Functional Significance of Autoantibodies in Inflammatory Demyelinating Diseases of the Central Nervous System

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ABSTRACT

Multiple Sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system which currently affects approximately 107, 000 people in the UK, with around 5,000 people being newly diagnosed each year. Here, in Scotland, is the highest disease incidence in the world with around 1 in 170 women in Orkney currently living with the disease. These statistics emphasise the importance of researching and understanding this disease, so as to develop better therapeutics to fight MS.

This thesis focused on the role of antibodies in MS, a widely studied area. There is already a great deal of published data supporting the presence and function of autoantibodies in disease pathogenesis. A more recent development was the discovery that a significant proportion of paediatric MS and acute disseminated encephalomyelitis (ADEM) patients have autoantibody responses directed against the extra-cellular domain of myelin oligodendrocyte glycoprotein (MOG). It has been shown that MOG-specific autoantibodies can induce demyelination in animal models but as yet, the clinical significance of these antibodies in human disease remains unknown. In animal models, MOG-specific antibodies exacerbate disease and mediate demyelination and it was hypothesis that this would also be the case in patients with multiple sclerosis. This hypothesis was investigated throughout this thesis via two main approaches:

(1) Explore the pathogenicity of patient derived IgG using a well-characterised *in vitro* bioassay.

(2) Based on the hypothesis determine the efficacy of depleting or tolerising MOG-reactive B cells *in vivo*.

To study this, the effects of patient sera, which were positive for MOG reactivity, were tested on myelinating neural cell cultures. These studies showed no correlation

between the presence of MOG antibodies and demyelination, therefore suggesting that these antibodies did not mediate myelin loss.

MOG-specific antibodies were also studied *in vivo* using MOG-induced experimental autoimmune encephalomyelitis (EAE), a model which reproduces many of the clinical and pathological features of paediatric MS. Two MOG-specific therapeutic approaches were analysed: (i) induction of antigen-specific tolerance using low doses of soluble MOG, and (ii) treatment with a MOG-specific B cell immunotoxin. They both demonstrated MOG-specific immunotherapies can be efficacious. However, increasing evidence indicated full clinical protection would require targeting both MOG-specific T and B cell dependent patho-mechanisms.

As these antibodies did not affect the myelin in the bioassay and the MOG-specific treatments had limited beneficial effect *in vivo*, it raised the question are these autoantibodies irrelevant to human disease? This was addressed by investigating the effects of low titres of MOG-specific antibodies in the absence of complement. The data presented in this thesis showed that autoantibodies, independent of complement, mediated myelin loss, microglial activation and induced chemokine production, processes which could all contribute to disease pathogenesis. Therefore, revealing a new perspective role for antibodies in MS.

AUTHOR'S DECLARATION

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.

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Figure 1. Acknowledgements.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Ab	antibody
AChR	acetylcholine receptor
ADCC	antibody dependent cellular cytotoxicity
ADEM	acute disseminated encephalomyelitis
Ag	antigen
AICD	activation-induced cell death
ATM	acute transverse myelitis
APC	antigen presenting cells
APL	altered peptide ligand
AQP4	aquaporin-4
BBB	blood brain barrier
BLAST	Basic Local Alignment Search Tool
BR	binding ratio
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CIA	collagen-induced arthritis
CIDP	chronic idiopathic demyelinating polyneuropathy
CIS	clinically isolated syndrome
CNS	central nervous system
CO_2	carbon dioxide
CSF	cerebral spinal fluid
C_T	cycle threshold
DAPI	4'-6-diamidino-2-phenylindole
DEPC	diethylpyrocarbohydrate
dH_2O	distilled water
DIV	days *in vitro*
DNA	deoxyribonucleic acid
DM+	differentiation media with insulin
DM-	differentiation media without insulin
DMEM	Dulbecco's Modified Eagle Medium
cDMEM	complete Dulbecco's Modified Eagle Medium
d.p.i.	days post immunisation
E	embryonic day
EAE	experimental autoimmune encephalomyelitis
ECDI	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
E. Coli	*Escherichia coli*
EDSS	expanded disability status scale
EDTA	ethylenediaminetetraacetic acid
EF-2	elongation factor 2
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ETA	*Pseudomonas aeruginosa* exotoxin A
EtOH	ethanol
FACS	fluorescence activated cell sorting

Fab	fragment antigen binding
FBS	foetal bovine serum
FCS	foetal calf serum
Fc	fragment crystallisable
FcR	fragment crystallisable receptor
FDA	U.S. Food and Drug Administration
g	relative centrifugal force
G418	geneticin
GalC	galactocerebroside
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GOI	gene of interest
H_2SO_4	sulphuric acid
HBSS	Hank's balanced saline solution
HC	healthy control
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IEF	isoelectric focussing
IFNγ	interferon-γ
IFNβ	interferon-β
Ig	immunoglobulin
IL-	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kiladaltons
L-15	Leibovitz medium
LB	L. Broth
LDAO	lauryldimethylamine-oxide
LN	lymph node
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
MAC	membrane attack complex
MAG	myelin associated glycoprotein
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
MFI	mean fluorescence intensity
MHC	minor histocompatibility complex
MG	myasthenia gravis
MOG	myelin oligodendrocyte glycoprotein
Mono ADS	one acquired demyelinating event
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis

NaH_3	sodium azide
NK	natural killer
NMDA	N-methyl D-aspartate
NMO	neuromyelitis optica
NOD	non-obese diabetic
NSM	neurosphere media
OCB	oligoclonal bands
OD	optical density
OIC	ovalbumin immune complex
O/N	overnight
ON	optic neuritis
OND	other neurological disease
OPCs	oligodendrocyte progenitor cells
OVA	ovalbumin
P	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PLL	poly-L-lysine
PLP	proteolipid protein
PM	plating media
PML	progressive multifocal leukoencephalopathy
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
PTx	pertussis toxin
qRT-PCR	quantitative real time polymerase chain reaction
RA	rheumatoid arthritis
rMOG	recombinant myelin oligodendrocyte glycoprotein
RPMI	Roswell Park Memorial Institute-1640 media
RRMS	relapsing remitting multiple sclerosis
RT	room temperature
TPE	therapeutic plasma exchange
s.c.	subcutaneous injection
SD	Sprague Dawley
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLE	systemic lupus erythematosus
SMI31	phosphorylated neurofilament
SPMS	secondary progressive multiple sclerosis
TIMP-1	tissue inhibitor of metalloproteinases-1
TNFα	tumour necrosis factor-α
VEGF	vascular endothelial growth factor

CHAPTER ONE

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 PAEDIATRIC MULTIPLE SCLEROSIS

1.1.1 Introduction to paediatric multiple sclerosis

Multiple Sclerosis (MS) was first described as a combination of ataxia, tremor and speech disturbances by Charcot (Charcot, 1868), but he himself credits a fellow professor, Jean Cruveilhier, for his earlier illustrations of lesions and clinical definition of the disease [*Anatomie pathologique du corps humain* (1835-42). (Field, 1980)]. Today MS is described as a chronic, inflammatory demyelinating disease of the central nervous system (CNS), characterised by the formation of demyelinated plaques of gliotic scar tissue associated with varying degrees of axonal injury and loss. This pathology is attributed to the effects of repeated episodes of focal inflammation and demyelination. These lesions may develop in virtually any region of the CNS and may lead to any of a wide range of motor, sensory and/or cognitive deficits. However, there is a predilection for lesions to develop in the optic tract, periventricular and spinal cord white matter, and cortical grey matter. Clinical deficits in MS are due to the culmination of acute inflammation, demyelination and axonal injury and loss.

Until recently MS was considered a disease of young adults, which were diagnosed in their second or third decade of life. In 1868 Charcot himself set 14 as the lowest age of disease onset, but less than 30 years later Eichorst confirmed a case of childhood MS in a 9 year old boy who, amongst other symptoms, presented with muscle weakness and paralysis affecting both legs before death (Eichorst, 1896). Since then a substantial body of evidence has accumulated confirming that MS can indeed manifest prior to puberty (Schupfer, 1902, Low and Carter, 1956). This included a large cohort of 125 children with disease onset below the age of 16 (Duquette et al., 1987). Despite these early findings, it is only in the past 10 to 20 years that any significant advances have been made in our understanding of this juvenile neurological disease (Hanefeld, 2007). However, paediatric MS is now

recognised as one of the most common juvenile diseases of the CNS and recent studies indicate it accounts for 3 to 5% of all cases of MS (Fernandez Carbonell and Chitnis, 2013).

1.1.2 Diagnosis of paediatric multiple sclerosis

In adults MS is diagnosed using the McDonald Criteria, which were initially presented in 2001 by the International Panel (McDonald et al., 2001). Data driven revisions of these criteria were made in 2005 (Polman et al., 2005), and again in 2010 (Polman et al., 2011), but the core criterion for a diagnosis of MS remains the dissemination of lesions in time and space. Other paraclinical criteria are based on magnetic resonance imaging (MRI) studies and analysis of cerebrospinal fluid. These can be used to support a diagnosis of MS, but making a definitive diagnosis in paediatric patients is more challenging than in adult-onset cases because the clinical presentation can be much more variable. This is a particular problem with respect to two other inflammatory demyelinating diseases of the CNS, acute disseminated encephalomyelitis (ADEM) and neuromyelitis optica (NMO). In children these diseases often mimic the clinical and radiological features of MS. The lack of clear diagnostic criteria to differentiate paediatric MS from other neurological conditions is not a trivial problem, as treatments that are beneficial in MS may be counter-productive in other diseases and *vice versa*. To resolve this problem the National MS Society (NMSS) established the International Paediatric MS Study Group to develop a common diagnostic consensus for childhood-onset MS (Krupp et al., 2013).

MS is now classified as paediatric when the first neurological signs of disease occur under the age of 18. This can occur in children as young as two, although disease is rarer in pre-pubertal children and accounts for only 20 - 30% of paediatric MS cases (Chitnis, 2013). A diagnosis of paediatric MS requires the patient to develop two clinical incidents of CNS demyelination separated in space and time (at least 30 days apart from each other), as required for the diagnosis of adult MS (Krupp et al., 2007). MRI scans play a pivotal role in the diagnosis of paediatric MS and may

even be used alone to meet the McDonald criteria providing three of the following features are present; 1) one gadolinium enhancing lesion or nine or more white matter lesions 2) three of more periventricular lesions 3) a juxtacortical lesion 4) an infratentorial lesion. MRI techniques still have a long way to go before being able to accurately distinguish MS from other neurological diseases, particularly as many juvenile demyelinating disorders feature variable and ill-defined lesions (Hynson et al., 2001, Chabas et al., 2010). However, MRI can also be used in conjunction with cerebrospinal fluid (CSF) analysis to confirm diagnosis (Yeh et al., 2009). The CSF must show either oligoclonal bands (OCBs) or an elevated IgG index (Krupp et al., 2007). Importantly the child's first clinical events must clearly exclude a diagnosis of ADEM, which is defined as a multifocal disease (full diagnostic criteria is discussed in section 1.2.1). Further revisions of the guidelines set out by the International Paediatric MS Study Group were made in 2013, which highlighted differences in the clinical presentation between younger (under the age of 12) and older children with MS. It highlighted the need at time of diagnosis to take into consideration the observation that earlier attacks are more "ADEM-like" (Krupp et al., 2013).

1.1.3 Paediatric multiple sclerosis pathogenesis and etiology

Studying paediatric MS provides a novel opportunity to address questions related to the role of environmental factors in disease development, as there is only a limited time interval between exposure to environmental factors believed to trigger disease development and diagnosis. Moreover, the immune mechanisms underlying disease development in childhood MS are broadly identical to those involved in adult onset disease. There are however subtle differences between paediatric and adult onset MS.

1.1.3.1 Pathogenesis and clinical presentation

Paediatric MS can present in two clinical phenotypes as defined by their disease course; (1) relapsing-remitting MS (RRMS) and (2) primary progressive MS (PPMS).

RRMS is characterised by repeated, unpredictable episodes of disability followed by periods of complete or partial recovery, whereas PPMS is characterised from its onset by accumulation of disability in the absence of obvious clinical relapses or remissions (Renoux et al., 2007). PPMS has several features that make it different from RRMS. In addition to the different disease course, there is also no female dominance in PPMS and the average age of onset is later (Koch et al., 2013). Children rarely develop PPMS, therefore paediatric disease is almost solely RRMS (98% compared to 84% in adult onset-MS). This phenotypic bias in children with MS to a relapsing-remitting disease course was first observed in 1987 (Duquette et al., 1987) and was recently confirmed (Gorman et al., 2009).

A MRI study investigating differences in lesions in paediatric MS patients compared early-onset (<11 years) and later-onset (≥11 years) MS plaques (Chabas et al., 2008). The results revealed that the overall numbers of lesions were similar between groups but the lesions were less well-defined and confluent in the younger patients. In addition 92% of the early-onset patients had a decrease in T2-bright lesion load at their second scan, compared to their initial scan, which was taken soon after the first clinical event. This was significantly more than in the older patient group, suggesting there are different pathological processes occurring in adolescent lesions. These results, although from a small cohort of patients, shed light on lesion dynamics in patients less than 11 years of age and have implications with respect to using MRI scans for diagnosis in these very young patients. The areas of the brain most affected also appear to differ between paediatric and adult patients, the former tending to develop less inflammation in the spinal cord but more in the posterior fossa of the brain (Mowry and Waubant, 2010). Taken together these data indicate lesions in early-onset paediatric MS have distinct characteristics that differentiate them from post-pubertal lesions which appear to resemble adult-onset MS lesions.

Studies analysing CSF samples revealed early-onset paediatric MS was associated with increased infiltration of white blood cells compared to post-pubertal patients

(Yeh et al., 2009, Chabas et al., 2010). Most markedly, there was a selective increase in the number of neutrophils observed in younger patients, as well as fewer OCBs and autoantibodies. Correspondingly, fewer young patients had an increased IgG index compared to the later-onset patient cohort (Chabas et al., 2010). Overall, these studies led to the proposal that disease activity in MS patients below the age of 11 was associated with a more pronounced innate immune component compared to post-pubertal and adult-onset patients.

1.1.3.2 Disease progression

Disease progression and disability outcomes are only slightly different in paediatric and adult onset MS. Although the short-term prognosis can be favourable, more than 50% of patients diagnosed with MS in childhood will develop secondary progressive MS (SPMS) associated with severe physical and/or cognitive disabilities by the age of 30 (Banwell et al., 2007, Renoux et al., 2007). Comparing time of conversion to SPMS with adult-onset MS, paediatric patients take approximately 10 years longer to develop SPMS than adult onset patients, but this end point is reached approximately 10 years earlier (Renoux et al., 2007). Moreover, they have a significantly higher rate of relapse compared to patients with adult-onset MS (Gorman et al., 2009), which could be an indication that there is more inflammatory activity occurring in the CNS of these younger patients. However, this does not result in a faster accumulation of disability compared to older patients. This observation has led to the suggestion that the functional and structural plasticity of the developing CNS in young MS patients' may compensate for damage caused by inflammatory demyelination.

