as in the case of NMO (Bradl et al., 2010). Passive transfer of patient derived immunoglobulins from patients with NMO (Bradl et al., 2009); MG (Toyka et al., 1975) or neuromyotonia (Shillito et al., 1995) reproduces the clinical features of these diseases in experimental animals. Unfortunately similar studies using MS immunoglobulins have been largely unsuccessful. In a paper by Zhou et al. the authors identified a single patient with high anti-MOG antibody titres which bound to MOG transfectants with high affinity. However when immunoglobulin from this patient was transferred into rats with EAE the animals developed a very minor disease especially when compared to a MOG monoclonal antibody (Zhou et al., 2006).

In this respect the study by Bradl et al. (2010) is particularly informative. In this case animals had to be immunised with 10mg of NMO IgG in order to induce disease. Similarly passive transfer of MG to mice required 10-11mg human IgG (Toyka et al., 1975). These antibody doses required to induce disease are astonishingly high. For example 10mg IgG is approximately 50% greater than the total circulating native IgG in the mouse (Santos, 1967). Therefore there is a discrepancy between the amount of patient derived antibody needed to induce disease in rodents and the pathogenic antibody titres detected in patients. In MG patients, AChR antibody titres associated with disease are much lower (~10-

20nM) (Lindstrom et al., 1976). This makes the bioassay an excellent screening strategy to detect pathogenic but we have still to address the problem of developing relevant *in vivo* models to investigate the effects of acute and chronic exposure to MS autoantibodies.

MS was long described as a disease of myelin characterised by primary oligodendrocyte loss and demyelination but with relative sparing of axons. However it is known understood that MS lesions are also associated with profound axonal loss (Trapp et al., 1998). Using our system we detected autoantibody responses which can mediate axonal injury, an observation not previously reported. A potential target for this axopathic response is Nfasc, in particular Nfasc186. Nfasc was first reported as a potential autoantigen by Mathey et al. who demonstrated that autoantibodies against Nfasc could be found in a large proportion of MS patients. The authors also reported that recognition of Nfasc186 exacerabates disease severity in EAE due to axonal injury (Mathey et al., 2007).

Using immunoaffinity chromatography we isolated the Nfasc155 specific antibody repertoire from MS and OND patients. When added to myelinating cultures we observed that in all MS cases these autoantibodies mediated complement dependent demyelination and in two cases this was accompanied by significant axonal injury. We observed demyelination in only two OND samples. Given that in the previous chapter we demonstrated that a Nfasc155 specific anti-sera mediated complete demyelination this was not unexpected, and we attribute the limited axopathic effect to cross reactivity with Nfasc186.

Unfortunately if was not possible to determine the site of antibody binding for this rrNfasc155 specific response. We were unable to see specific binding to any particular structure in live cultures due to high levels of background staining and uptake of IgG aggregates by microglia. It was also not possible to detect MAC deposition. However as demonstrated in the previous chapter when myelinating cultures are treated with low doses of monoclonal antibody (such as 100ng/ml of 8-18C5) we were unable to detect MAC deposition although treatment ultimately lead to complete demyelination. This may be the case for our purified patient antibodies i.e. the pathogenic component may be present at concentrations too low to visualise by immunofluorescence.

In one patient (MS5) removal of the rrNfasc155 reactive repertoire was sufficient to remove all pathogenic activity of the plasma IgG therefore in this patient there is a dominant pathogenic antibody component which recognises rrNfasc155. Myelin absorption of this sample significantly depleted the demyelinating activity therefore part of this response must be myelin associated. Axonal injury was also significantly reduced potentially due to adsorption of antibodies recognising both Nfasc155 and Nfasc186. A similar observation was reported in the previous chapter when A12/18.1 (pan-specific Nfasc mAb) was adsorbed with rat myelin resulting in a reduction in both demyelinating and axopathic activity.

We must be cautious when drawing conclusions regarding Nfasc responses in MS. To immunopurify the Nfasc specific repertoire we used recombinant rat Nfasc155 (rrNfasc155) this peptide was generated by a murine myeloma derived cell line (NSO) and therefore may contains post translational modifications not usually present when expressed by oligodendrocytes. Western blot analysis of the purified antibody binding to rrNfasc155 or deglycosylated rrNfasc155 revealed that the majority of our purified antibody reacted primarily with glycosylated motifs (especially in the OND group) although we were able to detect some reactivity to the peptide backbone. Therefore the antibody we purified is not homogeneous and must contain alternate specificities, some of which may be unrelated to Nfasc.

Our bioassay focusses on complement dependent effects. However *in vivo* there may be multiple pathogenic mechanisms. Clearly it is not possible to reproduce all adaptive and innate mechanisms within our *in vitro* model. Using this bioassay to model complement independent effects will be discussed in the next chapter.

6 Modelling chronic autoantibody mediated injury in the absence of complement.

6.1 Introduction

The previous chapters demonstrated that myelinating cultures can provide the basis of an *in vitro* screening strategy to detect antibodies that mediate acute complement dependent demyelination and axonal loss *in vitro*. The decision to initially focus on complement-dependent mechanisms was driven by reports that they play an important role in the development of demyelinating lesions in EAE (Linington et al., 1989; Storch et al., 1998) and the immunopathology of MS lesions (Lucchinetti et al., 2000).

However autoantibodies may also mediate demyelination and/or axonal injury via complement independent mechanisms. These include:

- *Antibody-dependent cellular cytotoxicity (ADCC).*

  Recognition of antigen/antibody complexes by Fc-gamma receptors results in the activation of cellular components of the innate immune system principally NK cells and polymorphonuclear neutrophils. This triggers a respiratory burst associated with the generation of free oxygen species ond/or the release cytolytic proteins from intracellular granules. Infiltrating neutrophils are prominent feature of some EAE models (Maatta et al., 1998; Zehntner et al., 2005) and are also observed in fulminate cases of MS (Johnson et al., 1990).

- *Opsonisation and phagocytosis.*

  Antibodies bind to the target leading to Fc-gamma receptor mediated phagocytosis of the antibody-antigen complex and tissue destruction. Demyelinating lesions in both MS and EAE contain numerous phagocytes (macrophages and microglia) containing Ig and myelin debris (Genain et al., 1999). However whether this represents clearance of opsonised debris or a primary immune attack on CNS myelin is uncertain.

- *Direct effect due to antibody binding.*

  Antibody binding to the cell surface may mediate direct functional effects as demonstrated by the phosphorylation of cellular stress response proteins and cytoskeletal changes induced in oligodendrocytes by MOG-specific antibodies (Marta et al., 2003; 2005).

Intriguingly previous studies using rodent organotypic myelinating cultures reported that that anti-myelin antisera inhibited myelin formation in vitro. There are reports in the literature that MS patient serum also contains myelination inhibitory factors although this issue is controversial (Seil, 1977; Caspary et al., 1977). We therefore decided to readdress this question using the myelinating cultures to investigate whether either MOG- or Nfasc-specific responses would have any pathogenic effect in the absence of exogenous complement.

6.2 Results

6.2.1 MOG specific antibodies disrupt myelin formation in vitro

To determine whether MOG-specific autoantibodies are able to effect myelin formation in the absence of an exogenous source of complement, myelinating cultures were grown in the presence of mAb Z2 (10μg/ml) from either 12, 18 or 24 DIV [Figure 6.1].

As described in chapter 3, at 12 DIV these cultures contain very few MOG+ cells and no myelin. Significant numbers of MOG+ oligodendrocytes and associated axonal ensheathment are only observed from 18 DIV onwards. Addition of Z2 from either 12 or 18 DIV resulted in an almost complete block of myelination as assessed at 24 and 30 DIV [Figure 6.1A &B]. In contrast, the ability of Z2 to inhibit myelination when present from 24 DIV onwards was partial. This observation suggests that in the absence of a source of complement the mAb Z2 inhibits the development of myelination competent oligodendrocytes, but does not mediate destruction of pre existing myelin sheaths [Figure 6.1C]. These antibody mediated effects on myelination were not associated with any changes in axonal density as assessed by SMI-31 immunoreactivity. Furthermore, no disruption of myelination was observed when cultures were grown in the presence of a control mouse IgG2a myeloma protein (10μg/ml).

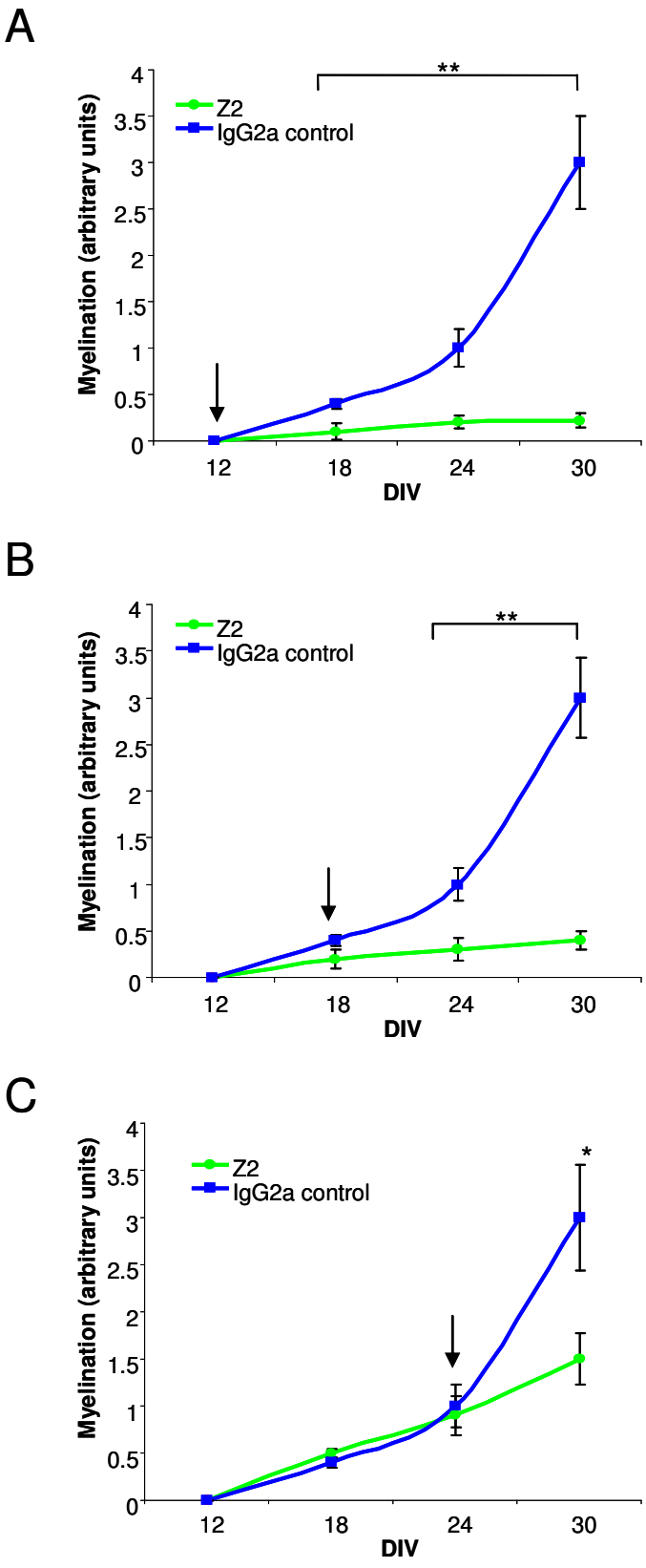


Figure 6.1: MOG specific antibodies can block myelination *in vitro*.
Myelinating cultures were incubated with 10µg/ml Z2 from 12 DIV **(A),** 18 DIV **(B)** or 24 DIV **(C)** until 30 DIV (antibody addition marked with an arrow). Analysis of immunochemical data reveals that in all cases long term incubation with Z2 can block myelin formation *in vitro.* In all cases axonal density was equivalent and unaltered in response to antibody treatment. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *p<0.05 **p<0.001 (t-test).

6.2.1.1 Inhibition of myelination by MOG specific antibodies is concentration dependent

To determine the concentration dependence of the effect of mAb Z2 on myelination in the absence of an exogenous source of complement, cultures were treated from 18 to 30 DIV with various Z2 concentrations ranging from 10µg/ml to 1ng/ml [Figure 6.2]. Axonal density and myelination were then quantified as described previously.

This experiment revealed that myelination in this culture system is very sensitive to the presence of this MOG-specific mAb. Significant inhibition was observed at 10ng/ml (66pM), which is lower than that required to obtain a similar degree of acute antibody-mediated, complement-dependent demyelination using this antibody in vitro (Chapter 4). Myelination in this culture system is therefore highly sensitive to the presence mAb Z2, being detected at antibody concentrations significantly lower than those reported for pathogenic autoantibody responses in both myasthenia gravis (Lindstrom et al., 1976) autoimmune mediated epilepsy/neuromyotonia (Irani et al., 2010)

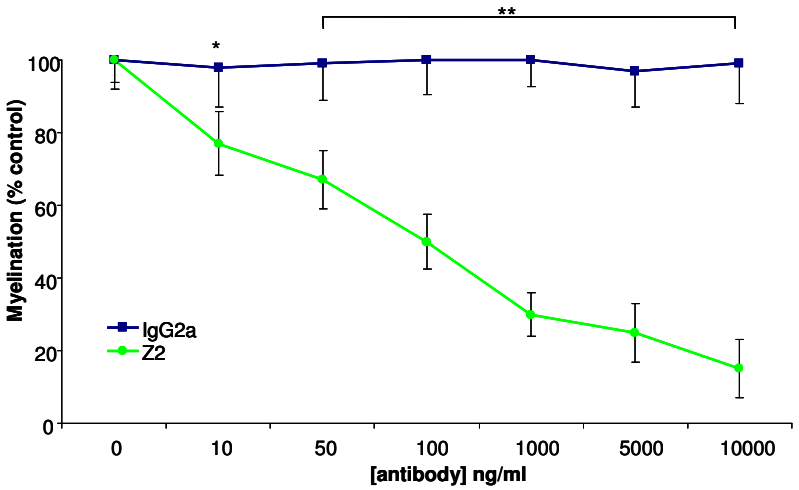


Figure 6.2: Disruption of myelination *in vitro* by MOG specific antibodies is concentration dependent.
To determine the antibody concentration range required to inhibit myelin formation myelinating cultures were treated from 18 DIV with various concentrations of Z2 (10µg/ml-10ng/ml) until 30DIV. Analysis of immunochemical data reveals that myelinating cultures are extremely sensitive to antibody treatment, as inhibition of myelin formation can be detected down to antibody concentrations ≥ 10ng/ml. In all cases axonal density was equivalent and unaltered in response to antibody treatment. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *p<0.05 **p<0.001 (t-test).

6.2.1.2 Inhibition of myelination by MOG specific antibodies is reversible

To determine whether exposure to mAb Z2 results in an irreversible block in myelination, cultures were treated with 1mg/ml Z2 between 12 and 18DIV, after which they were washed extensively and cultured in the absence of antibody for a further 12 days. As demonstrated above the presence of Z2 from 12 DIV onwards results in dramatic reduction myelination as assessed by immunoreactivity for PLP. However, withdrawl of Z2 from 18 DIV was followed by a partial recovery indicating this antibody mediated effect is not necessarily permanent. Myelination in these cultures was approximately thirty percent of that seen in cultures treated with an isotype control mAb [Figure 6.3].

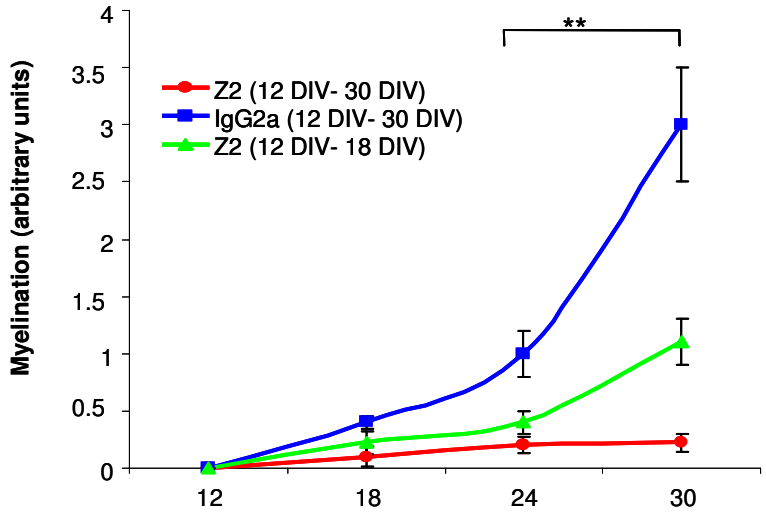


Figure 6.3: Inhibition of myelination caused by MOG specific antibodies is reversible.
To determine whether the block of myelin formation caused by MOG specific antibodies is permanent, cultures were treated with 1µg/ml Z2 from 12 DIV and antibody was withdrawn from the media at 18 DIV and cultured normally until 30 DIV. Analysis of immunochemical data demonstrates that at 24 DIV only a very small amount of has been myelin formed however by 30 DIV significant myelination has taken place. In all cases axonal density was equivalent and unaltered in response to antibody treatment. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *p<0.05 **p<0.001 (t-test).