1.1.3.3 Cognitive and social interaction deficits

Paediatric MS patients face significant cognitive impairment and they often start to develop neurophysiological symptoms at the critical time of schooling. The cause of these deficits has not yet been elucidated. It could be related to poor school attendance; around 40% of children with MS struggle with school and recreational

activities due to severe fatigue (Banwell et al., 2007), therefore disrupting their education and compromising their sociological development. On the other hand it could also be due to direct functional effects related to neurodegeneration, loss of neural connectivity and compromised development of white matter pathways required for cognitive tasks. These deficits will also have psychosocial effects. In a small cohort of children with MS, neuropsychological tests identified deficits specific to certain cognitive tasks in every patient (Banwell and Anderson, 2005). In particular they had problems with self-generated organisational strategies and tasks requiring efficient processing speed or working memory. These observations paralleled the children's increasingly poor performance in high school as these cognitive skills became more important in higher education. Interestingly, this study did not find a link between disease severity, quantified by the Expanded Disability Status Scale (EDSS), and cognitive impairment. Problems with cognitive function are documented in around 30% of children with MS and this percentage increased with decreasing age of disease onset (MacAllister et al., 2005, Yeh et al., 2011, Banwell and Anderson, 2005). The evident effect of MS on cognition makes early, effective treatment for these children even more crucial.

1.1.3.4 Epidemiology

Studies investigating links between ethnicity and paediatric MS interestingly showed that Hispanic children had a more severe disease and did not respond as well to therapies (Yeh et al., 2009). This pattern was not observed with African American patients, which is in contrast to adult MS studies. These investigations found that African Americans had an increased rate of disease progression, increased tissue damage and number of lesions (Kister et al., 2010, Weinstock-Guttman et al., 2010).

In addition to the differences in ethnic distribution of disease between adult and childhood disease, there are also differences in gender dominance. Prevalence of MS in children under the age of 10 or pre-pubertal shows no gender bias, but disease becomes more common in females around the age of puberty and

afterwards, suggesting a role for sex hormones in disease susceptibility (Chitnis, 2013). In adult onset MS it is much more prevalent in women (2:1 female to male ratio; (Whitacre, 2001)) and this ratio appears to be increasing as more recent population based studies indicate the female:male ratio is now around 3:1 (Orton et al., 2006, Maghzi et al., 2010).

1.1.4 Potential treatments of paediatric multiple sclerosis

With emerging evidence highlighting the significant impact of childhood MS on cognitive and physiological function the need for early and effective treatments tailored for these children is exceedingly important. Treatment of juvenile patients' needs careful consideration as with children as young as 2, short and long term side effects could be very different from adults. Thus far, there is no evidence showing that the current treatments used in adults have any detrimental effects in children. However, treatment of young patients is still in its infancy and as yet adverse effects and tolerability of long-term treatment has not yet been established. This is of particular concern as there is no data on how the treatments will affect processes like puberty and growth.

A retrospective collaborative study assessed the treatment of 258 children with RRMS, (Yeh et al., 2009). The study highlighted that it was important to start treatment as soon as possible, which unfortunately can be problematic as diagnosis is often difficult. The patients in this study were all treated initially with first-line therapies, interferon beta (IFNβ) or glatiramer acetate, which were well tolerated and were predominantly efficacious. Some patients had to switch to second-line disease modulating therapies (DMTs) or immunosuppressants as they had breakthrough relapses or MRI changes. In some cases treatments were used in combination. Overall, this study showed paediatric patients tolerated and responded well to adult MS treatment regimens.

As yet no DMTs are officially approved for paediatric patient use as patients under the age of 18 are usually not involved in clinical trials but due to the apparent

overlap in disease pathogenesis young patients are already receiving these treatments (Pohl et al., 2007). Currently there are 7 approved drugs for RRMS in adults: -

- IFNβ is available in two formulations; IFN-β1a (Rebif or Avonex) and IFN-β1b (Betaseron or Extavia), both of which inhibit T cell activation and decrease blood-brain barrier (BBB) permeability but have slightly different pharmokinetics. In 1993 IFN-β1b was the first drug to be licensed for treating MS. Since then all these IFNβ drugs have been approved for treatment of RRMS as they have been shown to reduce disease activity and clinical exacerbations (Lim and Constantinescu, 2010). A retrospective study investigating effects of IFN-β1a used at adult doses in paediatric-onset patients showed that the drug was well tolerated and that it reduced the annual relapse rate (Tenembaum et al., 2013).

- Glatimer acetate (Copaxone) is a synthetic co-polymer which has a very similar structure to myelin basic protein (MBP), a significant protein component of the myelin sheath. This drugs mode of disease suppression is not fully understood but is believed to modulate the immune response by blocking MBP-specific T cells (Scott, 2013). It has been approved for the treatment of RRMS and clinically isolated syndrome (CIS) patients.

- Natalizumab (Tysabri) is a humanised monoclonal antibody against the integrin $\alpha_4\beta_1$, which is an essential adhesion molecule required by the immune cell to migrate into the CNS. It first was approved by the FDA in 2006. Clinical trials showed that the drug reduced relapses, lesion load and decreased the risk of disability progression (Polman et al., 2006a). They also showed that IFN-β1a treatment plus natalizumab was a more effective treatment than IFN-β1a alone (Polman et al., 2006b).

- Mitoxantrone (Novantrone) is a cytotoxic agent with immunosuppressive properties. This drug is believed to suppress inflammation through a variety

of ways; inhibition of T cell activation, decreased T and B cell and macrophage cell division, defective antigen presentation and reduction of macrophage-mediated demyelination and cytokine production (Lim and Constantinescu, 2010). Clinical studies revealed it supressed disease in worsening RRMS patients and SPMS patients and has been approved for treatment of both (Scott and Figgitt, 2004).

- Fingolimod (Gilenya) is a drug targeting the sphingosine 1-phosphate receptor which is needed for immune cells to leave the lymph nodes; therefore this treatment retains cells in these lymphoid structures inhibiting them from entering the CNS. Clinical trials with this drug showed significant decreases in relapse rates (Devonshire et al., 2012). It is currently FDA approved for the use in patients with RRMS.

- Teriflunomide (Aubagio) is a pyrimidine synthesis inhibitor, which inhibits the enzyme dihydroorotate dehydrogenase essential for DNA synthesis, therefore reduces lymphocyte proliferation and activation. It was approved by the FDA in 2012 for the treatment of RRMS after a successful phase III clinical trial (O'Connor et al., 2011).

- Dimethyl fumarate (Tecfidera™) was approved by the FDA in 2013 for a first-line therapy in RRMS patients. Two large phase III trials showed reduction in relapses, MRI lesions and disability progression (Fox et al., 2012, Gold et al., 2012). How it suppresses disease is still unknown but it is thought to be due to the enhancement of an antioxidant response via activation of nuclear factor (erythroid-derived 2)-like 2 (Scannevin et al., 2012).

Progressive forms of MS have few treatment options as only Mitoxantrone and IFN-β1b are licensed for the treatment of SPMS and as yet there are no FDA approved treatments for PPMS. There are however many drugs currently in development. Monoclonal antibodies under investigation include Alemtuzumab,

Rituximab and Daclizumab, which are against CD52 on all lymphocytes, CD20 on B cells and CD25 on activated T cells, respectively. Other treatments include plasma exchange, which is only used in severe cases of adult MS. Therapeutic plasma exchange for the treatment of paediatric patients has only been documented in one case (Takahashi et al., 1997). The patient responded markedly to the treatment, suggesting it may be an effective, alternative therapy for certain children with severe MS.

The future of clinical trials needs to be multinational and have clear guidelines on defining adequate therapies to improve disease control. In addition, further research needs to investigate advanced ways to differentiate paediatric MS from other juvenile neurological disorders, which can have very similar presentation.

1.2 OTHER JUVENILE DEMYELINATING DISEASES

1.2.1 Acute disseminated encephalomyelitis (ADEM)

ADEM is defined as a monophasic immune-mediated clinical event that affects multifocal areas of the brain and spinal cord leading to a polysymptomatic disease, which includes some type of encephalopathy. It features widespread demyelination centred on the white matter of the brain and spinal cord, with particular damage occurring in the perivenous region (Wender, 2011). ADEM can occur at any age but is predominantly a juvenile disease with diagnosis commonly occurring between the ages of 5 and 8 (Tenembaum et al., 2007). Unlike MS, ADEM studies have shown that disease is more prevalent in boys rather than girls (Murthy et al., 2002, Tenembaum et al., 2002).

Although the definitive cause of ADEM is unknown it is commonly preceded by a viral infection or immunisation, which has been reported in around 70-77% ADEM cases (Tenembaum et al., 2007). After onset the disease progresses rapidly over

hours and peaking within days. Due to lack of biomarkers the disease is diagnosed based on clinical presentation and radiology. A follow-up study of 84 ADEM patients showed prominent presenting features were acute hemiparesis, unilateral of bilateral long tract signs and a change in mental state (Tenembaum et al., 2002). Other symptoms included visual loss because of optic neuritis, impairment of speech and seizures. Like MS, the initial symptoms were dependent on the area of the brain being damaged.

Diagnosis of ADEM relies heavily on neuroimaging. These lesions are normally large and many, with an asymmetric pattern. Follow-up MRI scans are crucial in establishing if the disease is monophasic or multiphasic. ADEM is predominantly a monophasic disease but there have been cases of recurrent or multiphasic courses, which produces difficulties in determining ADEM from childhood MS. There is a great need to be able to definitively distinguish between MS and the multiphasic ADEM phenotype, most importantly for therapeutic reasons, as ADEM patients are treated with corticosteroids whereas MS patients require early treatment with immunomodulators to reduce disease activity and future disability.

Currently MS cases are frequently misdiagnosed as ADEM initially (9.5% - 27%) (Dale et al., 2000, Leake et al., 2004), which means these patients are missing out on getting the early treatment they need. This highlighted a great need to have clear guidelines defining and distinguishing these very similar disseminated demyelinating disorders. The International Paediatric MS Study Group was formed to address this issue. It concluded that mulitphasic ADEM requires subsequent clinical events to occur either within 3 months of initial event or 1 month after cessation of treatment and to involve new anatomic areas of the CNS with full or partial resolution of initial lesions (Krupp et al., 2007, Krupp et al., 2013). In addition to this it must also satisfy standard ADEM criteria of being polysymptomatic, involving encephalopathy. Recurrent ADEM is a reoccurrence of the original disorder and was defined the same as multiphasic ADEM with the exception of no new areas of brain being involved.

Also highlighted in this report was that CSF results often show increases in protein and white blood cells counts, which is less common in MS. Interestingly OCBs are not a common feature in ADEM, unlike MS, but can still be present (Menge et al., 2005a). Overall there is still a great need to identify more reliable biologic markers so diagnosis is not dependent on clinical and radiological features as these are not definitive for each demyelinating disorder.

1.2.2 Neuromyelitis optica

Neuromyelitis optica (NMO) is another paediatric demyelinating disorder of the CNS. It is defined by the amalgamation of monophasic or recurrent episodes of optic neuritis and longitudinally extensive transverse myelitis (LETM) (Banwell et al., 2008). This is evident as clinical features commonly include severe optic neuropathy with fixed visual loss of 20/200 or more and muscular weakness succeeding an acute event (Krupp et al., 2007). The criteria for diagnosis of NMO include; optic neuritis, acute myelitis and two of the three; 1) spinal cord MRI monolesion extended over three vertebral segments, 2) brain MRI not in accordance with diagnostic criteria for MS, 3) anti-aquaporin-4 (AQP4) IgG seropositive (Krupp et al., 2007, Krupp et al., 2013). A unique feature of this disease is its association with a definitive antibody target –AQP4, a regulatory water channel protein. This was a fantastic breakthrough that has significantly aided NMO diagnosis. In adults anti-AQP4 antibodies (termed NMO-IgG) are detected in a significant group of NMO patients (73%) (Lennon et al., 2004, Jarius et al., 2007). Historically it was debated that NMO was a variant of MS but the identification of NMO-IgG has defined NMO as a distinct disease entity.

NMO is considered an antibody-mediated disease. Pathological evidence strongly suggests that these autoantibodies play a role in disease as immunohistological studies have shown deposition of immunoglobulins and complement (Wingerchuk, 2006). In addition to this, antibody and B cell targeted therapies such as plasma exchange and Rituximab have been partially effective (Kim et al., 2013a, Kim et al., 2013b). There is evidence that the AQP-4 antibodies may cause pathology, these

include; loss of AQP4 from spinal cord lesions (Roemer et al., 2007), disruption of the BBB, astrocyte and oligodendrocyte loss/injury (Jarius et al., 2008, Saikali et al., 2009, Wrzos et al., 2014). However, it remains to be fully determined if these autoantibodies are pathologically relevant. Studies *in vitro* where patient-derived IgG was injected intrathecally with complement into naïve animals led to pathological lesions associated with antibody-mediated damage after 7 days (Asgari et al., 2013, Asavapanumas et al., 2014). In addition these studies also showed there was also loss of AQP4 and glial fibrillary acidic protein (GFAP) (an astrocyte marker). Therefore, evidence reinforcing a pathologic role for these antibodies in disease.

The NMO-IgG status was investigated in paediatric NMO in the hope of this also being a biologic marker for the childhood disease. A study of 17 children with definitive NMO revealed 8 to be NMO-IgG seropositive (47%). Therefore, these autoantibodies are present but not as prevalent in the childhood disease, highlighting NMO-IgG is not as useful for the diagnosis of children as it is for adults (Banwell et al., 2008). However, children with the relapsing NMO phenotype have much higher frequencies of NMO-IgG than the monophasic, 78% and 12.5%, respectively.

Another difficulty in NMO diagnosis is with lesion characteristics. In adults, longitudinally extensive spinal cord lesions are a very useful diagnostic tool for differentiating between NMO and MS. Unfortunately in childhood MS they can also present this type of lesion (Hahn et al., 2004), therefore it is not specific to NMO. In conclusion, similar to the other juvenile disorders described previously NMO can present as a spectrum of disorders and resemble features associated with childhood ADEM and MS (Lotze et al., 2008).