6.2.2 Effects mediated by Nfasc specific autoantibodies in the absence of complement

6.2.2.1 Pan-Nfasc antibodies mediated complement independent demyelination and axonal loss

As antibodies to Nfasc155 were reported to block myelination *in vitro* (Charles et al., 2002), it was anticipated that the pan-Nfasc mAb A12/18.1 would have a similar effect. This hypothesis was tested by adding A12/18.1 (10μg/ml) to myelinating cultures from 12, 18 DIV and 24 DIV onwards. In each case axonal density and myelination was determined as described previously [Figure 6.4].

Surprisingly the presence of A12/18.1 from 12 or 18 DIV not only resulted in inhibition of myelination but also decreased SMI-31 immunoreactivity by approximately half compared to control cultures. This loss of SMI-31 positive structures was observed within six days of addition of A12/18.1, but was far less pronounced in those cultures treated from 24 DIV onwards. In this case axonal loss was associated with an inhibition of myelination but there was no concomitant loss of pre-existing myelin sheathes [Figure 6.4C]. This suggests that those axons/neurons that have already been myelinated are resistant to the complement-independent effects of A12/18.1.

As previously demonstrated in chapter 4, antibody treatment in the presence of exogenous complement induces pronounced axonal loss and demyelination but no acute injury was detected after 16hrs in the absence of complement.

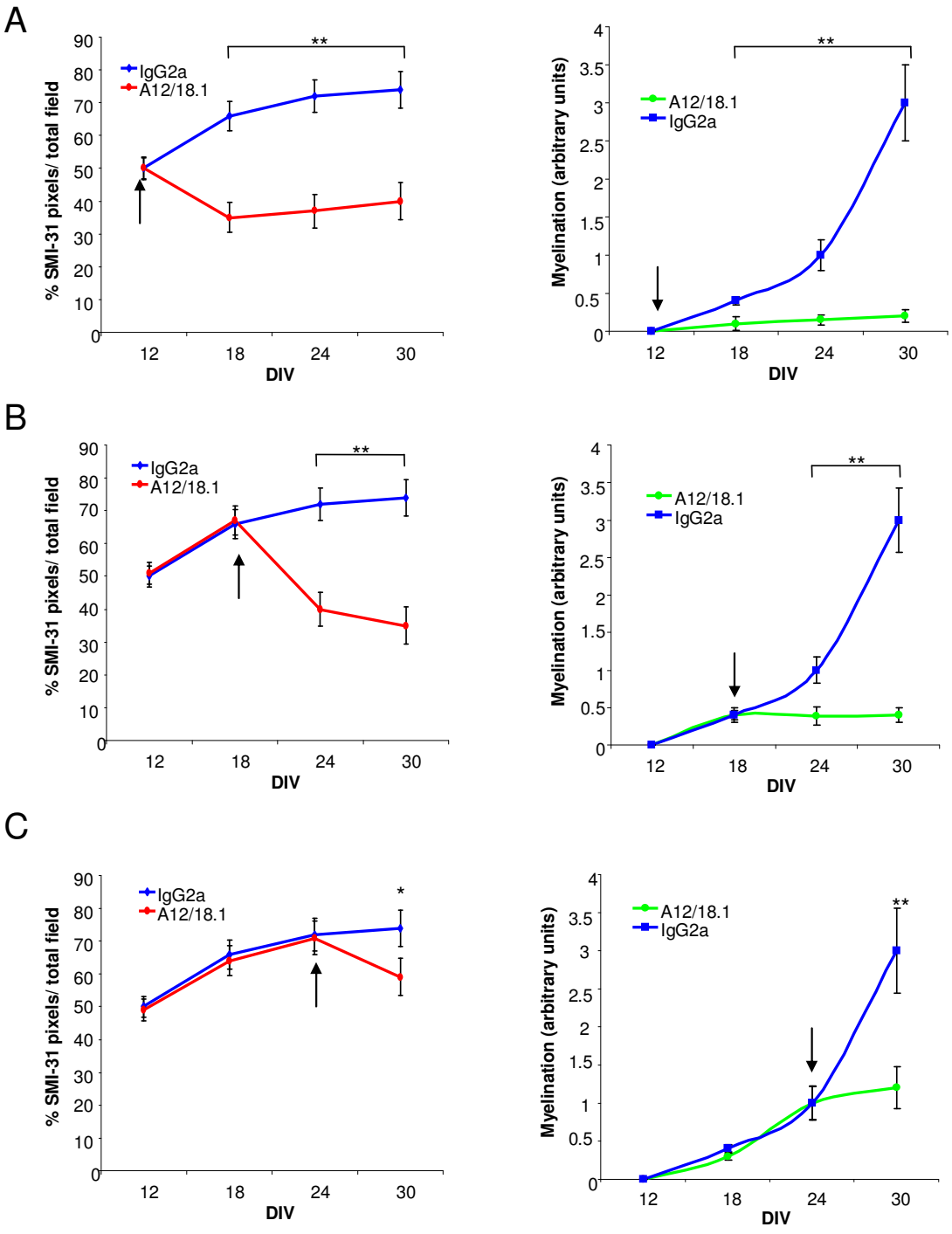


Figure 6.4: Nfasc specific antibodies can inhibit myelin formation and mediate axonal injury in the absence of complement.
Myelinating cultures were incubated with 10µg/ml A12/18.1 from 12 DIV **(A),** 18 DIV **(B)** or 24 DIV **(C)** until 30 DIV (antibody addition marked with an arrow). Analysis of immunochemical data reveals that in all cases long term incubation with A12/18.1 can block myelin formation *in vitro* (green line). Strikingly inhibition of myelination is accompanied by a loss of axons, which can be detected after 6 days of antibody addition (red line). Early antibody addition (from 12 DIV or 18 DIV) mediates a significant loss of axons (~40%) and complete block of myelination. Antibody addition much later (24 DIV) mediates axonal injury and block of myelination however to a much lesser extent than in earlier treatments. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *p<0.05 **p<0.001 (t-test).

6.2.2.2 Concentration dependence of A12/18.1 mediated effects

The concentration dependence of effects mediated by A12/18.1 in the absence of exogenous complement on axons and myelination were investigated by treating myelinating cultures with 10μg/ml to 1ng/ml of antibody from 18 DIV onwards and then quantifying myelination and axonal density twelve days later (30 DIV) [Figure 6.5]. Significant inhibition of myelin formation was observed at mAb concentrations $\geq$ 50ng/ml, whilst significant axonal loss was on at concentrations $\geq$100 ng/ml indicating the latter is less sensitive to the complement independent effects of the pan-Nfasc antibody.

6.2.2.3 Effects mediated by A12/18.1 are irreversible

To determine whether the effects of A12/18.1 on axonal survival and myelination were reversible myelinating cultures were treated with 1μg/ml of the antibody between 12 and 18 DIV. The cultures were then washed extensively to remove unbound antibody and cultured for a further 12 days in the absence of A12/18.1. Quantification of axonal loss and myelination revealed that unlike the effect of the MOG-specific mAb Z2 on myelination, axonal loss and failure of myelination induced by A12/18.1 were irreversible [Figure 6.6]. The failure of these cultures to exhibit any evidence of myelination suggests early treatment with the antibody eliminates all potentially myelination competent axons.

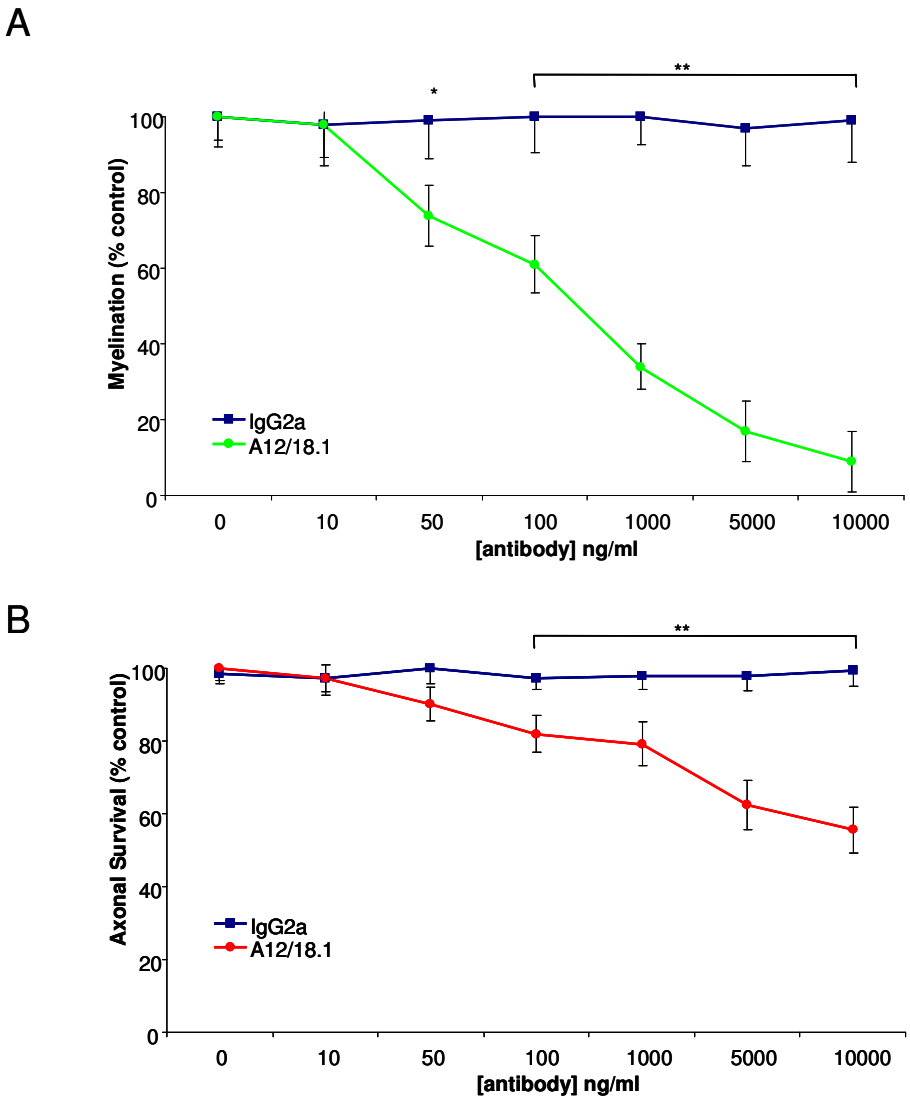


Figure 6.5: *In vitro* effects mediated by Nfasc specific antibodies are concentration dependent.
To determine the antibody concentration range required to inhibit myelin formation, myelinating cultures were treated from 18 DIV with various concentrations of A12/18.1 (10µg/ml-1ng/ml). Analysis of immunochemical data reveals that myelinating cultures are extremely sensitive to antibody treatment as inhibition of myelination can be detected down to antibody concentrations ≥ 10ng/ml. Axonal loss was less sensitive however axopathic activity remains at antibody concentrations ≥ 100ng/ml. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *$p<0.05$ **$p<0.001$ (t-test).

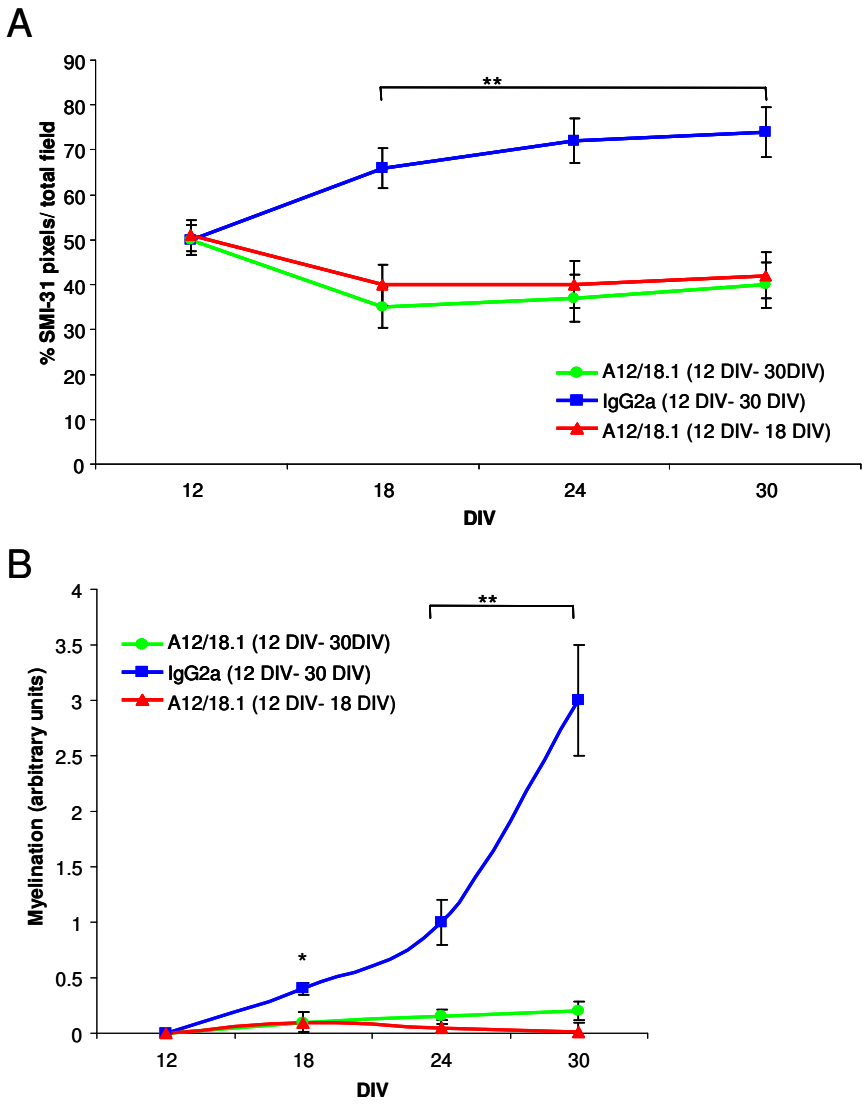


Figure 6.6: Pathogenic effects on axons and glia mediated by Nfasc specific antibodies are irreversible.
To determine whether pathology caused by Nfasc specific antibodies is permanent cultures were treated with 1µg/ml A12/18.1 from 12 DIV and antibody was withdrawn from the media at 18 DIV and cultured normally until 30 DIV. Analysis of immunochemical data demonstrates that there is no recovery of myelination **(A)** or any replenishment of axons **(B)** at 24 DIV or 30 DIV. Values plotted are the average of three independent experiments performed in triplicate (± S.E.M), *p<0.05 **p<0.001 (t-test).

6.2.3 Complement independent activity of patient derived IgG in vitro

The observation that antibodies recognising axons and/or myelin can have profound complement independent effects on myelination and axonal survival promoted us to reassess the concept that some MS patients develop myelination inhibiting autoantibody responses (review: Seil et al., 1977). Purified IgG preparations from seven patients (MS1-7) were added to myelinating cultures from 18 DIV onwards at a concentration of 50μg/ml. Analysis of the extent of myelination revealed that twelve days later six of the seven IgG preparations had inhibited myelination to varying extents [Figure 6.7A], whilst one (MS5) had also induced a fifty percent reduction in SMI-31 immunoreactivity [Figure 6.7B]. No pathogenic effect was observed when cultures were treated with either a patient derived IgG preparation that exhibited no complement-dependent effects *in vitro* (MS6) or a commercially available IgG preparation obtained from healthy donors.