1.2.3 Idiopathic acute transverse myelitis

Acute transverse myelitis (ATM) is an immune-mediated disorder that targets the spinal cord and is associated with a rapid onset of motor, sensory and autonomic dysfunction. It can exist in three different forms:-

- As an isolated, monophasic entity (idiopathic)
- As part of a multifocal CNS demyelinating disorder e.g. ADEM, MS and NMO
- As part of a multi-systemic disease e.g. systemic lupus erythematosus (SLE)

At first presentation of the patient they are diagnosed under the umbrella term of ATM. Initial steps are to exclude cases with associated diseases. Remaining patients with no identifiable cause are termed idiopathic ATM. These patients must present evidence of spinal cord involvement, through either an enhancing spinal cord lesion, increased white blood cell count or increased IgG index in the CSF (Wolf et al., 2012). In addition, time till peak disability must be between 4 hours and 21 days. Reasons for onset of idiopathic ATM are unknown, as with the other demyelinating diseases discussed in this thesis, but inflammation is accompanied by cellular infiltration into the parenchymal and perivascular region associated with myelin loss and neuronal damage (Kerr and Ayetey, 2002). Approximately 20% of ATM cases occur in children, with disease incidence peaking between the ages of 0-2 and 5-17 (Banwell et al., 2009, Pidcock et al., 2007).

Commenting on the literature as a whole, ATM along with ADEM and NMO can all present very similar clinical characteristics to paediatric onset MS. This contributes to the difficulty of making the correct diagnosis, essential for early and effective treatment of these patients.

1.3 MULTIPLE SCLEROSIS – A T CELL-MEDIATED AUTOIMMUNE DISEASE?

Virtually all our mechanistic concepts of adult MS are derived from an animal model; experimental autoimmune encephalomyelitis (EAE). The pathology of MS is still incompletely understood but is thought to involve the convergence of genetic susceptibility and undefined environmental factors that initiate autoimmune T cell and B cell responses directed against self CNS antigens. The concept that MS is an autoimmune disease derives primarily from studies in EAE that demonstrate this "MS-like" disease in terms of its pathology and clinical course can be induced in rodents and other species by breaking self-tolerance to CNS myelin antigens. This has shaped the development of therapeutic strategies for MS over the past thirty years, resulting in a range of disease modifying treatments that significantly reduce disease activity and frequency of relapses by disrupting the development of inflammatory response within the CNS.

1.3.1 Evidence of $CD4^+$ T cells in multiple sclerosis

EAE studies led to MS generally being considered as a $CD4^+$ T_H1 cell dependent disease of the CNS. An important study performed in 1981 adoptively transferred T cells specific to MBP, a major CNS myelin protein, into rats (Ben-Nun et al., 1981). This highlighted effector T cells as possessing a central role in EAE as the T cells induced neuroinflammation in the CNS. Subsequently adoptive transfer of syngenic $CD4^+$ T cells specific for a wide variety of different myelin and non-myelin CNS antigens was shown to induce focal inflammatory infiltrates in the brain and spinal cords of susceptible species (Zamvil et al., 1985), indicating this arm of the immune response may play a major role in the development of MS lesions (Lassmann et al., 2001).

EAE and MS pathology have a similar cellular composition in the active lesions (Lassmann, 1983). Early immunohistological studies of MS lesions from patients have identified the cellular infiltration of T cells over three decades ago, indicating

their role in MS pathology (Prineas and Wright, 1978, Traugott et al., 1983). An in depth collaborative study investigating the cells present in active MS lesions elucidated that T cells were ubiquitously found in all lesions of MS patients (Lucchinetti et al., 2000). Despite this, the lesions could be segregated into four distinct patterns. Pattern I was dominated by T cell and macrophage mediated inflammation and was evident in 12.3% of patients (Table 1.2). Evidence of T cells has also come from studies isolating T cells from the blood and CSF of MS patients, which revealed myelin-reactive T cells to be present (Allegretta et al., 1990, Chou et al., 1992, Soderstrom et al., 1993).

1.3.1.1 T_H1 T cells in MS

T_H1 cells have been the main focus of T cell studies in MS. Many features observed in MS lesions indicate a T_H1-mediated inflammatory process particularly that MS pathology is similar to the pathology observed in T_H1-mediated EAE. They predominantly cause damage and inflammation by activating macrophages via the secretion of interferon-γ (IFNγ) (Hendriks et al., 2005). Early studies in MS showed that numbers of IFNγ–secreting cells were higher in patients compared to controls (Olsson et al., 1990). In addition, the expression patterns of pro-inflammatory chemokines and chemokine receptors identified in MS lesions resembles that observed in EAE (Sorensen et al., 1999). However, since the discovery of T_H1 cells T cell subsets have become increasingly more complex (Figure 1.1).

1.3.1.2 T_H17 T cells

Having presumed T_H1 cells to be the main drivers of MS pathogenesis it was then realised that there were other $CD4^+$ T cell subsets involved; in particular IL-17 secreting T_H17 cells. A fundamental study by Cua et al. (2003) highlighted T_H17 cells role in EAE due to the discovery of IL-23, a structurally related cytokine to IL-12, both having the subunit p40. Their experiments revealed that IL-23 was responsible for EAE, and not IL-12, which drives T_H17 and T_H1 differentiation, respectively (Yamane and Paul, 2013) (Figure 1.1). T_H17 drives pathogenesis

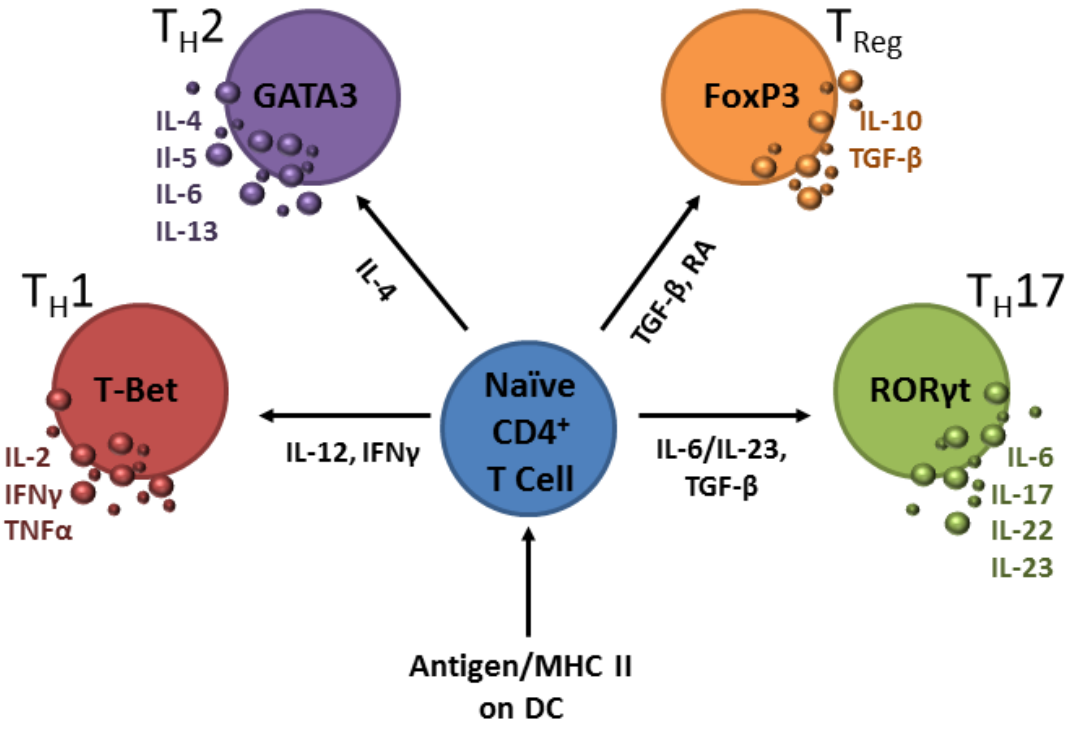


Figure 1.1. Summary of the major $CD4^+$ T cell subsets.

Naïve CD4+ T cell is activated via its T cell receptor by its cognate antigen-derived peptide presented in the context of MHC class II (MHC II) on a dendritic cell (DC). The T cell then proliferates extensively and differentiates into a distinct T helper (T_H) cell subset depending on the cytokine milieu; T_H1, T_H2, T_{Reg} or T_H17 cells. These four subsets have distinct transcription factors and secrete unique cytokines, therefore providing them each with a different function in the immune response. Diagram was adapted from Yamane and Paul (2013).

primarily through the secretion of IL-17, but also through other pro-inflammatory cytokines like IL-6 (Langrish et al., 2005). Induction of EAE through passive transfer of T_H17 has suggested they may have a role in neuroinflammation (Langrish et al., 2005).

Further evidence in EAE studies showed using IL-17 neutralising antibody and IL-$17^{-/-}$ mice led to amelioration of disease (Hofstetter et al., 2005, Komiyama et al., 2006). Studies in MS have not been conclusive but IL-17 producing cells are present in active lesions and in the peripheral blood of MS patients (Tzartos et al., 2008, Fletcher et al., 2010).

1.3.1.3 T_H2 T cells

It is commonly assumed that T_H2 cells play an immuno-modulatory role in disease and that skewing the T cell response this way would have beneficial therapeutic effects, however this approach has been problematic (Hohlfeld, 1997). EAE studies have provided evidence as to why this might be, they identified that the transfer of T_H2 cells into immunodeficient mice can induce disease with similar lesions to that observed in NMO patients (Lafaille et al., 1997). In addition, the presence of eosinophils and granulocytes at active MS lesion sites is also suggestive that a potentially harmful T_H2 immune response is occurring (Lassmann et al., 2001, Rook et al., 2000). These studies contribute to the increasing heterogeneity and complexity observed in EAE and MS pathogenesis.

1.3.1.4 T regulatory T cells

The final major subset of $CD4^+$T cells is $FoxP3^+$ T regulatory T cells (T_{Regs}) (Figure 1.1). These cells have an important role in disease modulation, predominantly through the production of immunosuppressing cytokine IL-10 (Fujio et al., 2010). EAE models have shown that T_{Regs} are capable of modulating neuroinflammation, but the extent of their role in MS is still unclear as histological studies of post mortem brain tissue did not find any T_{Reg} cells in active lesions (Venken et al.,

2010). However, the relevance of all these T cell subsets *in vivo* is questionable as dissecting the roles of these different subsets in disease is compromised by their plasticity (Bluestone et al., 2009, Mucida and Cheroutre, 2010).

1.3.2 Evidence of $CD8^+$ T cells

The adoptive $CD4^+$ T cell transfer animal studies led to many predictions being been made about disease mechanisms occurring in MS lesions however it now appears that the pathogenesis of MS lesions may be more complex than originally presumed (Lassmann and Ransohoff, 2004). Firstly, immunohistological analysis of T cell subsets present in MS lesions found that $CD8^+$ T cells outnumbered $CD4^+$ T cells (Booss et al., 1983), and several other pathological studies have identified that clonal expansion is more prominent in $CD8^+$ T cell compared to $CD4^+$ T cells (Monteiro et al., 1995, Babbe et al., 2000). These data taken together indicate $CD8^+$ T cells also play a significant role in disease pathogenesis (Johnson et al., 2007, Denic et al., 2013). Experiments in EAE showed $CD8^+$ T cells were capable of inducing a severe form of EAE through studies using the adoptive transfer of enriched MOG-specific $CD8^+$ T in C57BL/6 mice (Sun et al., 2001). Another investigation recorded disease severity in CD4 and CD8 deficient mice after being immunised with MOG. This experiment led to both groups developing a milder disease course, suggesting both cell groups have a function in disease pathogenesis (Abdul-Majid et al., 2003).

In general, the observation that T cells have a major role in MS has led to and shaped the development of many successful FDA approved T cell targeted therapies including IFNβ, glatiramer acetate and mitoxantrone, which are thought to specifically target T cells. In addition fingolimod, natalizumab and teriflunomide target lymphocytes and immune cells generally. These therapeutic approaches, however, are limited by non-responding patients, adverse effects and their inadequate efficacy in PPMS patients.

1.4 B CELLS IN MULTIPLE SCLEROSIS

Although adoptive transfer of CNS-specific T cells provides an effective animal model of neuroinflammation in MS, it fails to recapitulate all the pathological features of MS. In particular disease activity in rat and marmoset models of T cell mediated EAE is not associated with extensive demyelination, a pathological hallmark of MS in humans. It is now recognised these models failed to develop significant primary demyelination because they lack autoantibodies targeting the myelin surface. This was resolved by the development of a 'two-hit' model using the co-transfer of T cells and pathogenic antibodies, which increased EAE severity and primary demyelination (Linington et al., 1988, Genain and Hauser, 2001). The experiment revealed that to reconstruct the immunopathology observed in MS these models required encephalitogenic T cells to inflame and breech the blood brain barrier (BBB), which allowed primary demyelinating autoantibodies to gain access to the CNS. These studies rekindled interest in B cell research in MS.

1.4.1 Evidence of B cells in multiple sclerosis

There is a wealth of evidence supporting a role for B cells in the pathology of MS, which is summarised in Table 1.1 and will be discussed in more detail below.

1.4.1.1 Intrathecal synthesis of antibodies

Intrathecal antibody production is used in the diagnosis of MS and analysis of CSF offers a novel insight into what immune mechanisms are acting in the CNS. OCBs are visualised using isoelectric focusing (IEF) and IgG immunoblotting (Luque and Jaffe, 2007). Preliminary studies by Kabat first described the presence of antibodies in the CSF of patients with MS (Kabat et al., 1942). Four decades later studies investigating a large patient cohort showed that greater than 90% of these patients had OCBs present in their CSF (Ebers and Paty, 1980). This feature is now a standard diagnostic tool for MS in addition to MRI scans (McDonald et al., 2001, Krupp et al., 2013). Subsequent studies have further tried to determine the clinical

Table 1.1. Evidence of B cell presence and role in patients with multiple sclerosis.
This table details significant findings over the past 60 years that support B cells presence and importance in MS disease pathogenesis. Table adapted from Boster et al., 2010.

Evidence of B cell presence and role in MS patients	
Key Point of Evidence	Key Publications
Intrathecal synthesis of immunoglobulin found in CSF	Kabat et al., 1942. Eber and Paty, 1980.
Immunoglobulin and complement deposited in MS plaque	Luccinetti et al., 2008.
B cells and plasma cells present in CSF and MS lesions	Prineas et al., 1978 Corcione et al., 2004
Identification of MOG-specific antibodies in MS lesion	Genain et al., 2002
Clonally expanded B cells in CSF which have undergone somatic hypermuation	Qin et al., 2003 Obermeier et al., 2008
Ectopic B cell follicles and germinal centres in meninges	Serafini et al., 2004 Magliozzi et al., 2007

significance of these OCBs but this still remains debated (Link and Huang, 2006). In one study one thousand CSF samples were screened in an attempt to find a correlation between the patient OCB profile and a specific disease phenotype but none was found (Ebers and Paty, 1980). However, other investigations showed an association between an increased number of OCBs and poorer disease prognosis (Zeman et al., 1996, Villar et al., 2002). The antigen specificity of these OCBs remains unknown although reports include viral and autoantigen targets.