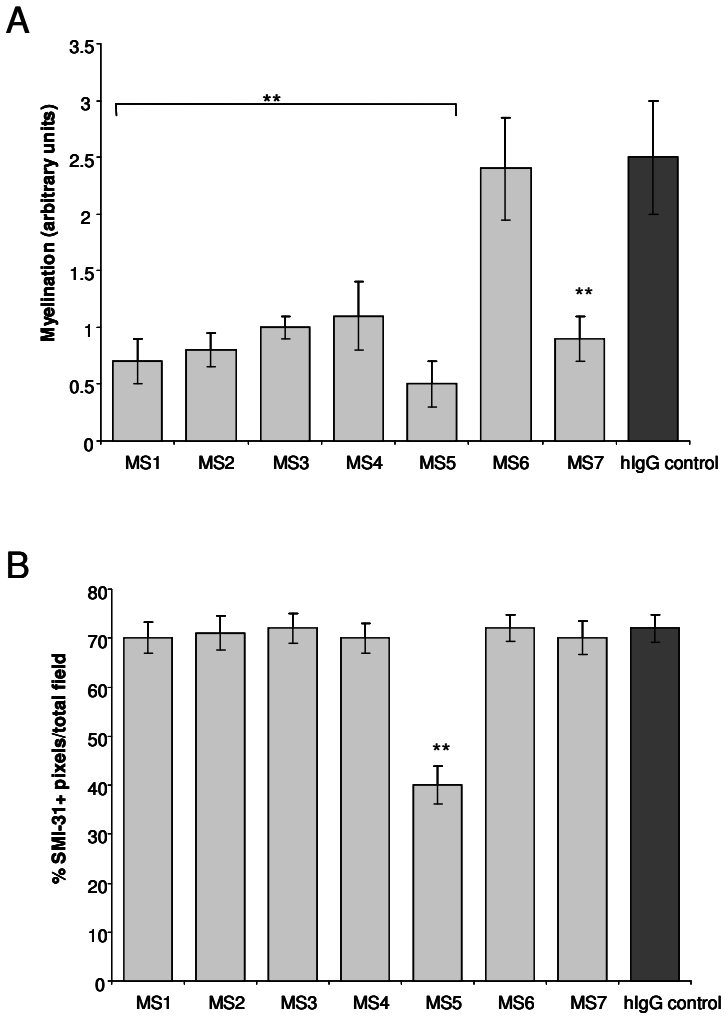


Figure 6.7: In the absence of complement, long term incubation with IgG purified from MS patients can inhibit myelination and mediate axonal injury *in vitro.*
At 18 DIV myelinating cultures were incubated with 50µg/ml IgG purified from MS patients until 30 DIV. Myelin/ oligodendrocytes were stained for PLP and axons labelled using SMI-31. Analysis of immunochemical data reveals that six of seven samples tested were able to inhibit myelin formation *in vitro* **(A)**. One patient (MS5) also exhibited axopathic activity leading to a loss of approximately 40% of total axons **(B)**. Pathogenic activity was not seen in cultures treated with control IgG preparations (hIgG). Values plotted are the average of two independent experiments performed in triplicate (± S.E.M), *$p<0.05$ **$p<0.001$ (t-test).

6.3 Discussion

This chapter demonstrates that autoantibody responses directed against myelinated axons can mediate profound complement-independent effects *in vitro,* as demonstrated by the ability of a MOG-specific mAb to inhibit myelination and a pan-Nfasc mAb to induce axonal loss. The antibody-mediated effect on myelination exhibited significant intra and inter assay variability (Cv=36%) [Table 6.1A], but was significant compared to parallel cultures treated with an irrelevant mAb of the same isotype down to a concentration of 10ng/ml (Figure 6.2). Axonal loss induced by mAb A12/18.1 was less variable (Cv= 10.6%) [Table 6.1B] but compared to antibody-mediated inhibition of myelination required higher concentrations of antibody only being significant at concentrations > 50ng/ml.

Table 6.1: Variability of complement independent MOG specific antibody mediated injury
Values shown are an average from three coverslips ± standard deviation after standardising to control values. Coefficient of variance was calculated as standard deviation/ mean and expressed as a percentage.

A

10µg/ml Z2 (18 DIV- 30 DIV)			
	Exp 1	**Exp 2**	**Exp 3**
Myelination (arbitrary unit)	0.29 ± 0.11	0.15 ± 0.05	0.2 ± 0.07
Coefficient of variance (%)	37.9	33.3	35.0

B

10µg/ml A12/18.1 (18 DIV- 30 DIV)			
	Exp 1	**Exp 2**	**Exp 3**
Axon density (% SMI-31^{+}/ total field)	40.9 ± 4.1	38.2 ± 4.9	41.4 ± 3.7
Coefficient of variance (%)	10.0	12.8	8.9

Unfortunately due to time constraints I was unable to characterise the mechanisms responsible for these antigen-specific effects on myelination and axonal survival. A number of obvious experiments using Fab and F(ab)2` antibody fragments would help elucidate the relative importance of Fc dependent effector pathways and the microglial cell response, as well as the effects of antigen cross-linking at the cell surface. It will also be important to determine that the observed effects are not dependent on low levels of endogenous complement components produced by the cultures themselves (Stevens et al., 2007; Thomas et al., 2000).

It is already known that treatment of cultured oligodendrocytes with a MOG-specific mAb is sufficient to trigger an influx of calcium and active the MAPK/Akt signalling pathways (Marta et al., 2003). Activation of these pathways is associated with cell survival and regulation of apoptosis in other systems (Datta et al., 1997; Bonni et al., 1999) and is implicated in regulation of OPC differentiation (Chew et al., 2010). The most obvious effect on cultured oligodendrocytes was retraction of processes and loss of myelin like sheets. These effects were independent of MOG partitioning into lipid rafts, but if the bound antibody was cross-linked at the cell surface not only did MOG partition into lipid rafts, but stress response proteins and elements of the cytoskeleton were also phosphorylated (Marta et al., 2005). Our in vitro studies suggest that whilst these effects inhibit myelination they do not have a detrimental effect on pre-existing myelin sheaths (Figure 6.1, 24 DIV). Moreover, the effect in the cultures was not permanent. We speculate that this is due to the continued presence of MOG- OPCs that once the antibody is removed, can still differentiate into myelinating oligodendrocytes. However in the absence of data on the fate of MOG+ cells following antibody treatment we can not exclude the involvement of a pre-existing pool of mature oligodendrocytes.

A previous report indicated that Nfasc155-specific antibodies can also inhibit myelination and in this case the antibody is believed to act by blocking the interaction of Nfasc155 with Caspr/contactin complexes on the axonal surface (Charles et al., 2002). It was assumed the pan-Nfasc mAb A12/18.1 would mimic this effect, but in this case failure of myelination was secondary to irreversible axonal loss. Why this occurs remains obscure. One possibility is that microglial activation by bound antibody may trigger an oxidative burst, the release of pro-

inflammatory cytokines or disrupt local glutamate homeostasis, all of which may lead to neuronal cell death (Matute et al., 2011; Gehrmann et al., 1995; Mrak and Griffith, 2005).

The demonstration that patient-derived antibodies mediate similar effects suggests that complement-independent effector mechanisms could play a significant role in the immunopathogenesis of MS. However the observation that antibody exposure can result in an irreversible loss of some neurons may have far wider implications. Our results clearly indicate the developing CNS contains a subset of neurons including those destined to produce myelination-competent axons that are susceptible to injury by Nfasc-specific antibodies. If present in pregnant mothers we suggest that these antibodies may cross the placenta to eliminate Nfasc+ neurons in the developing foetus. This could have profound consequences not only with respect to the immediate post natal period but also into adulthood due to perturbed white matter tract development. This hypothesis warrants further investigation as there is increasing evidence that white matter tract abnormalities are associated with a number of important psychiatric conditions including schizophrenia (Kubicki et al., 2007; Peters et al., 2010) but the underlying mechanisms are completely unknown.

7 General Discussion

Over the past 50 years circumstantial evidence has accumulated suggesting autoantibody-dependent mechanisms are involved in the pathogenesis of MS, however their clinical significance remains controversial. This is largely because of the lack of formal evidence that patients develop pathogenic autoantibody responses that actively participate in lesion formation. The demyelinating potential of some MS sera was first demonstrated *in vitro* in the 1960s, but doubts were then raised as whether this was actually an antibody-mediated phenomenon (review: Caspary, 1977). These pioneering attempts using myelinated CNS tissue cultures to identify pathogenic autoantibodies in patient sera were ultimately abandoned due to combination of factors including:

- The presence of antibody-independent myelinotoxic factors in some sera.

- The lack of objective measures to determine myelin/axonal loss.

- The identification of demyelinating responses in patients with other neurological diseases.

The broad aim of this thesis was to exploit subsequent advances in tissue culture, antibody purification and imaging techniques to develop a reliable bioassay that can be used to determine whether MS is associated with a pathogenic autoantibody response against myelinated axons.

This goal was achieved and the bioassay used to demonstrate the presence of demyelinating and in a small number of cases axopathic autoantibodies in patients with MS.

7.1 Myelinating cultures as a tool to screen for the presence of pathogenic autoantibodies in clinical samples- a critical reappraisal.

In order for myelinating cultures to be used as a reliable tool to identify demyelinating and/or axopathic autoantibodies in clinical samples they must satisfy a number of criteria, in particular:

- They must reproduce the structural organisation of myelinated fibres in the CNS.
- Variability in axonal density and myelination should be minimal.
- Large numbers of cultures should be available for intermediate to high through put screening studies.
- Analysis must be rapid and based on objective measures.

As described in chapters 3 and 4 the myelinating cultures clearly satisfy these criteria. They are characterised by the presence of numerous compact myelin sheaths associated with paranodal axo-glial junctional complexes and nodes of Ranvier that reproduce the topological organisation of these structures *in vivo*. Moreover large numbers of cultures can be prepared at any one time (~ 150 – 200/E15 litter) that exhibit limited variability with respect to axonal density and myelination. This makes them suitable for use in high throughput screens to identify pathogenic autoantibodies in clinical samples. However data analysis while semi automated remains labour intensive; for example to analyse the 43 sera described in chapter 5 required acquiring and analysing approximately 8000 images. The reproducibility of the assay suggests the number of images/cultures used in the analysis can be reduced significantly, but nonetheless the current method of outlining myelin sheaths by hand is extraordinarily time consuming. This problem is being addressed by designing an algorithm that will differentiate intact myelin sheaths from immunoreactive myelin debris and cell bodies. Ultimately the aim is to develop this into a program that will automatically process two colour immunofluorescent images and generate total pixel data and

calculated myelin densities automatically. This will not circumvent the time required to acquire the images in the first place and visual inspection of the cultures is important to control for pathogenic effects that may not be picked up by an automated program.

The other major limitation of using this culture system to detect pathogenic autoantibodies is that it is derived from rodent not human tissue. Ideally it would be preferable to use myelinating human cultures, but to attempt this using primary human tissue would be both ethically and technically taxing. However recent advances in generating human neurons and glia derived from adult (Othman et al., 2011) and embryonic CNS tissue (Satoh et al., 1994; Jana et al., 2007), as well as various stem cell populations suggests that generating a humanised myelinating culture model may be feasible in the near feature.

One possible alternative to using this dissociated myelinating culture system in order to identify pathogenic autoantibodies are organotypic slice cultures derived from post-natal cerebellum or spinal cord. These have the advantage that they reproduce the three dimensional architecture of white matter tracts in the adult CNS, but suffer from several limitations with respect to their suitability as a screening tool to identify pathogenic autoantibodies. The major drawbacks are that only a limited number of cultures (6 - 8) can be generated from each donor, there is significant slice to slice variation in myelination and that whilst these cultures reproduce the three dimensional architecture of white matter this it self restricts diffusion of antibody to its target (Harrer et al., 2009). However these organotypic cultures provide a powerful *in vitro* model in which to study remyelination not possible dissociated cell systems (Zhang et al., 2011).

7.2 The significance of identifying pathogenic autoantibody responses in patients with MS

The possibility that autoantibodies contribute to the immunopathogenesis of MS has been discussed for many years, but the specificity of this response remains unknown. The results described in this thesis suggest why this remains a complicated issue. Quite simply it appears that pathologically relevant i.e demyelinating or axopathic responses are restricted to a subset of patients and may actually be relatively rare in patients with typical relapsing remitting disease. If this is the case, then any strategy that screens patients at random using either candidate antigen or proteomics based approaches is unlikely to succeed, a problem that will be exacerbated if there are multiple pathogenic targets (review: Reindl et al., 2006). The formal demonstration that pathogenic autoantibody responses specific for myelin-associated antigens are present in certain patients will stimulate a new generation of far more focused studies to define their specificity. The results presented in this thesis suggest these demyelinating and/or axopathic autoantibody responses are a specific feature of MS, as these activities were not detected in IgG preparations obtained from patients with other neurological diseases (mainly Gullian-Barré Syndrome and chronic idiopathic demyelinating polyneuropathy) or healthy controls. Our study focussed purely on the pathogenic properties of patient-derived IgG. A number of studies have reported MS specific IgM responses against a number of antigens in particular MOG (review: Reindl et al., 2006). Therefore screening of alternative immunoglobulin isotypes using our bioassay may be informative.

It may be anticipated that these responses may ultimately not prove to be strictly MS specific, as pathogenic MOG-specific autoantibodies have been implicated in the pathogenesis of demyelination in ADEM, whilst neuron-specific autoantibodies are implicated in a number of other neurological diseases.

However, the real significance of this study lies in the implications it has with respect to improving the clinical management of MS. The demonstration that antibody-dependent mechanisms are involved in other diseases such as myasthenia gravis and NMO has resulted in rapid improvements in diagnosis and treatment. However it is too early to say whether this will also be the case for

MS patients found to be seropositive for axopathic and/or demyelinating antibodies, but complement inhibitors, high dose IgG, plasma exchange and immunoabsorption are just some of the treatments now used to suppress antibody-mediated effects in other diseases.

Which of these treatments may be most appropriate to use in MS patients will depend on precisely how these autoantibodies contribute to disease pathogenesis. The focus of this study was complement-mediated effects, but we cannot rule out a role for ADCC mediated by microglia or infiltrating monocytes and NK cells. Moreover, in concordance with previous studies (Bornstein and Raine, 1970) antibodies can also inhibit myelination and mediate axonal injury in the absence of an exogenous source of complement *in vitro*, but further studies are required to assess the potential relevance of these complement independent effects in patients.

7.3 Future Directions

The demonstration that some patients with MS develop axopathic and/or demyelinating autoantibody responses raises two immediate questions:

- What is the clinical significance of this response?
- Which antigens are targeted by these pathogenic responses?

7.3.1 Clinical studies

Although this pilot study investigated only a small number of patients, a trend was detected indicating that pathogenic autoantibody responses were most prevalent in patients with steroid non-responsive relapses. This observation suggests that in certain patients these antibodies may have evolved to become the dominant mechanism responsible for acute neurological deficits in these patients at this particular stage of disease. However whilst severe steroid non-responsive relapses are relatively rare, neuropathological studies can be interpreted as indicating antibodies are involved in lesion formation in the majority of patients (Lucchinetti et al., 2000).

To resolve this dichotomy the following clinical studies should be carried out:

- Determine the frequency of pathogenic responses in patients with respect to disease duration and clinical subtype e.g. RRMS compared to SPMS etc.

- Investigate the prognostic significance of these pathogenic autoantibody responses. Long term studies including regular MRI scans and clinical investigations will determine if the presence of a high pathogenic antibody titre is predictive of a poor prognosis.

- In patients with severe steroid non-responsive relapses investigate the relationship between the presence/titre of pathogenic autoantibodies and clinical response to plasma exchange or other treatments that target antibody-mediated effects.

- Investigate potential correlates between lesion immunopathology and axopathic and/or demyelinating serum autoantibody responses in patients where lesion biopsies are available.

7.3.2 Identification of target antigens

Although a pathogenic autoantibody response can now be identified in some patients its specificity is unknown. One approach to resolve this question is to use immobilised recombinant antigens to selectively deplete IgG preparations of antibody recognising defined candidates such as MOG or Nfasc. In one case (MS5) the axopathic and demyelinating IgG response was completely absorbed by recombinant rat Nfasc155, however in the remaining patients this was not the case. It is therefore unlikely that Nfasc155 is a dominant target for pathogenic autoantibodies in MS. Indeed in the absence of evidence that the antibodies isolated from this patient bind Nfasc155 *in situ* the role of this autoantigen in disease pathogenesis must be discussed with care. An alternative approach is to use the bioassay to identify populations of seropositive and negative patients and then use a proteomics based approach to identify potential candidates (review: Fathman et al., 2005).

The classical method relies on 2D-polyacrylamide gel electrophoresis and resolves proteins based on their molecular weight and isoelectric point. Spots of interest are then digested enzymatically and the resulting peptides analysed by mass spectrometry. However some proteins are readily resolved by 2D gel electrophoresis or are present in the gels at levels below the techniques detection threshold. More recently “shotgun” proteomics approaches have been introduced in which the composition of immune complexes formed when IgG preparations are incubated with a relevant target is analysed by mass spectrometry (review: Kinoshita et al., 2006). The demonstration that isolated myelin will absorb pathogenic antibodies present in some patients indicates this approach may be applicable to MS. However it should be stressed this technique will not identify a specific target, but rather produce a hierarchy of “hits” for each donor. These will not only (hopefully) include the relevant target(s) but also any other molecules that remain associated with the target in the immune precipitate plus other non-specific contaminants. It will therefore be important

to screen a significant number of patients and define strategies to select and validate potential candidates.

An alternative is microarray based screening of antigen libraries in which IgG from patients and appropriate controls are screened against either a panel of suspected antigens or the entire proteome expressed and deposited on microarray slides. Disease specific biomarkers are identified by comparing patient data to controls [Figure 7.1]. This approach has been used to some notable success in other autoantibody mediated diseases including autoimmune pancreatitis (Frulloni et al., 2009), alopecia (Lueking et al., 2003), rheumatoid arthritis (Lueking et al., 2003). A number of disease specific biomarkers in ovarian (Hudson et al., 2007) and prostate cancer (Wang et al., 2005) and Alzheimer's disease (Reddy et al., 2011) have also been identified using this method. However, this methodology may fail to detect pathologically relevant responses to conformation dependent targets. Moreover, it is also necessary to establish validation techniques for each candidate. The importance of this was demonstrated by the study reported by Kanter et al. in 2006 that used a lipid microarray to screen for disease associated antibody responses in MS. This study identified a disease associated response to sulphatide and then used the sulphatide-specific mAb O4 to validate the pathogenic potential of this response.

It was notable that the pathogenic antibodies identified in the course of this project were clearly not sulphatide specific as they spared large numbers of O4+ cells in the myelinating cultures.