1.4.1.2 Evidence of clonally expanded B cells in the CNS of MS patients

It is now evident that OCBs are derived from a clonally expanded B cell population sequestered in the CNS. To confirm this a study analysed the variable region of the IgG heavy chain genes of B cells isolated from the CSF of patients who had experienced clinical episodes suggestive of MS (Qin et al., 2003). This revealed that a substantial number of the patients' B cells were clonally expanded and had also undergone somatic hypermutation, suggestive of positive selection via their B cell receptors. Subsequent investigations revealed that CSF Ig proteomes corresponded with the B cell transcriptomes. This indicated that the CNS sequestered B cells were responsible for the intrathecal antibody synthesis

observed, as opposed to peripheral B cells (Obermeier et al., 2008). These studies present strong evidence indicating that B cells are expanding and synthesising antibodies as part of a local antigen-driven response. This suggests that the CNS of patients with MS provides a niche that can support the long-term survival and differentiation of B cells.

Early immunohistological studies highlighted the presence of B cells in the CNS of MS patients. Investigations analysed plaques from ten patients with MS and confirmed the presence of immunoglobulin-containing cells (Esiri, 1977). Further studies showed large numbers of plasma cells appeared to be particularly associated with patients who had chronic MS (Prineas and Wright, 1978). More recent investigations have focused on determining what B cell subsets are present in patient with MS. Using molecular studies, B cells present in MS lesions have been analysed providing evidence that they were clonally expanded (Owens et al., 1998, Baranzini et al., 1999). B cell subsets have also been investigated in the CSF of MS patients which revealed the CSF to be enriched with a B cell subset found in secondary lymphoid organs and memory B cells (Corcione et al., 2004). Therefore, again promoting the idea that the B cell response compartmentalised within the CNS during neuroinflammation is capable of the mimicking of peripheral B cell differentiation.

1.4.1.3 Ectopic follicles in the CNS

The source of these clonally expanded B cells is still under debate and unlikely to be derived directly from the periphery. An immunohistological study of post-mortem brain and spinal cord samples from patients with MS were stained for B cells, T cells and follicular dendritic cells. They found these cells and CXCL13, a chemokine constitutively expressed in the follicles of lymphoid tissue, was continually expressed in the cerebral meninges, suggesting this area has ectopic germinal centres (Serafini et al., 2004). Other investigations found lymphotoxin-α, CXCL12 and CXCL13 in the CSF of MS patients, all crucial mediators for lymphoid neurogenesis, (Corcione et al., 2004). It has also been discovered that B

lymphocytes can release lymphotoxin-α (Fu et al., 1998, Lorenz et al., 2003), and further studies showed that it was secreted selectively by the memory B cell subset (Duddy et al., 2007).

Interestingly, in this study these ectopic structures were only found in SPMS patients and not RRMS (Serafini et al., 2004). Another study investigated the presence of B cell follicles in PPMS and SPMS patients, which again revealed that these ectopic lymph node structures only occurring in SPMS samples (Magliozzi et al., 2007). This suggests that B cell follicles are associated with a more progressive disease phenotype. These ectopic germinal centres are also found in other progressive autoimmune diseases like rheumatoid arthritis and Sjogren's syndrome (Weyand and Goronzy, 2003, Salomonsson et al., 2003).

1.4.1.4 Evidence of therapeutic effects of B cell depletion

Although current first line treatments for MS use broad immunosuppression to relieve disease symptoms the need for more specific treatments and the knowledge that B cells have a function in MS has led to B cell depletion emerging as a new treatment for this neurological disease.

Initial studies in this field used rituximab, a monoclonal chimeric anti-CD20 antibody. It was first approved to treat rheumatoid arthritis and lymphoma and then researchers turned to investigate its effects in MS. These studies discovered that rituximab can be efficacious in RRMS patients (Hauser et al., 2008). The initial clinical trial in RRMS patients demonstrated very promising results, where patients in the treatment group exhibited a significant decline in the number of gadolinium-enhancing lesions and a reduction in relapses (Hauser et al., 2008). Importantly there were no significant safety issues with these trials. Intriguingly, these studies also revealed that although rituximab decreased disease these clinical effects preceded any decrease in antibody serum levels, suggesting the clinical effect of B cell depletion was independent of any direct effects on antibodies. Analysis of patient CSF 6 months after treatment using flow cytometry showed a diminution

of B cells, which was also associated with a reduction in T cells (Cross et al., 2006). Rituximab was also trialled in PPMS but overall comparison between the placebo and treated group showed no significant improvement in disease. However, analysis of subgroups showed significant benefits in younger patients (< 51 years old) (Hawker et al., 2009).

These very promising results fuelled the development of a second generation of anti-CD20 monoclonal antibodies; ocrelizumab and ofatumumab (Deiss et al., 2013). Encouraging results in RRMS patients have been seen with ocrelizumab, which is a humanised anti-CD20 monoclonal antibody (Kappos et al., 2011). The study showed that gadolinium-enhancing lesion numbers were reduced 89% in the treated group. Another anti-CD20 monoclonal antibody under investigation is ofatumumab, which is a fully human monoclonal antibody (Castillo et al., 2009). A phase II study of in RRMS patients has just been published, which showed B cells were selectively diminished in parallel with suppression of lesion activity (Sorensen et al., 2014). This new drug was also well tolerated with no adverse effects.

These studies all support the role of B cells in MS disease pathogenesis, as depletion of $CD20^+$ B cells resulted in a significant decrease in active lesion numbers. The mechanisms by which this disease suppression is induced is still unclear (Hawker, 2008). It has become apparent that the clinical benefits are not due to the removal of antibodies as antibody-secreting plasma cells are $CD20^-$, therefore are not depleted and serum Ig levels remain unaffected. However, B cells do have other significant functions in disease pathogenesis.

1.4.2 The functional significance of B cells in multiple sclerosis

Studies on the functional role of B cells in MS have traditionally concentrated on their antibody production and patho-mechanisms associated with these antibodies (which will be discussed in section 1.5) but in addition to this they also have important functions independent of antibody production (Boster et al., 2010).

Interest has focused on two major mechanisms of B cell-mediated immune modulation.

1.4.2.1 Production of cytokines by B cells in MS

B cells can secrete a range of cytokines that play a role in modulating immune responses. It has been found that B cells produce different cytokines depending on their environment. They can be induced to produce IFNγ when cultured with T_H1 cells (Harris et al., 2005a), or IL-4 when cultured with T_H2 cells (Harris et al., 2005b). This dichotomy has been described as two different B cell effector subsets, namely, Be1 and Be2, where each subset produces a distinct cytokine profile (Harris et al., 2000). B cells have also been shown to produce IL-6 which was shown to contribute to EAE severity as when B cell-specific IL-6 production was deleted EAE disease burden was ameliorated (Barr et al., 2012). In addition to this T_H17 cells were decreased suggesting B cell secreted IL-6 was supporting T_H17 cells. They have also been shown to produce other pro-inflammatory cytokines such as TNFα, IL-12, and LTα, a cytokine involved in lymphogenesis (Lund, 2008). Overall, these data suggest that B cell cytokines play a role in supporting effector T cell differentiation, thereby potentiating disease.

In contrast to their pro-inflammatory functions very early studies provided evidence of B cells having a regulatory function. These studies in guinea pigs revealed that B cells could delay hypersensitivity responses (Katz et al., 1974, Neta and Salvin, 1974). Later, Janeway and colleagues performed the first experiment in EAE models to reveal an increased disease severity in B cell deficient mice (Wolf et al., 1996). Therefore, suggesting that these cells are needed to regulate disease. Further studies showed that B cells modulated the immune system through supressing effector T cells (Akdis and Blaser, 2001), and it has been identified that they do this predominantly via the secretion of IL-10 (Fillatreau et al., 2002, Matsushita et al., 2010). However, they can also modulate the immune response through a variety of other ways like inhibiting cytokine secretion from monocytes and macrophages (Fiorentino et al., 1991). B cells may also mediate immune

suppression via TGF-β1 secretion, which has been shown to modulate T_H1 cells (Tian et al., 2001).

There have been multiple studies attempting to characterise this regulatory population. One study defined these B cells as a small population of splenic B cells which are phenotypically distinct, $CD1d^{high}CD5^{+}$ (Yanaba et al., 2008, Matsushita et al., 2008). Most recently it has been identified that B_{Regs} may be a subset of plasma B cells and in addition to IL-10 also secrete immunosuppressing IL-35 (Shen et al., 2014). Interestingly, studies in MS patients showed that their B cells were skewed as their ability to produce IL-10 was significantly reduced (Duddy et al., 2007), suggesting that the reduction in regulatory B cells may contribute to disease severity.

1.4.2.2 B cells are efficient antigen-presenting cells

For T cells to adopt their encephalitogenic effector functions in an immune response they must first encounter their cognate antigen in the periphery, then migrate to the CNS and be presented with it again. B cells are thought to play an important role in driving disease via antigen presentation to primed cognate T cells in the CNS. It is now established that B cells are exceedingly efficient antigen presenting cells (APCs) and can potentiate disease by promoting antigen-specific pro-inflammatory T cell responses (Lehmann-Horn et al., 2013). Antigen-specific B cells are specialised in processing and presenting protein antigen, which they uptake via their B cell receptor (Lanzavecchia, 1985, van der Veen et al., 1992).

Experimental studies suggest that disease reduction in EAE is most likely due to the loss of antigen-specific B cells that act as highly efficient APCs for their cognate antigen. Furthermore, T cell priming and proliferation was greatly impaired in B cell deficient mice, suggesting that T cells were dependent on B cell antigen presentation (Rivera et al., 2001). This study also showed that B cell presentation was necessary for T cell priming when there were low levels of antigen available. Subsequent investigations reinforced the crucial role of B cells APC function in EAE

by using mice deficient in MHC II in B cells only. This revealed that these mice were resistant to MOG-induced EAE (Molnarfi et al., 2013). They also made a transgenic mouse with MOG-specific B cell receptors, which could not secrete antibody. These mice were not resistant to MOG-induced EAE, again indicating B cells have a pivotal cellular function during disease pathogenesis which is independent of autoantibody-mediated inflammation.

B cell antigen presentation may not only serve to activate antigen-specific T cells but could also establish a feedback loop. Research showed products were secreted by the activated T cells, CD40L and IL-4, which directly induced changes in the B cells, which appeared to be crucial for their ability to process and present antigen to T cells (Harp et al., 2008). Although the exact mechanisms underlying the clinical benefits observed with Rituximab and other anti-CD20 drugs are still not clear, it is thought that the effects are due to loss of B cell antigen presentation function (Weber et al., 2010). Here, B cell depletion led to a decrease in MOG-specific T_H1 and T_H17 cells during MOG-induced EAE. This suggests that antigen presentation by these activated B cells induced T cell differentiation into pro-inflammatory effector T cells. Overall, it is now apparent that B cells have an important role as APCs during neuroinflammation, aiding T cell activation and polarisation, which should be taken into account when investigating B cell therapies.

1.5 AUTOANTIBODIES IN MULTIPLE SCLEROSIS

1.5.1 Evidence of antibody-dependant mechanisms in MS

In addition to the presence of OCBs and intrathecal synthesis of antibodies previously discussed, there are several other observations that implicate autoantibodies in the pathology of MS. These are derived from a number of sources:-

- Immunoglobulin deposition in MS lesions

- The benefits of therapeutic plasma exchange (TPE)

Evidence of immunoglobulin deposition and complement activation in MS lesions was described in a crucial paper by Lucchinetti et al. In this international collaborative study large numbers of autopsy and biopsy material was acquired to try and identify immuno-pathologic correlates to demyelination (Lucchinetti et al., 2000). Previous investigations had involved only small cohorts, resulting in very heterogeneous outcomes. Therefore, this large, collaborative effort revealed that despite all lesions ubiquitously showing infiltration of T cells and macrophages there were four distinct patterns that existed within these lesions (Table 1.2). The pattern varied between patients but was consistently found within an individual patient. This was a fascinating discovery as it reflected the clinical heterogeneity seen in the diverse presentations of MS and also offered insights into how to treat individual patients. Interestingly the most common pattern was pattern II, deposition of antibodies and complement activation, which was found in around 50% of the patients in the study. Thus, this study highlighted antibody and complement-mediated demyelination as an immuno-dominant mechanism in disease pathogenesis.

The interpretation of this study remains controversial and debated by other researchers but the results remain a pivotal finding of great importance with many subsequent studies being based on it. One study to dispute these findings was Barnett et al. (2009), as they observed the presence of co-deposition of antibodies and activated complement (pattern II) in autopsy tissue from patients with other neurological diseases as well as MS patients, therefore presenting it as a non-specific feature and questioning the exclusivity of this pathology to MS. In addition to this, another study in contrast to Lucchinetti's did not find any heterogeneity in active demyelinating lesions amongst patient brain tissue samples (Breij et al., 2008). They uniformly observed activated complement and antibodies present in

all their autopsy samples, suggesting there are no different subtypes of lesions in MS patients.

It is currently not known if these four lesion patterns are relevant to paediatric MS but in one rare case where a biopsy sample was attained showed the patient had pattern I lesion with infiltration of foamy macrophages and T cells. The sample did not show any evidence of immunoglobulin or complement deposition and activation (Hoche et al., 2011).

Evidence for the role of antibodies in disease also comes from the beneficial clinical effects observed in a subset of MS patients after therapeutic plasma exchange (TPE). This process involves the removal of blood from a patient and

Table 1.2. Heterogeneity of MS lesions described by Luccinetti et al., 2000.
This table defines the four patterns observed in autopsy and biopsy material studied by Luccinetti et al. Table adapted from Luccinetti et al., 2000.

	Immunological features	Location of demyelination	Oligodendrocytes	Protein targeted	Remyelination	% of total patients
Type I	Macrophages and T cells	Centered around veins Defined lesion	High density	All	Yes	12.3
Type II	Macrophages and T cells Immunoglobulin and complement deposition	Centered around veins Defined lesion	High density	All	Yes	53.4
Type III	Macrophage/microglia and T cells	Concentric rings at periphery of lesion. Ill defined lesion	Loss of OLGs by apoptosis	>> MAG	No	30.1
Type IV	Macrophages and T cells	Defined lesion	Some loss of OLGs	All	No	4.1

then plasma is separated from blood cells and replaced without the pathogenic antibodies and other harmful immune factors (Schroder et al., 2009). TPE is commonly used and a well-established therapy in other neurological diseases with autoantibody involvement such as myasthenia gravis and Guillain-Barré syndrome, but is not as well described in MS treatment.

Clinical studies investigating the effects of TPE with SPMS patients, with EDSS score varying from 5 to 6.5, revealed that this approach could help stabilise and reduce subsequent neurological defects in this patient group, who commonly don't respond as well to other treatment regimens (Grapsa et al., 2008). In fact many studies have shown efficacy with TPE in progressive patients who haven't responded to conventional therapies like IFNβs or steroid methylprednisolone (Khatri, 2009, Trebst et al., 2009, Meca-Lallana et al., 2013). TPE was well tolerated and had a clear beneficial effect on these steroid-unresponsive RRMS patients (Trebst et al., 2009). However, TPE does not work in every patient case and this is likely due to the heterogenic nature of MS. A retrospective study of patients who were treated with TPE discovered a very interesting correlation (Keegan et al., 2005). They revealed that patients with pattern II lesions, i.e. with complement and antibody deposition, responded much more favourably to the treatment than patients with other lesion classifications. Therefore this study related TPE efficacy with the presence of autoantibodies.

The pathological relevance of antibodies in MS has been questioned *in vitro* using myelinating cultures and treating them with patient derived IgG and complement (Elliott et al., 2012). This study demonstrated that approximately 30% of the MS patients had antibodies that had demyelinating properties *in vitro* and that some patient IgG samples also mediated axonal loss. Myelin loss was only seen in a subset of patients, which offers an explanation as to why TPE and B cell depleting treatments are only effective in some patients. These pathogenic antibodies were targeting antigens on myelinating oligodendrocytes and myelin sheaths. Although the most likely antibody target was MOG, demyelination still occurred after the

MOG-specific repertoire had been depleted. This study provided evidence that autoantibodies could have a demyelinating role in MS pathogenesis.

1.5.2 Potential targets for autoantibody mediated demyelination

Experimental studies demonstrate autoantibodies targeting epitopes exposed on the surface of oligodendrocytes and their myelin sheaths exacerbate demyelination and disease severity in EAE. The identity of antigen(s) recognised by pathogenic, in this case demyelinating, autoantibodies in MS remains controversial with many antigens being implicated (Table 1.3). The crucial point to bear in mind is that in order to induce primary demyelination any relevant target must be accessible to the antibody in the extracellular milieu. However, it should be documented that antibody responses recognising epitopes which become exposed once demyelination is initiated may have an indirect role in mediating further tissue damage.

The molecular composite of the CNS oligodendrocyte myelin continuum is now well characterised. Compact internodal myelin is a multi-lamellar structure derived from the oligodendrocyte plasma membrane containing 20-30% protein (dry weight) (Figure 1.2). Due to its multi-lamellar structure not all of its proteins and lipids are readily accessible to autoantibodies. Potentially pathogenic antibodies will have far greater access to the outermost components of the myelin sheath and associated membrane which are enriched in a larger number of proteins and glycolipids.

There are a variety of screening protocols implicated to identify potential candidate antigens, ranging from direct analysis of serum or CSF to generating recombinant antibodies from clonally expanded plasma cells from MS patients. Despite great efforts the elusive antibody target(s) in MS is still unknown but many candidate antigens have been identified in the process.

Table 1.3. Candidate autoantibody targets in multiple sclerosis.

Antigen	Evidence in MS and EAE	Publications
GalC	Anti-GalC Abs in RRMS patients. Pathogenic in EAE studies.	Raine et al., 1981; Moore et al., 1984; Fierz et al., 1988; Menge et al., 2005b.
Gangliosides	Anti-GD2, GM3, GM4 and GD1a Abs are found in serum and CSF of MS patients.	Kasai et al., 1986; Sadatipour et al., 1998; Mata et al., 1999; Marconi et al., 2006.
HSPs	Abs against HSP70 in CSF MS studies. Possible role in neuroprotection.	Chiba et al., 2006; Reviewed in Mansilla et al., 2012.
KIR4.1	Seropositive adult and paediatric MS patients. Pathogenic in EAE models.	Srivastava et al., 2012; Kraus et al., 2014.
MAG	Selective MAG loss in MS lesions. Some anti-MAG reactivity in MS CSF studies	Itoyama et al., 1980; Baig et al., 1991; Lucchinetti et al., 2000.
MBP	MBP Ab response detected in serum and CSF of MS patients.	Warren and Catz, 1994; Reindl et al., 1999; Egg et al., 2001.
MOG	Anti-MOG Abs found in MS lesions. Seropositive adult and paediatric MS patients. Pathogenic in EAE models.	Genain et al., 1999; McLaughlin et al, 2009; Reviewed in Reindl et al., 2013.
Neurofascin	Changes in Nfasc distrubution in MS lesions. Anti-Nfasc-155/186 Abs found in MS serum. Anti-Nfasc-186 pathogenic in EAE models.	Howell et al., 2006; Maier et al., 2007; Mathey et al., 2007; Lindner et al., 2013.
Neurofilament	Anti-light and heavy neurofilament Abs detected in CSF and serum of MS patients. Increased levels of Abs associated with increased disease burden.	Fialova et al., 2013a; Fialova et al., 2013b; Amor et al., 2014; Gresle et al., 2014.
PLP	PLP-specific Abs in MS CSF studies. O10 hybridoma cells mediate demyelination.	Sun et al., 1991; Warren and Catz, 1994; Rosenbluth and Schiff, 2009.
Sulfatide	Sulfatide-specifc abs in CSF/serum of MS patients. O4 hybridoma cells mediate demyelination.	Ilyas et al., 2003; Rosenbluth et al., 2003; Kanter., 2006; Brennan et al., 2011.

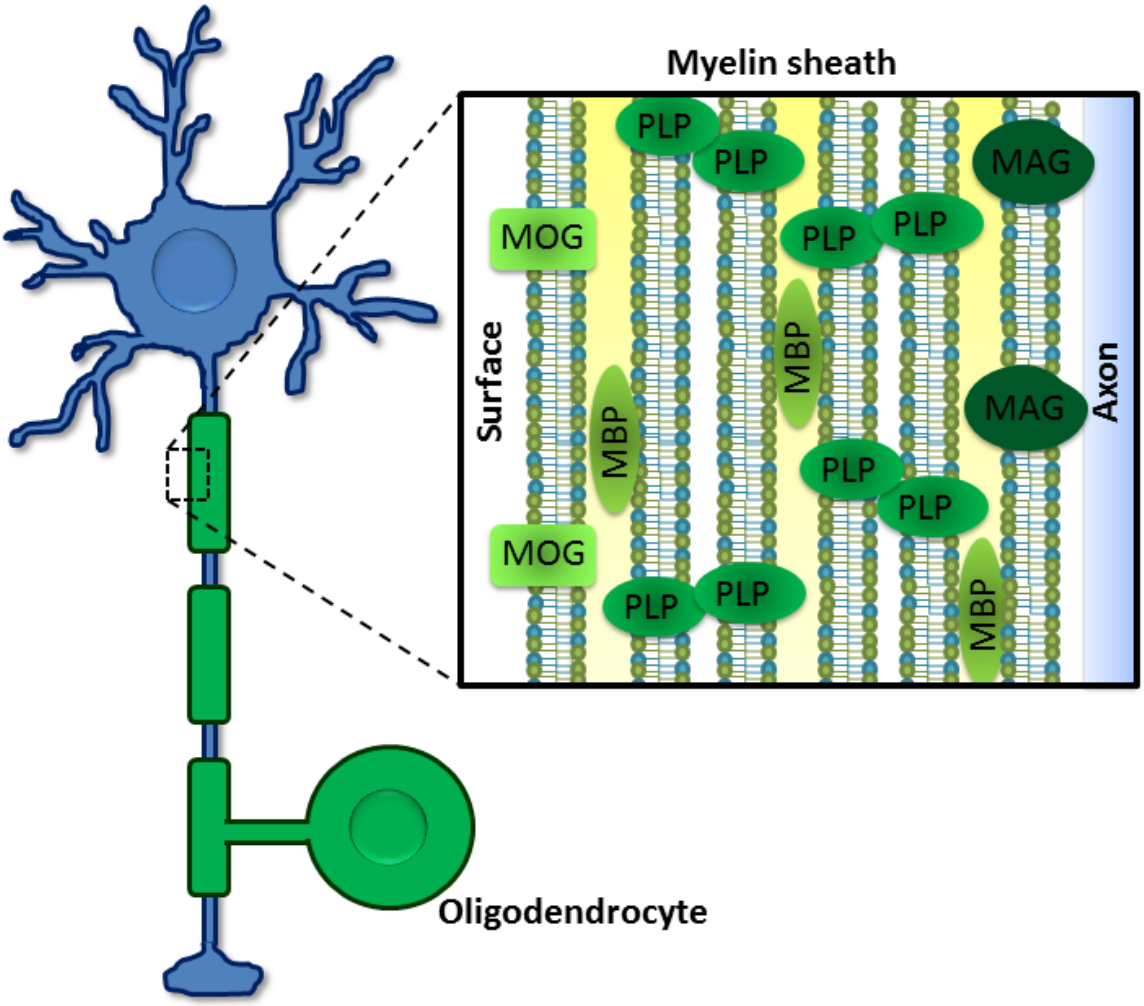


Figure 1.2. The composition of the myelin sheath within the central nervous system.
This myelin sheath composite diagram summarises the main proteins in the myelin sheath, all of which are candidate targets for the immune response during MS, including oligodendrocytes and axons. The myelin sheath is a multi-layer membrane synthesised by oligodendrocytes. It is mainly composed of lipids (80%); cholesterol (not represented), phospholipids (small light green circles) and glycosphingolipids (blue circles). 30% of the sheath is made up of proteins including; MAG, myelin-associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; MBP, myelin basic protein. MOG is the only protein found on the outermost surface of the myelin sheath, therefore making it a candidate antigen for primary demyelination. PLP is a transmembrane protein although may transiently be expressed on the surface during development. MBP makes up 30% of the myelin membrane. It is located in the cytoplasm apposition (yellow), and is required for the compaction of the cytoplasmic glia surfaces at the major dense lines. MAG is located at the periaxonal side of the myelin sheath. Figure adapted from Hemmer et al. (2002).

1.5.2.1 Myelin lipids as pathogenic targets in MS

The lipid content of myelin is made up of three components; cholesterol, phospholipids and glycolipids in a ratio of approximately 2:2:1 (Podbielska and Hogan, 2009, Greer, 2013). Glycolipids constitute 27.5% of the dry weight of the myelin sheath and consist of three main constituents; sulfatides, galactocerebroside (GalC) and gangliosides.

Sulfatide is enriched in the CNS myelin sheath and is expressed on the outermost surface, therefore leaving it exposed to autoantibody attack. Sulfatide and GalC play an essential role in the maintenance and function of CNS myelin as demonstrated in studies using genetically modified mice in which the enzyme essential for their biosynthesis (ceramide galactosyltransferase) is knocked out (Bosio et al., 1996, Bosio et al., 1998). These mice showed conduction abnormalities due to dissolution of the myelin sheath. Many research groups have reported the presence of anti-sulfatide IgM and IgG antibodies in CSF and/or serum from MS patients (Ryberg, 1978, Ilyas et al., 2003, Kanter et al., 2006, Brennan et al., 2011). Pathologic potential of this response was demonstrated by focal demyelination as a result of the implantation of O4 monoclonal antibody secreting hybridoma into the spinal cord of rats (Rosenbluth et al., 2003). Further animal studies also showed that co-immunisation with sulfatide and myelin peptide induced a more severe EAE than with myelin peptide alone and that the addition of anti-sulfatide antibodies increased disease severity (Kanter et al., 2006). However, sulfatide antibody reactivity has also been detected in other autoimmune diseases (Aotsuka et al., 1992, Ilyas et al., 1991), and in healthy controls (Avila et al., 1993).

GalC accounts for around 25% of the myelin lipid and is also situated on the outer surface of the myelin bilayer, so like sulfatide, is exposed to a pathogenic antibody attack. Studies in human disease have shown the presence of GalC-specific autoantibodies in RRMS patients, compared to CIS, SPMS and PPMS (Menge et al., 2005b). Again, their role in immune-mediated demyelination is unknown but EAE

studies have revealed that GalC has encephalitogenic properties (Raine et al., 1981, Moore et al., 1984), and that anti-GalC antibodies can augment EAE (Fierz et al., 1988), therefore suggesting that these antibodies are potentially pathogenic.

A very complex group of glycolipids is the gangliosides, which are sialic acid-bearing glycolipids present in the myelin sheath in many different structural forms. They are of interest as they are one of the main lipids found on the surface of the myelin sheath. Anti-ganglioside reactivity has been detected in numerous MS studies; namely anti-GD2, GM3, GM4 and GD1a (Kasai et al., 1986, Sadatipour et al., 1998, Mata et al., 1999, Marconi et al., 2006). However, their relevance in disease is still unknown.

1.5.2.2 Major proteins as pathogenic targets in MS

Numerous antibody responses to myelin associated proteins have been reported in MS (Table 1.3). However, only a few candidates are of particular interest due to having significant supporting evidence. MBP was originally thought to be a major autoantibody target as it comprises around 30% of the myelin sheath; however it is only present within compact myelin, therefore is not readily accessible. The most important protein is MOG and it is a well characterised target for antibody-mediated demyelination in EAE. Recently, MOG-specific antibodies have been found to be present in a subgroup of MS patients (McLaughlin et al., 2009, Reindl et al., 2013b)(MOG will be discussed in detail in the next section).

A very exciting autoantibody target most recently identified is the potassium channel KIR4.1, which is expressed on glial cells and is thought to function in oligodendrocyte and myelin development (Neusch et al., 2001), and maintaining the electro-chemical gradient in the cell membrane of astrocytes (Kucheryavykh et al., 2007). It was demonstrated that 47% of the MS patients analysed had KIR4.1 reactive antibodies (Srivastava et al., 2012). They further went on to show that 24 hours after anti-KIR4.1 patient derived antibodies were injected into the cisterna magna of mice there was disruption of GFAP architecture, loss of KIR4.1 staining

and deposition of C9neo, a marker of complement activation, suggesting these antibodies have pathogenic potential *in vivo*. More recent studies analysing brain tissue from MS patients showed evidence of an immune response being mounted against KIR4.1 (Schirmer et al., 2014). Its reactivity has also been investigated in paediatric demyelinating disorders, which observed that 57.5% patients had serum antibodies to KIR4.1 (Kraus et al., 2014). Therefore, these data highlight KIR4.1 as a new, potentially important antibody target in both adult MS and in juvenile demyelinating disorders.