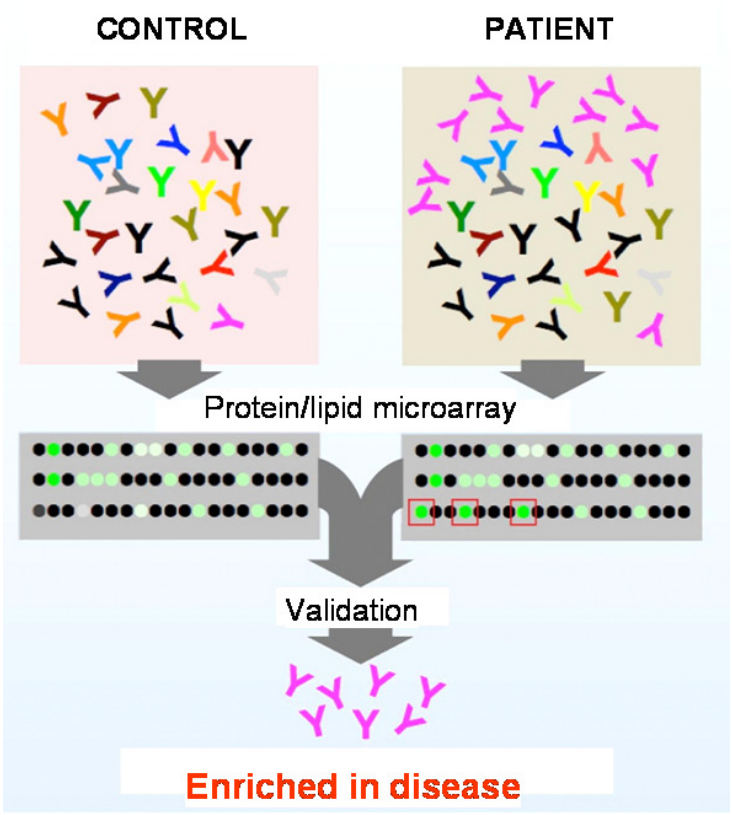


Figure 7.1: Comparative screens of case and control serum to identify disease biomarkers.

The Y-shaped figures represent IgG antibodies. The figure depicts hypothetical binding of an antibody present at high levels in an autoimmune serum sample, but not in a healthy serum sample, binding to three molecules on a microarray. After probing with a fluorescently labelled secondary antibody, this would produce a much higher intensity at these three spots on the array (indicated in by red boxes) after exposure to the autoimmune serum sample than the healthy control serum sample. Figure adapted from Reddy et al. 2011.

7.4 Final Conclusions

Autoantibodies have been implicated in the pathogenesis of MS for many years. The data presented in this thesis provides formal evidence that this response actually exists by identifying axopathic and/or demyelinating autoantibodies in a subset of MS patients. This is an extremely important observation which enhances our conceptual understanding of the pathogenesis of MS and certainly warrants further investigation. Identifying autoantibody responses specific to MS will have great implications in the clinical management of the disease ranging such as their use as diagnostic or prognostic markers. From the results of studies on the role and clinical significance of AQP-4 specific antibodies in NMO we predict that this will be a complex undertaking. However I am confident that advances in laboratory techniques, as well as our conceptual understanding of the disease will clarify the role of antibody-dependent mechanisms in MS within the next ten years.

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Appendix

Materials

All reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

General buffers & solutions

Phosphate buffered saline (10X)

1.4M NaCl (80g)

0.015M KH_2PO_4 (2g)

0.027 KCl (2g)

0.19M $Na_2HPO_4.2H_2O$ (29g)

Made up to 1L with dH_2O and diluted 1 in 10 for use.

Sodium Hydroxide (1M)

Dissolve 40g sodium hydroxide pellets in 1L distilled H2O.

Hydrochloric acid (1M)

Slowly add 88.8ml concentrated hydrochloric acid to 900ml distilled H2O. After addition of acid make up to 1L with distilled H2O.

Immunocytochemistry

Paraformaldehyde solution (4%)

Paraformaldehyde (4g)

Add 1M NaOH until solution becomes clear.

Make up to 100ml with 1x PBS and store in 5ml aliquots at -20°C.

Bouins fixative

Saturated picric acid (30ml)

Formaldehyde (10ml)

Glacial acetic acid (2ml)

0.5% Triton X-100

Solubilise 0.5ml triton X-100 in 100ml distilled H2O and store at 4°C.

Blocking Buffer

1% Bovine serum albumin (1g)

10% Normal Goat Serum (10ml)

0.3M glycine (2.3g)

Make up to 100ml with 1x PBS and stored in 5ml aliquots at -20°C.

ELISA

Wash buffer

0.05% Tween-20 (0.5ml)

Make up to 1L with 1X PBS.

Secondary antibodies

Horseradish peroxidase conjugated secondary antibodies were purchased from Invitrogen (UK).

Citric Acid (0.1M)

Dissolve 21g sodium citrate in 1L distilled H2O.

Sodium Phosphate (0.2M)

Dissolve 28.4g in 1L distilled H2O.

Substrate solution

1 substrate tablet OPD

28ml 0.1M Citric acid

32ml 0.2M Na_2HPO_4

Once the tablet has fully dissolved, add 20µl H_2O_2 to activate prior to use keep at 4°C and protect from light.

Sulphuric acid (4M)

Slowly add of 222ml concentrated sulphuric acid (95-98%) to 778ml of dH_2O.

SDS-PAGE

Resolving gel buffer (4X)

250ml 1.5M Tris pH 8.8

10ml 10% SDS

400ml dH_2O

10% SDS (w/v)

Add 100g SDS in 900ml dH_2O and heat to 68°C to dissolve the crystals. Adjust to pH 7.2 with 1N HCl and make to 1L.

10% APS (w/v)

Add 1g ammonium persulphate in 10ml dH_2O. Store at -20°C.

Stacking gel buffer

250ml 1.5M Tris pH 6.8

10ml 10% SDS

400ml dH_2O

Resolving gel (15%)

5ml 40% acrylamide/bis solution 19:1 (Biorad, USA)

2.5ml 4X resolving gel buffer

2.39ml dH_2O

100µl 10% APS

10µl TEMED

Stacking gel (6%)

1.3ml 40% acrylamide/bis solution 19:1 (Biorad, USA)

2.5ml stacking buffer

6.1ml dH_2O

Sample loading buffer (3X)

2.4ml 1M tris-HCl pH 6.8

3ml 20% SDS

3ml 100% glycerol

1.6ml β- mercaptoethanol

0.006g bromophenol blue

Store at 4°C.

Running buffer (10X)

30.3g Tris base

144g glycine

10g SDS

Make up to 1L with dH_2O

Protein G affinity chromatography

Binding buffer

20mM NaH_2PO_4 (137g)

Make up to 500ml with dH_2O and adjust to pH7 using 1M NaOH.

Elution Buffer

0.1M glycine (75g)

Make up to 500ml with dH_2O and adjust to pH3 using 1N HCl.

Tris-HCl (1M)

Dissolve 60.5g tris base in 500ml with dH_2O and adjust to pH9 using 1N HCl.

Primers

Nfasc155 (Forward): 5`-CAGTGGAACCGCGTCTACTC

Nfasc155 (Reverse): 5`- ACCACAACCATCTCCAGCTT

Nfasc186 (Forward): 5`- TCTCCCTCAGTGCCAGGAC

Nfasc186 (Reverse): 5`-TGGGATAGATGGGAACTGTTG

ß-actin (Forward): 5`-TTGTAACCAACTGGGACGATATGG

ß-actin (Reverse): GATCTTGATCTTGATGGTGCTGCTAGG

Myelin purification

Sucrose (0.32M)

Dissolve 109.5g of sucrose in 1L distilled H_2O with 1x protease inhibitor cocktail.

Sucrose (0.80M)

Dissolve 273g of sucrose in 1L distilled H_2O 1x protease inhibitor cocktail.

Cell culture stock solutions

Poly-L-lysine

25mg of poly-L-lysine hydrobromide

Dissolve in 6.25ml sterile H_2O. Sterilise by filtering through a 0.22µ filter and store at -20°C in 66µl aliquots.

For coating flasks/ coverslips add one aliquot/20ml. Ensure flasks/coverslips are thoroughly rinsed and air dried before use.

Collagenase

Dissolve 20mg collagenase (Invitrogen, UK) to 20ml Leibovitz L-15 media (Invitrogen). Sterilize using a 0.22µ filter and store at -20°C.

SD (soybean trypsin inhibitor)

Dissolve 13mg trypsin inhibitor (soybean), 1mg DNAse I (bovine pancreas) and 75mg BSA fraction V in 25ml Leibovitz L-15 media (Invitrogen). Sterilize using a 0.22µ filter and store at -20°C.

Glial cell culture media

Neurosphere media

Glucose (30%)

Dissolve 30g of D-glucose to 100ml sterile H2O with constant stirring. Filter sterilise through a 0.22µ filter and store at -20°C.