Other antigens may not normally be accessible in mature myelin sheaths but may be expressed on the outermost surface of the oligodendrocyte myelin continuum during myelination/remyelination. Potential targets here include; PLP, neurofascin and myelin-associated glycoprotein (MAG).

PLP is the main protein component of the CNS myelin, which makes up around 17% of the protein constituent. It plays a role in the spacing of myelin lamellar at the intraperiod lines (Figure 1.2). There is not a vast amount of evidence supporting PLP autoantibodies in MS, however, B cells secreting PLP-specific antibodies were detected more frequently in the blood and CSF of MS patients compared to control patients (Sun et al., 1991). However, another study found that PLP antibody reactivity was only present in a very small percentage of MS patients (1%) (Warren and Catz, 1994). Implantation of O10 hybridoma cells, which secrete an antibody recognising a surface exposed PLP epitope, into the CNS of adult and juvenile rats demonstrated that anti-PLP antibodies can mediate demyelination (Rosenbluth and Schiff, 2009). In addition, in juvenile rats this was associated with a widening of the intraperiod dense lines which was attributed to incorporation of IgM between PLP-containing surfaces of the myelin sheath.

MAG is a minor protein of the myelin sheath, which makes up less than 1% of the CNS myelin composition. It is a type I trans-membrane glycoprotein located in the periaxonal myelin sheath. Therefore, in healthy myelin it is sequestered making it

an unlikely target for primary demyelination (Quarles, 2007). However, preliminary immunocytochemistry studies of MS lesions indicated a selective loss of MAG at the edges of developing plaques (Itoyama et al., 1980, Johnson et al., 1986). This was also observed in subsequent immunohistochemistry studies by Lucchinetti et al. (2000), which showed that a subset of MS patients had preferential loss of MAG around the edge of lesions (pattern III, (Table 1.2)). Despite this, only a few, early studies of small cohorts have been able to detect a MAG-specific antibody response in patients. One CSF study showed MAG-specific antibody binding was significantly higher in MS patients than in control patients (Wajgt and Gorny, 1983). Another group analysed the specificity of antibodies secreted by B cells isolated from the CSF of 25 patients with MS (Baig et al., 1991). This study revealed approximately half of the MS cohort contained B cells secreting IgG antibodies recognising MAG. However, in both studies some anti-MAG reactivity was also observed in control patients, suggesting this was not a disease specific response.

Autoantibodies present in MS patients are not just reactive against myelin, there are also axon-specific antibody targets (Derfuss et al., 2010). Neurofascin is a cell adhesion molecule and exists in two isoforms, neurofascin-155 (Nfasc-155) and neurofascin-186 (Nfasc-186). Nfasc-155 is produced by oligodendrocytes and is sequestered at the paranodal axo-glial junction and is required for correct organisation of the paranodal junction (Vyshkina and Kalman, 2008). On the other hand, Nfasc-186 is a neuronal product and is located at the node of Ranvier where it is exposed to the extracellular milieu (Sherman et al., 2005). Studies in MS lesions revealed changes in the expression and distribution of Nfasc-155 suggesting it might be an autoantibody target during disease pathogenesis (Howell et al., 2006, Maier et al., 2007). Investigations in MS patients using ELISA detected Nfasc-155 and Nfasc-186 autoantibodies in around a third of the serum samples (Mathey et al., 2007). Subsequent observations of these Nfasc-specific antibodies *in vitro* and *in vivo* revealed that Nfasc-186-specific antibodies led to inhibition of axonal conduction, axonal injury and exacerbation of EAE (Mathey et al., 2007). Another group showed that the adoptive transfer of serum from hNfasc-186

primed rats exacerbated EAE compared to serum from hNfasc-155 and complete Freund's adjuvant (CFA) controls and that this increase in disease was associated with axonal injury (Lindner et al., 2013). These data collectively indicate that Nfasc-186-specfic antibodies could induce antibody-mediated damage in MS.

1.6 Myelin Oligodendrocyte Glycoprotein as an immune target in Paediatric Multiple Sclerosis

1.6.1 MOG: A historic perspective

MOG is a type I membrane protein which is expressed exclusively in CNS myelin. Its structure consists of an extracellular IgV-like domain, a transmembrane domain and a short cytoplasmic tail (Figure 1.3). MOG was first identified as a minor CNS glycoprotein in 1984 (Linnington et al., 1984), and over the past 30 years the role of MOG in MS has been researched extensively. This interest is based on animal experiments where it was initially highlighted as an immuno-dominant target for demyelinating antibodies in EAE (Linnington et al., 1984). This reflects its exposure on the outer most surface of the myelin/oligodendrocyte continuum, where it is available to bind MOG-specific antibodies present in the extracellular milieu (Linington et al., 1989). This feature sets it apart from the other CNS antigen, which are sequestered within the myelin sheath and normally inaccessible to antibodies.

Further studies showed that MOG not only provides a target for demyelinating autoantibodies, but also contains T cell epitopes capable of triggering encephalitogenic $CD4^+$ and $CD8^+$ T cell responses (Mony et al., 2014). This ability of MOG to induce demyelinating autoantibody and encephalitogenic T cell responses in experimental animals make it a unique component of the CNS myelin, and account for its ability to recapitulate the MS-like disease induced by immunisation with whole myelin or spinal cord homogenates in rats and marmosets (Genain et al., 1995). MOG-induced variants of EAE in the mouse are now the most commonly

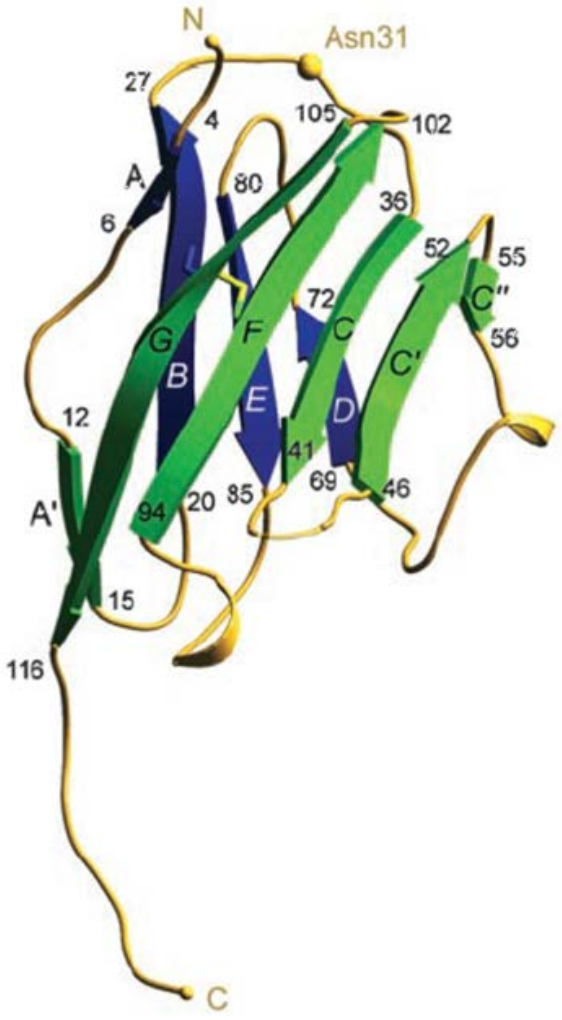


Figure 1.3. Myelin oligodendrocyte glycoprotein structure.
Ribbon diagram of overall myelin oligodendrocyte (MOG) structure taken from Breithaupt et al. (2003). This figure demonstrates MOGs extracellular IgV-like structure comprising of two anti-parallel β–sheets (blue and green), and its short cytoplasmic tail.

used animal model for MS, but it should be noted that not all animal strains/models are associated with a demyelinating MOG-specific autoantibody response, including MOG_{35-55}-induced EAE in C57BL/6 mice (Bourquin et al., 2003).

It is also currently unknown why this anti-MOG response develops. One attractive hypothesis is through molecular mimicry with dietary or microbe components, e.g. butyrophilin1a1 (BTN1a1) and Epstein-Barr virus (EBV), respectively. BTNa1 is mammalian milk protein, which expresses a high level of homology with the extracellular domain of MOG. Studies have proven molecular mimicry exists between MOG and BTNa1 and MS patients have BTN-specific antibody responses, suggesting BTN1a1 dietary exposure could modulate future immune responses to MOG (Stefferl et al., 2000, Guggenmos et al., 2004).

Demyelinating MOG-specific antibodies appear to bind to conformational-dependent, exposed epitopes of the protein (Brehm et al., 1999). This was formally demonstrated following elucidation of the crystal structure complex formed when the Fab region of the demyelinating MOG-specific mouse mAb 8-18C5 bound to MOG (Breithaupt et al., 2003). This experiment showed MOGs dominant binding epitope involved amino acid residues 101-108, which account for 65% of the total contact area. The introduction of single point mutations in this region reduced cognate recognition of MOG by polyclonal MOG-specific sera by >90% and completely inhibited its recognition by 9 out of 10 monoclonal antibodies, showing its importance.

1.6.1.1 The MOG-specific T cell response identified in MS

Later investigations have researched the encephalitogenic MOG-specific T cell repertoire and subsequently mapped the T cell epitopes for a variety of rat and mouse strains. Predominantly MHC class II restricted epitopes have been identified; MOG_{35-55} in C57BL/6J mice (Mendel et al., 1995), MOG_{8-21} and $_{35-55}$ in Biozzi ABH mice (Amor et al., 1994), and MOG_{79-96} in DBA/1 mice (Abdul-Majid et al., 2000). In addition to this a MOG epitope, MOG_{35-55}, has also been identified

which is recognised by MHC class I restricted $CD8^+$ T cells in C57BL/6J mice (Sun et al., 2001).

In MOG-induced models of EAE the MHC class II T cell restricted response is crucial as this is required to initiate an inflammatory response in the CNS and disrupt the BBB function; the latter being a pre-requisite if MOG-specific autoantibodies are to gain access into the CNS and induce demyelination. In addition they are also essential for the initial activation of B cells that ultimately results in the secretion of high affinity anti-MOG IgG antibodies.

1.6.2 MOG-specific antibodies are present paediatric MS patients

Originally, studies investigating the MOG response in human disease led to conflicting results due to the use of different methodologies (Table 1.3). Early studies attempting to detect MOG-specific autoantibodies in patients used solid-phase immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and western blotting and used antigen derived from a variety of sources including MOG isolated from human white matter (Xiao et al., 1991), human MOG expressed in *E.Coli* (Reindl et al., 1999) and recombinant mouse MOG expressed in transfected mammalian cells (Gaertner et al., 2004). These assays lacked the specificity required to differentiate between pathogenic and non-pathogenic MOG-specific autoantibodies and resulted in confusion as to the clinical significance of this MOG-specific autoantibody responses in MS (Kuhle et al., 2007).

This problem was resolved by the introduction of cell based assays that used live MOG transfected cell lines as a target to identify autoantibodies that bound MOG in its native conformation at the cell surface (Haase et al., 2001). This methodology revealed that previous reports of MOG reactivity as detected by ELISA or western blotting in patients and controls were mostly irrelevant with respect to demyelination, as it was unable to bind to the native protein. Indeed it is now recognised that potentially pathogenic MOG-specific autoantibodies are only

Table 1.3. Different methodologies used to detect anti-MOG antibodies in MS and other CNS disorders.

Technique	Antigens	Patient sample tested	Publications
ELISA	MOG from human white matter	Plasma and CSF from adult MS patients and controls	Xiao et al. 1991
	Mouse MOG	Serum from adult MS patients and controls	Gaertner et al. 2004
	Human MOG	CSF from adult MS patients, OND and controls	Reindl et al. 1999
	Refolded rat MOG	Serum and CSF from adult MS patients and controls	Gori et al. 2011
ELISA (DELFIA)	Refolded human MOG	Purified IgG from MS lesions, serum and CSF from adult MS patients and controls	O'Connor et al. 2005
Western Blot	Human MOG	Plasma from adult MS patients and controls	Lindert et al. 1999
MOG tetramer radioimmunoassay	Tetramers of human MOG extracellular domain	Serum and CSF from pediatric ADEM and MS patients and adult MS patients and controls	O'Connor et al. 2007
Cell-based assay	Human MOG transfected cell lines	Serum and CSF from paediatric patients with ADEM, MS or ONDs and from adults with MS and controls	O'Connor et al. 2007; Brilot et al. 2009; McLaughlin et al. 2009; Lalive et al. 2011; Di Pauli et al. 2011; Probstel et al. 2011

detected in a small number (4 to 5%) of cases of adult onset MS, although the frequency is much higher in paediatric MS and ADEM patients, around 20% and 40%, respectively (McLaughlin et al., 2009, Brilot et al., 2009).

The clinical significance of this MOG-specific autoantibody response in paediatric inflammatory demyelinating diseases remains unproven, but MOG-specific antibodies with similar properties mediate demyelination and exacerbate disease severity in animal models of MS (Linington et al., 1988). Early studies demonstrated MOG-specific antibodies are not only associated with myelin damage in marmosets with EAE, but also in some cases of MS providing circumstantial evidence that the MOG-specific antibody response is involved in lesion formation (Raine et al., 1999, Genain et al., 1999). Moreover, McLaughlin et al. (2009) reported IgG1 as the predominant isotype of the MOG-specific antibodies detected in patients with MS, thus providing evidence that they are in theory capable of mediating complement-dependent demyelination.

Taken together these observations identify MOG as a potential candidate for the development of antigen-specific therapies that might prove beneficial in paediatric MS and ADEM, an approach that may circumvent the many adverse effects associated with current treatments that rely on far broader immunosuppression.

1.7 The use MOG-Specific Therapies in the Treatment of Multiple Sclerosis

Current immunotherapies are based on broad suppression of the immune system but an alternative approach is to develop autoantigen-specific therapies, which requires the identification of appropriate antigen candidates. Recent studies on paediatric MS patients have revealed a high incidence of MOG-specific autoantibody responses (McLaughlin et al., 2009, Brilot et al., 2011), identifying this myelin antigen as a prime target for the development of an autoantigen-

specific therapy. The preliminary analysis of two such MOG-specific therapeutic approaches are discussed in detail in this thesis; (i) treatment with a MOG-specific B cell immunotoxin and (ii) induction of antigen-specific tolerance using low doses of soluble MOG. These chapters demonstrate that MOG-specific immunotherapies could be efficacious for the future treatment of patients with MS.

1.8 THESIS AIMS

In response to the large body of evidence supporting the presence of myelin-specific autoantibodies, particularly targeted to MOG, in MS disease pathogenesis, a primary goal of this thesis was to elucidate the function of these antibodies using an established *in vitro* bioassay (Elliott et al., 2012).

In parallel to this, two therapeutic approaches were investigated *in vivo* in animal models, which targeted the anti-MOG response;

(i) MOG-specific B cell immunotoxin

(ii) Low doses of soluble MOG

These aims were addressed individually in the hope of furthering our knowledge on autoantibody function in MS and in antigen-specific therapies with the aspiration of elucidating a novel treatment for people with MS.

CHAPTER TWO

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 ANIMAL EXPERIMENTS

2.1.1 Animals

2.1.1.1 Mice

Throughout this study female DBA/1j mice were used, which were between 7-8 weeks old. The mice were maintained at the University of Glasgow Central Research Facility.

All mice were purchased from Harlan Laboratories (Blackthorn, UK). All animal care and procedures were in accordance with the Animals Scientific Procedures Act, 1986, under a project license (No. 60/4314) and personal license (No. 60/12872), issued by the UK Home Office and with approval from the University of Glasgow Ethical Review Process Applications Panel.

2.1.1.2 Rats

Sprague Dawley (SD) rats were used in this study. They were maintained at the University of Glasgow Central Research Facility.

The rats were purchased from Harlan Laboratories, UK. All animal care and procedures were the same as section 2.1.1.1.

2.1.2 Induction of experimental acute encephalomyelitis (EAE) in DBA/1j mice

2.1.2.1 MOG_{1-125} induced EAE

Recombinant MOG_{1-125} (rMOG) (purified as per section 2.7.1) was diluted with phosphate buffered saline (PBS), and then mixed to make an emulsion with an equal quantity of incomplete Freund's adjuvant (IFA) (Sigma Aldrich, Dorset, UK), which was supplemented with 3 mg/mL of heat-inactivated *Mycobacterium*

tuberculosis H37RA (Difco Laboratories, Detroit, MI) by forcing the two solutions through a 24G connector bridging two syringes. Mice were injected subcutaneously (s.c.) at base of tail with 50 µg/100 µL.

2.1.2.2 MOG_{79-96} induced EAE

The MOG encephalogenic peptide, MOG_{79-96} (Cambridge Research Biochemicals, Billingham, UK), was prepared and injected as in section 2.1.2.1. Mice were given an additional intraperitoneal (i.p.) injection of 200 µg Pertussis Toxin (PTx) (List Biological Laboratories, Surrey, UK) on day of immunisation and 2 days post immunisation (d.p.i.).

2.1.3 Treatment of DBA/1j mice with EAE

2.1.3.1 MOG-ETA' treatment

Mice were immunised with rMOG as per section 2.1.2.1. Mice were then treated with i.p. injections at 48 hour intervals from 2 d.p.i. onwards with either 25 µg of an active or control immunotoxin, MOG-ETA' or Bo9-ETA', respectively. These immunotoxins were developed by Professor Stefan Barth's group in Aachen, Germany (Barth et al., 2000, Nachreiner et al., 2008).

2.1.3.2 Soluble MOG treatment

Mice were immunised with rMOG as per section 2.1.2.1. Mice were then treated i.p. with 25 µg rMOG, 25 µg MOG_{79-96} or PBS at 48 hour intervals from 2 d.p.i. onwards.

2.1.4 Clinical assessment of EAE

EAE was scored personally on a daily basis by the following scale: 0, no disease; 0.5, partial paralysis of tail without ataxia and/or pilo-erection; 1.0, flaccid paralysis of tail or ataxia and/or significant weight loss (≥1g); 2.0, impaired righting reflex or flaccid tail and ataxia; 3.0, partial hind limb paralysis; 3.5, same as above

but with full paralysis of one leg; 4.0, full hind limb paralysis; 5.0, full hind limb paralysis with forelimb involvement or moribund; 6.0, dead. A score of 5.0 was a humane endpoint for euthanasia. Other disease measurements have been used to assess EAE. These include the following; the incidence of EAE, cumulative and mean maximal scores, number of days with severe EAE, deaths as a result of severe EAE, and clinical scores and weights for each day. Cumulative scores were calculated by summing daily scores for each mouse. Mean maximal scores used the most severe EAE score from each mouse, including mice that did not get EAE which had a score of 0. For mean number of days with severe EAE, the threshold for severe EAE was a clinical score ≥2. Mice were scored by the same person throughout the experiments to minimise variation.

2.1.5 Isolation of blood, spinal cord, spleen and lymph nodes

Mice were culled by asphyxiation using CO_2. Blood was taken by cardiac puncture of the left ventricle. Mice were perfused with ice cold PBS and spinal cords flushed out by hydrostatic pressure using an 18G needle and Roswell Park Memorial Institute-1640 media (RPMI) (Sigma Aldrich). Sub-inguinal and para-aortic lymph nodes (LN) and spleens were taken and transferred into RPMI.

2.1.5.1 Preparation of spinal cord for FACS analysis

Spinal cords were triturated then enzymatically broken down using Collagenase D (Roche, Burgess Hill, UK) and DNAse (Sigma Aldrich) (both at 1 mg/mL) for 30 minutes at room temperature (RT) to make a single cell suspension. Cells were washed with RPMI then filtered through 70 µm cell strainers (BD Biosciences, Franklin Lakes, NJ, USA). Leukocytes were then isolated using a density gradient centrifugation by resuspending cells in 30% Percoll® (Sigma Aldrich), 70% RPMI and layering this over 70% Percoll®, 30% PBS and centrifuging for 20 minutes at 2000g. The interface was then washed and resuspended in FACS buffer (1% foetal calf serum (FCS) (Sigma Aldrich), 0.02% Sodium azide (NaH_3), 5 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich) in PBS).

2.1.5.2 Preparation of spleen and lymph nodes for FACS

Tissues were mechanically disrupted using 1 mL syringe plungers and 40 µm cell strainers (BD Biosciences). Sub-inguinal and para-aortic LNs from each mouse were pooled together for this step. Spleens were then resuspended in 1 mL Ack Lysis Buffer (Gibco Life Sciences, Paisley, UK) and incubated on ice for 1 minute. The resulting single-cell suspension was then washed and resuspended in FACS buffer at a density of 2 $x10^6$/mL.

2.2 EX VIVO ANALYSIS OF EAE

2.2.1 Flow cytometry for tissues

Cells were prepared as in section 2.1.5. All cells were then incubated for 20 minutes on ice with Mouse Fc Block™ (BD Biosciences) at a dilution of 1/100. To identify different immune cell populations cells were incubated on ice for 20 minutes in the dark with fluorescent-labeled antibodies to Ly6G, CD1d, CD8, CD11b, CD25, B220, CD5 and CD4 (eBioscience, Hatfield, UK); CD45, CD3 or Ly6C (BD Biosciences). These markers were chosen to detect T cells, B cells, monocytes and neutrophils. FoxP3expression, to identify regulatory T cells, was assessed using a FoxP3 stain kit (#77-5775-40, eBioscience) as described by the manufacturer. Cells were then washed and resuspended in 200 µL FACS buffer. Full samples were acquired and recorded on the MACSQuant (Miltenyi Biotech, Bergisch Gladbach, Germany) and data analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

2.2.2 Restimulation of spleen and LN cells

2.2.2.1 Proliferation assay

Antigen-specific proliferation was determined using single cell suspensions from LNs prepared in section 2.1.5.2. Cells were cultured in triplicate in complete Dulbecco's Modified Eagle's Medium (cDMEM (10% FBS, 1% Penicillin and

Streptomycin, 1% L-Glutamate, 1% Sodium Pyruvate, 1% non-essential amino acids, 50nM β-Mercaptoethanol (1000x) in DMEM media); Sigma Aldrich) at a concentration of 2 x10^6 cells/mL in 96-well plates (Corning Life Sciences, Amsterdam, The Netherlands). They were then restimulated with either rMOG (6, 20 and 60 µg/mL) or ionomycin (350 ng/mL) (Invitrogen, Paisley, UK) and phorbol 12-myristate 13-acetate (5 ng/mL) (PMA; Invitrogen) to provide a positive control. Controls cultures were pulsed at 24 hours and MOG_{1-125} stimulated cells at 48 hours with 0.4 µCi per well of [^{3}H] thymidine (GE Healthcare, Buckinghamshire, UK). Cells were harvested 24 hours later on to Printed Filter A mats (Perkin Elmer, Waltham, MA, USA), scintillation fluid (Perkin Elmer) added and incorporation of [^{3}H] thymidine determined using a MicroBeta TriLux (PerkinElmer).

2.2.2.2 Cytokine assay

LN cells were harvested as per section 2.1.5.2. Cells were resuspended in cDMEM at a density of 2 x10^6/mL. Cells were then cultured in 6-well plates (Corning Life Sciences) with or without 20 µg/mL rMOG for 72 hours. Supernatants were harvested and centrifuged at 300g for 5 minutes then aliquoted and stored at -20°C. Thawed supernatant cytokine secretion was measured using a Proteome Profiler Mouse Cytokine Array (#ARY006, R&D Systems, Abingdon, UK) according to manufacturer's guidelines. Results were quantified using ImageQuant Total Lab software (Amersham Bioscience, Freiburg, Germany).

2.3 CELL CULTURE

2.3.1 O4, O10 and Z2 hybridomas

Hybridomas were cultured in CELLine® cell culture flasks (BD Biosciences). Media compartment contained RPMI supplemented with 10% foetal bovine serum (FBS), 1% Pen/Strep, 1% L-Glutamate, 1% sodium pyruvate, 1% non-essential amino acids (NEAA), 0.1% β-mercaptoethanol. Cell compartment was inoculated with 2 x10^6 cells/mL. Cells were split twice a week and supernatant removed, and replaced

with fresh media. Supernatants were centrifuged at 800g for 5 minutes and stored at -20°C until antibody purification, described in section 2.7.2. Hybridomas were maintained at 37°C/5% CO_2.

2.3.2 Transfectant cell lines

2.3.2.1 Culture of MOG transfected cells

Antibody binding to the native extracellular domain of MOG was detected by flow cytometry using mouse MOG transfected LTK cells as described previously (Brehm et al., 1999). Full length human MOG transfected TE 671 cells and mock-transfected controls were kindly donated by Professor Edgar Meinl (Ludwig-Maximilian's University, Institute of Clinical Neuroimmunology). Cells were grown as adherent monolayers in vented 75 cm^2 culture flasks (Corning Life Sciences) in DMEM selection media supplemented with 10% FBS and 2 mg/mL G418 (Promega, CA, USA). When cells were approaching confluence they were detached from flasks using a cell scraper (Greiner Bio-One Ltd, Gloucestershire, UK) and transferred to a 50 mL falcon tube. They were then centrifuged at 400g for 5 minutes, resuspended in fresh media and seeded into new culture flasks which were maintained at 37°C/5% CO_2. Negative TE 671 cells were cultured the same, but in absence of G418. Full length mouse MOG transfected LTK cells and negative LTK cells were cultured as per TE 671 cells.

2.3.2.2 Enrichment of MOG positive transfected cells

MOG TE and MOG LTK cells were detached from flask using a cell scraper and centrifuged at 400g for 5 minutes. Cells were resuspended in 100 µL MACS buffer (PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA) with 1 µL Z2 (2 mg/mL) and incubated on ice for 20 minutes. Cells were washed twice by adding 2 mL MACS buffer then centrifuging at 400g for 5 minutes. Cells were then resuspended in 180 µL MACS buffer and 20 µL anti-mouse IgG MicroBeads (Miltenyi Biotec) and incubated at 4°C for 20 minutes. Cells were washed as above once. Finally, cells were resuspended in a total of 1 mL MACS buffer and applied to the LS column

(Miltenyi Biotec) in magnetic field through a 40 µm cell strainer. LS column was then washed three times with 3 mL MACS buffer. Column was then removed from magnetic field and 5 mL of MACS buffer added to elute MOG positive cells with plunger. Cells were centrifuged at 400g for 5 minutes and resuspended in $G418^+$ cDMEM selection media and seeded into vented 75 cm^2 culture flasks.

2.3.3 Generation of myelinating cultures

This sophisticated culture system was first described by Sorensen et al. (2008). Put simplistically, the 'myelinating cultures' (termed as such throughout this thesis) involve a monolayer of astrocytes, to which dissociated embryonic spinal cord cells are seeded, containing oligodendrocyte progenitor cells (OPCs), microglial cells and spinal astrocytes. Therefore, the myelinating cultures incorporate all the cells found in the CNS, meaning this compact system can be used to mimic an intact CNS with which one can explore the interactions of these endogenous cells with exogenous factors.

2.3.3.1 Culturing of neurospheres derived from the corpus striatum

Neurospheres were harvested from the corpus striatum of 1 day old (P1) SD rat brains. Postnatal pups were euthanised by i.p. injection of euthathal in accordance to UK Home Office regulations (project license no. 60/4314, personal license no. 60/12872). First, the cerebellum was removed then the brain was cut mid-sagitally along the corpus callosum into two cerebral hemispheres with a scalpel. The corpus striatum was then carefully dissected out from each hemisphere and placed in Leibovitz L-15 Media (Invitogen). Approximately 3 brains were used per flask. Tissue was broken up by gentle trituration using a glass Pasteur pipette then centrifuged at 140g for 5 minutes. Pellet was resuspended in 2 mL DMEM/F12 (1:1) (Gibco Life Sciences), supplemented with hormone mix, 30% glucose (Sigma Aldrich), 7.5% $NaHCO_3$, 1 M HEPES (Sigma Aldrich), 1% L-Glutamine, 1% pen/strep; collectively termed neurosphere media (**NSM**), and seeded into a 75 cm^2 non-coated flask (Corning Life Sciences) in a total volume of 20 mL NSM. Flasks were

subsequently supplemented with 20 ng/mL of epidermal growth factor (EGF) (Peprotech, UK). Neurospheres were incubated at 37°C/7% CO_2. Growth took approximately 7 days before neurospheres were ready to generate astrocytes.

2.3.3.2 Preparation of astrocytes from neurospheres

Glass coverslips (13mm) (VWR International, Leicestershire, UK) first had to be coated in 13.3 µg/mL poly-l-lysine (Sigma Aldrich) by incubating them for 1 hour at 37°C, then washed extensively with sterile water. One coverslip per well were plated out into a 24-well plate (Corning Life Sciences) and left to dry O/N.