Sodium hydrogen carbonate (7.5%)

Gradually dissolve 7.5g $NaHCO_3$ to 100ml sterile H2O. Filter sterilise through a 0.22µ filter and store at -20°C.

DMEM/F12 (10X)

Measure 400ml sterile H2O in to a sterile 1 litre beaker. Dissolve 1 pot of DMEM powder (high glucose, Invitrogen), which usually makes up 5 litres and allow to fully dissolve. To this mix add 5 pots of F12 (usually 1L/pot) (Invitrogen). Bring final volume up to 500ml and filter through a 0.22µ filter and store at -20°C.

Hepes (1M)

Dissolve 23.8g HEPES and add to 80ml sterile H2O, once fully dissolved bring final volume up to 100ml. Sterilise by filtration through a 0.22µ filter and store at -20°C.

Putrescine

Weigh 96.6mg putrescine and add to 100ml of sterile H2O once dissolved store in 25ml aliquots at -20°C.

Selenium

Dissolve 1mg sodium selenate in 1.93ml sterile H2O (3mM) and store at -20°C.

Progesterone

Dissolve 1mg progesterone in 1.59ml 95% ethanol and store at -20°C.

Hormone mix (10X)

Firstly prepare 1X DMEM/F12 as follows:

10X DMEM/F12	25ml
30% Glucose	5ml
7.5% $NaHCO_3$	3.75ml
1M Hepes	1.25ml
Sterile H2O	187.5ml

To this solution add:

250mg apo-human transferrin

6.25ml human insulin (recombinant)

25ml 600µM putrescine (final concentration 60µM)

25µl 3mM sodium selenate

25µl progesterone

Epidermal growth factor (EGF)

Dissolve 1mg of recombinant murine EGF (Peprotech) in 1ml of sterile H2O store at -20°C. Use at a final concentration of 4ng/ml (4µl EGF/ 20ml neurosphere media).

Neurosphere media

Neurosphere media

Sterile H2O	185ml
DMEM/F12 (x10)	25ml
Hormone Mix (x10)	25ml
30% Glucose	5ml
7.5% $NaHCO_3$	3.75ml
1M HEPES	1.25ml
L-Glutamine (200mM)	2.5ml

Astrocyte growth media

Astrocyte Media (DMEM + 10% FBS)	
DMEM (1g/L glucose, Invitrogen)	179ml
Foetal bovine serum	20ml
L-Glutamine (200mM)	1ml

Plating media

Plating Media	
DMEM (4.5g/L glucose)	50ml
Horse Serum	25ml
HBSS	25ml

Differentiation media (DM)

Biotin

To make a 1mg/ml stock: dissolve 100mg biotin in 100ml 1M NaOH. Dilute 1 in 100 with sterile H_2O, filter using a 0.22µ filter and store at -20°C. Use at a final concentration of 10µg/ml.

Hydrocortisone

Dissolve 100mg of hydrocortisone (H2O soluble form) in 27.6ml sterile H2O. Dilute 1 in a 1000 by adding 20µl to 19.88ml of sterile H2O to create a 10µM stock. Filter through a 0.22µ filter and store in 250µl aliquots at -20°C.

Insulin

Dissolve 100mg insulin (bovine pancreas) in 200ml 10mM HCl/H_2O (final concentration 500µg/ml). Filter through a 0.22µ filter and store in 2ml aliquots at -20°C. Use at a final concentration of 10µg/ml (1ml/ 50ml media).

N1 supplement (100X)

N1 supplement containing: 0.5 mg/ml human transferrin (partially iron-saturated), 0.5 µg/ml sodium selenite, 1.6 mg/ml putrescine, and 0.73 µg/ml progesterone.

Differentiation media

Differentiation media + Insulin (50ml)

DMEM (4.5g/L glucose)	47.25ml
Biotin (1mg/ml)	50µl
N1 Supplement (100X)	500µl
Hydrocortisone (10µM)	250µl
Insulin (0.5mg/ml)	1ml

Differentiation media – Insulin (50ml)

DMEM (4.5g/L glucose)	48.2ml
Biotin (1mg/ml)	50µl
N1 Supplement (100X)	500µl
Hydrocortisone (10µM)	250µl

Hybridoma cell culture media

Z2 and 8-18C5 hybridoma

RPMI complete + 10% FBS

RPMI (1640)	429.5ml
Foetal bovine serum	50ml
L-glutamine (200mM)	5ml
Non-essential aas (100X)	5ml
Penicillin/streptomycin	5ml
Sodium Pyruvate (100mM)	5ml
β- mercaptoethanol (50mM)	500µl

A12/18.1 hybridoma

DMEM complete + 10% FBS

DMEM (4.5g/L glucose, Invitrogen)	424.5ml
Foetal bovine serum	50ml
L-glutamine (200mM)	5ml
Non-essential aas (100X)	5ml

L-Asparagine (2.5M)	5ml
Sodium Pyruvate (100mM)	5ml
Penicillin/streptomycin	5ml
β- mercaptoethanol (50mM)	500µl

Transfected cell media

Untransfected HeLa cells

DMEM complete + 10% FBS	
DMEM (4.5g/L glucose)	429.5ml
Foetal bovine serum	50ml
L-glutamine (200mM)	5ml
Non-essential aas (100X)	5ml
Sodium Pyruvate (100mM)	5ml
Penicillin/streptomycin	5ml
β- mercaptoethanol (50mM)	500µl

Transfectants

DMEM complete + 10% FBS with G418	
DMEM (4.5g/L glucose)	429.5ml
Foetal bovine serum	50ml
L-glutamine (200mM)	5ml
Non-essential aas (100X)	5ml
Sodium Pyruvate (100mM)	5ml
Penicillin/streptomycin	5ml
G418	5ml
β- mercaptoethanol (50mM)	500µl

Statistics

All calculations were performed using Microsoft Excel. Values were calculated using the formulae defined below;

Mean

$$\bar{x} = \sum_{i=1}^{n} x_i / n$$

Standard deviation

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Standard error of the mean (S.E.M)

$$s = \frac{\sigma}{\sqrt{n}}$$

T-Test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1^2} + \frac{s_2^2}{n_2^2}}}$$

Coefficient of Variance

$$\varepsilon = \frac{\sigma}{\bar{x}}$$

Myelin Quantification Macro (written by John Annan)

```
//set the thresholds here
thresh_red = 33;
thresh_green = 79;
thresh_blue = 128;

//get the name of the open image, and the folder it came from
iTitle=getTitle;
imageDirectory = getDirectory("image");
print (imageDirectory);
print ("Image name,Red Black,Red White,Green Black,Green
White,Blue Black,Blue White")

//build a list of all images in the folder
fList=getFileList(imageDirectory);
selectWindow(iTitle);
run("Close");

//start the loop to open files
for (i=0; i<fList.length; i++){
     //check that file is a valid image
     if (endsWith(fList[i],"tif")) {
     //if it is valid, open it
     open(imageDirectory + fList[i]);

     //build names of colour component images
     iTitle=getTitle;
     iTitleRed=iTitle + " (red)";
     iTitleGreen=iTitle + " (green)";
     iTitleBlue=iTitle + " (blue)";


     //split image into colour components
     run("RGB Split");

     //get red and blue areas and threshold

     string="image1=["+iTitleGreen + "] operation=AND
image2=[" + iTitleBlue+"] create";
     run("Image Calculator...", string);
     iTitleAND = getTitle;
     setThreshold(thresh_blue, 255);
     run("Threshold", "thresholded remaining black");
     run("Invert");

     //measure areas
     getStatistics(area, mean, min, max, std, histogram);
     n_blue_black=histogram[0];
     n_blue_white=histogram[255];

     //close windows
     selectWindow(iTitleAND);
```

```
        run("Close");
        selectWindow(iTitleBlue);
        run("Close");


        //get red threshold
        selectWindow(iTitleRed);
        setThreshold(thresh_red, 255);
        run("Threshold", "thresholded remaining black");
        run("Invert");

        //measure areas
        getStatistics(area, mean, min, max, std, histogram);
        n_red_black=histogram[0];
        n_red_white=histogram[255];

        selectWindow(iTitleRed);
        run("Close");

        //get green threshold
        selectWindow(iTitleGreen);
        setThreshold(thresh_green, 255);
        run("Threshold", "thresholded remaining black");
        run("Invert");

        //measure areas
        getStatistics(area, mean, min, max, std, histogram);
        n_green_black=histogram[0];
        n_green_white=histogram[255];

        selectWindow(iTitleGreen);
        run("Close");

        //display results
        print
(iTitle+","+n_red_black+","+n_red_white+","+n_green_black+","
+n_green_white+","+n_blue_black+","+n_blue_white);

        }
}
```