Neurospheres were transferred into 50 mL falcon tubes and centrifuged at 140g for 5 minutes. Resulting pellet was resuspended in 12 mL DMEM + 10% FBS per 24-well plate (typically five or six 24-well plates were made from 1 flask depending on cell density). Then 500 µL added to each well and volume made up to 1 mL with additional media. Astrocytes were incubated at 37°C/7% CO_2 for approximately a week until the astrocytes were confluent.

2.3.3.3 Isolation of embryonic spinal cord

SD female rats were euthanised on embryonic day E15.5 (day of plugging denoted as E0.5) by overdose of CO_2 in accordance to UK Home Office regulations. The abdominal skin and fur was sterilised with 70% ethanol then a V-shaped cut was made through the abdominal wall and the gravid uterus was removed. It was placed in a petri dish containing ice cold Hank's balanced salt solution (HBSS) without Ca^+ and Mg^+ (Gibco Life Sciences) where each embryo was removed from the amniotic sac. Using microscopy the embryos were decapitated whilst taking care not to remove the cervical flexure. Next, the spinal cord was exposed by carefully removing the top layer of skin then gently removed from the embryo. Any attached meninges and dorsal root ganglia were detached to avoid contamination of peripheral nerve cells.

Spinal cord tissue was then partly dissociated by trituration with a glass Pasteur pipette then enzymatically digested with 2.5% trypsin (Sigma Aldrich) and 1% collagenase I (Invitrogen) in HBSS, 4-5 spinal cords per 1 mL HBSS. Cells were incubated for 15 minutes at 37°C and reaction stopped by addition of 2 mL SD solution per 1 mL HBSS (soybean trypsin inhibitor with DNAse I; Sigma Aldrich). After further trituration to form a single cell solution the cells were centrifuged at 200g for 5 minutes and resuspended in plating media (PM) containing 50% low glucose DMEM (Gibco Life Sciences), 25% horse serum (Sigma Aldrich), 25% HBSS and 2 mM L-Glutamine to a concentration of 3 x10^6 cells/mL.

Coverslips with the neurosphere derived astrocyte monolayer, section 2.3.3.2, were removed from their 24-well plates and put into 35 mm petri dishes, 3 coverslips per dish. Then 50 µL of the dissociated spinal cord solution (total of 150, 000 cells) was pipetted onto each coverslip. Cells were left to attach for 2 hours at 37°C, after which 450 µL PM and 600 µL high glucose DMEM (Gibco Life Sciences) supplemented with 0.5 mg/mL insulin (Sigma Aldrich), 10 ng/mL biotin (Sigma Aldrich), 0.5X N1 supplement (Sigma Aldrich), 50 nM hydrocortisone (Sigma Aldrich); collectively termed differentiation media plus insulin (DM+) was added to each petri dish. Cultures were maintained at 37°C/7% CO_2 for 28-30 days. They were fed three times a week by removing 500 µL and adding 600 µL fresh DM+. From day 12 *in vitro* (DIV) and onwards cultures were fed differentiation media without insulin, DM-, to promote myelination.

2.4 MYELINATING CULTURE ASSAYS

2.4.1 Complement-dependant demyelination assay

24-28 DIV myelinating cultures were incubated for 16 hours at 37°C with mouse or patient sera, using 2% rat serum as exogenous source of complement. 10 µg/mL Z2, rat serum alone and DM- media alone were used as controls. Mouse and patient sera were heat-inactivated for 10 minutes at 56-59°C before use.

2.4.1.1 Human patient serum samples used in complement-dependent demyelination assays

Two cohorts of patient sera samples were kindly donated to allow the study complement dependent demyelination in the myelinating cultures.

The larger cohort of 17 patient samples was kindly donated from Markus Reindl, Innsbruck Medical University, Austria. The study was approved by the ethical committee of Innsbruck Medical University (study numbers AM3041A and AM4059). All patients and controls gave written informed consent to the study protocol. Samples were blinded by Markus Reindl and after analysis demographic data (age, sex), clinical diagnosis and anti-MOG antibody status were provided. The demographic and clinical diagnosis of these patients is described in Table 2.1.

The smaller cohort of 5 paediatric patient samples was kindly donated from Amit Bar-Or from the Montreal Neurological Institute, Canada. All samples were collected following the appropriate and informed assent, in accordance with the Canadian Pediatric Demyelinating disease protocol. Both protocol and the assent documents were approved by the Hospital for Sick Kids research ethics board. Demographic and clinical diagnosis for the 5 patients is given below (Table 2.2). Samples were also blinded by Amit Bar-Or and patient details supplied after analysis. Samples from all cohorts were stored -80°C.

Table 2.1. Patient details of samples from Innsbruck Medical University used in complement-dependent demyelination study.
This table provides details of the age, sex and clinical diagnosis of the patient serum samples kindly donated by Markus Reindl from Innsbruck Medical University. *Abbreviations:MS: multiple sclerosis, ADEM: acute demyelinating encephalomyelitis, OND: other neurological disease, ON: optical neuritis, HC: healthy control.*

Patient ID	Diagnosis	Gender	Age
MS1	Adult MS	Male	31
MS2	Adult MS	Female	51
MS3	Adult MS	Female	34
MS4	Adult MS	Female	24
MS5	Adult MS	Female	40
MS6	Adult MS	Female	29
ADEM1	Adult ADEM	Male	47
ADEM2	Pediatric ADEM	Female	10
ADEM3	Pediatric ADEM	Female	3
ADEM4	Pediatric ADEM	Male	14
OND1	Pediatric ON	Male	13
OND2	Adult Myelitis	Female	49
HC1	-	Female	29
HC2	-	Female	26
HC3	-	Female	25
HC4	-	Female	31
HC5	-	Female	34

Table 2.2. Patient data of sera samples provided by Montreal Neurological Institute for complement-mediated demyelination study.
This table displays the demographic and clinical data of the patient sera samples kindly donated by Amit Bar-Or from Montreal Neurological Institute. *Abbreviations: Mono ADS: one acquired demyelinating event, ADEM: acute demyelinating encephalomyelitis.*

Patient ID	Diagnosis	Gender	Age
1	Mono ADS	Male	13
2	Mono ADS	Male	10
3	Mono ADS	Female	1
4	ADEM	Female	7
5	ADEM	Female	7

2.4.2 Complement-independent treatment of myelinating cultures

The effects of treating 18 DIV myelinating cultures with antibody minus complement were explored in a variety of ways. They were all incubated at 37°C:

- Investigating concentration effects using a range of antibody concentrations, incubated for 24 hours.
- Time course experiments using 20 µg/mL of antibody and incubating for 24 hours, 48 hours and 10 days.
- Examining long-term treatments using a ten day model treating from 18 DIV to 24 DIV.

Antibodies specific for myelin proteins and other cell surface markers were explored along with their isotype controls (details supplied in Table 2.3). Antibody purification was performed as described in section 2.7.2. All antibodies and ovalbumin (OVA) were buffer exchanged with Amicon Ultra-0.5 mL, 50kDa, centrifugal units (Millipore) to remove any low molecular weight contaminants. They were then diluted in sterile PBS (Sigma Aldrich).

Table 2.3. List of reagents used to treat the myelinating cultures in this study.

Antibodies	Host	Company	Isotype	Antibody Target
Z2	Mouse	Linington Laboatory	IgG2a	MOG
O4	Mouse	Linington Laboatory	IgM	Sulphatide
AA3	Rat	Linington Laboatory	IgG	PLP
O10	Mouse	Linington Laboatory	IgM	PLP
O4	Mouse	R&D Systems	IgM	Sulphatide
Ovalbumin	Rabbit	Acris Antibodies	IgG	Ovalbumin

Isotype Control Antibodies	Host	Company
IgG2a	Mouse	Sigma Aldrich
IgG1	Mouse	Sigma Aldrich
IgM	Mouse	Sigma Aldrich
IgG	Rat	Abcam

Proteins	Host	Company
Lipopolysaccharide	Mouse	Sigma Aldrich
Ovalbumin	Chicken	Stratech Scientific Limited

2.4.2.1 Treatment of myelinating cultures with OVA immune complexes in the absence of complent

OVA protein and OVA antibody used at 10 µg/mL and 100 µg/mL (product details in Table 2.3), respectively, were incubated together for 30 minutes at RT to make the OVA complexes. Then a titration of volumes were added to the cultures, which already contained 1 mL of normal feeding media; 6.25 µL, 12.5 µL, 25 µL, 50 µL.

2.4.2.2 Treatment of myelinating cultures with patient sera in the absence of complement

The effects of antibodies minus complement was also investigated using patient derived IgG. A cohort of 20 patients was collected from patients at the Southern General Hospital (Glasgow, UK), University of Heidleberg (Germany) and the University Clinic Grosshardern (Munich, Germany). Collection from each site was carried out using a protocol approved by their Institutional Review Board. All patients gave written informed consent to the study protocol. Demographic information and clinical diagnosis is detailed in Table 2.4. Samples were stored -80°C.

Table 2.4. Clinical data of patient cohort selected for complement-independent study.
This table provides the clinical and demographic details of the patient IgG samples used in the complement-independent study. *Abbreviations: SPMS: Secondary progressive MS, RRMS: relapsing-remitting MS, GBS: Guillain Barre Syndrome, CIDP: chronic inflammatory demyelinating disorder, NMO: neuromyelitis optica, CIS: clinically isolated syndrome, MG: Myasthenia Gravis.*

Patient ID	Diagnosis	Gender	Age
MS1	SPMS	Female	43
MS2	RRMS	Female	33
MS3	RRMS	Male	57
MS4	RRMS	Female	44
MS5	RRMS	Male	50
MS6	RRMS	Male	49
MS7	RRMS	Female	41
MS8	RRMS	Male	38
MS9	RRMS	Female	22
MS10	RRMS	Male	36
MS11	RRMS	Female	35
OND1	GBS	Male	58
OND2	GBS	Male	77
OND3	CIDP	Female	50
OND4	CIDP	Female	62
OND5	CIDP	Female	79
OND6	NMO	Female	47
OND7	Tranverse Myelitis	Male	21
OND8	CIS	Male	63
OND9	MG	Female	65

2.4.3 Immunofluorescent staining of myelinating cultures

2.4.3.1 Staining to visualise myelin

Cultures were fixed with 4% PFA for 20 minutes, washed in PBS, then permeabilised with 0.5% Triton X-100/PBS (Sigma Aldrich) for 10 minutes, and washed again. After cultures were blocked with blocking buffer (1% BSA, 10% horse serum, in PBS) for 30 minutes at RT, cultures were incubated for 45 minutes with either anti-MBP or anti-PLP in addition to anti-SMI31 to visualise the myelin and neurites/axons, respectively (Table 2.5). After extensive washing, cover slips were stained with fluorochrome-conjugated anti-mouse IgG2a AlexaFluor 488 or anti-rat IgG AlexaFluor 488, depending on primary antibody, and anti-mouse IgG1 AlexaFluor 568 (Table 2.5). Cultures were incubated for 15 minutes at RT in the dark. Unbound secondary was removed by washing with PBS followed by distilled H_2O then mounted in Mowiol® 4-88 plus DAPI (Sigma Aldrich).

2.4.3.2 Staining to visualise microglial cells

Cultures were fixed, permeabilised and blocked as in section 2.4.3.1. Afterwards microglial cells were visualised with anti-IBA1 antibodies and activation status was detected with anti-ED1 antibodies (Table 2.5). Cultures were washed and labelled with secondary antibodies anti-rabbit IgG AlexaFluor 568 and anti-mouse IgG1 IgG AlexaFluor 488 for Iba1 and ED1, respectively (Table 2.5). Cultures were washed and mounted in Mowiol® 4-88 plus DAPI as in section 2.4.3.1.

2.4.3.3 Immunochemistry using cell surface markers

Myelinating cultures were stained with AA3 or O10 antibodies to visualise PLP positive mature oligodendrocytes and myelin sheaths. Primary antibodies were diluted in DMEM media and incubated with the myelinating cultures at 4°C for 30 minutes. The cultures were then fixed and blocked as in section 2.4.3.1. After washing, cultures were then stained with secondary antibodies anti-rat IgG AlexaFluor 488 or anti-mouse IgM AlexaFluor 488 (Table 2.5).Unbound secondary

antibody was removed by washing with PBS followed by distilled H_2O then mounted in Mowiol® 4-88 plus DAPI.

Table 2.5. List of primary and secondary antibodies used for immunochemistry in this study.

Primary antibody	Antigen	Cellular location	Isotype	Dilution	Source
AA3	Proteolipid protein	Intracellular	Rat IgG	1/100	Gift from Prof. Sue Barnett
Anti-MBP	Myelin basic protein	Intracellular	Mouse IgG2a	1/200	Chemicon
Anti-Iba1	Iba1 on Microglia	Intracellular	Rabbit IgG	1/1000	Wako
ED1	CD68 on Microglia	Intracellular	Mouse IgG1	1/100	Abcam
SMI-31	Phosphorylated neurofilament	Intracellular	Mouse IgG1	1/1500	Abcam
O10	Proteolipid protein	Cell Surface	Mouse IgM	1/100	Hybridoma

Isotype	Conjugated fluorochrome	Dilution	Source
Anti-rat IgG	488 (green)	1/400	Molecular Probes
Anti-mouse IgG2a	488 (green)	1/400	Molecular Probes
Anti-rabbit IgG	568 (red)	1/400	Molecular Probes
Anti-mouse IgG1	488 (green) or 568 (red)	1/400	Molecular Probes
Anti-mouse IgM	488 (green)	1/400	Molecular Probes

2.4.4 Quantification of myelinating cultures fluorescent images

2.4.4.1 Quantification of myelinated axons

To measure remaining myelin, quantification was performed using 10 randomly acquired images from each of the three coverslip, resulting in 30 images per treatment (10X magnification; Olympus BX51 fluorescent microscope). Axonal density and myelination were determined using a pipeline (written by Steve Mücklisch, https://github.com/muecs/cp) in CellProfiler (Anne E. Carpenter and Thouis (Ray) Jones, Broad Institute Imaging Platform, MIT, USA). The pipeline

processes each image by extracting the green and red channels. The green channel is corrected for background illumination, and filters out any cell bodies and artifacts by their shape. A binary threshold was then applied to both red and green channels to get total number of pixels per field (Figure 2.1). Values calculated by pipeline were then exported into Excel where values were expressed in one of two ways;

$$\textbf{\% myelinated axons = Total axon density / Total myelin density}$$

$$\textbf{\% myelin loss} = \frac{\textbf{\% myelinated axons of treatment}}{\textbf{\% myelinated axons of control}} \times 100$$

This calculation was done for the 10 images per coverslip then a mean value for all three coverslips per treatment was generated from that.

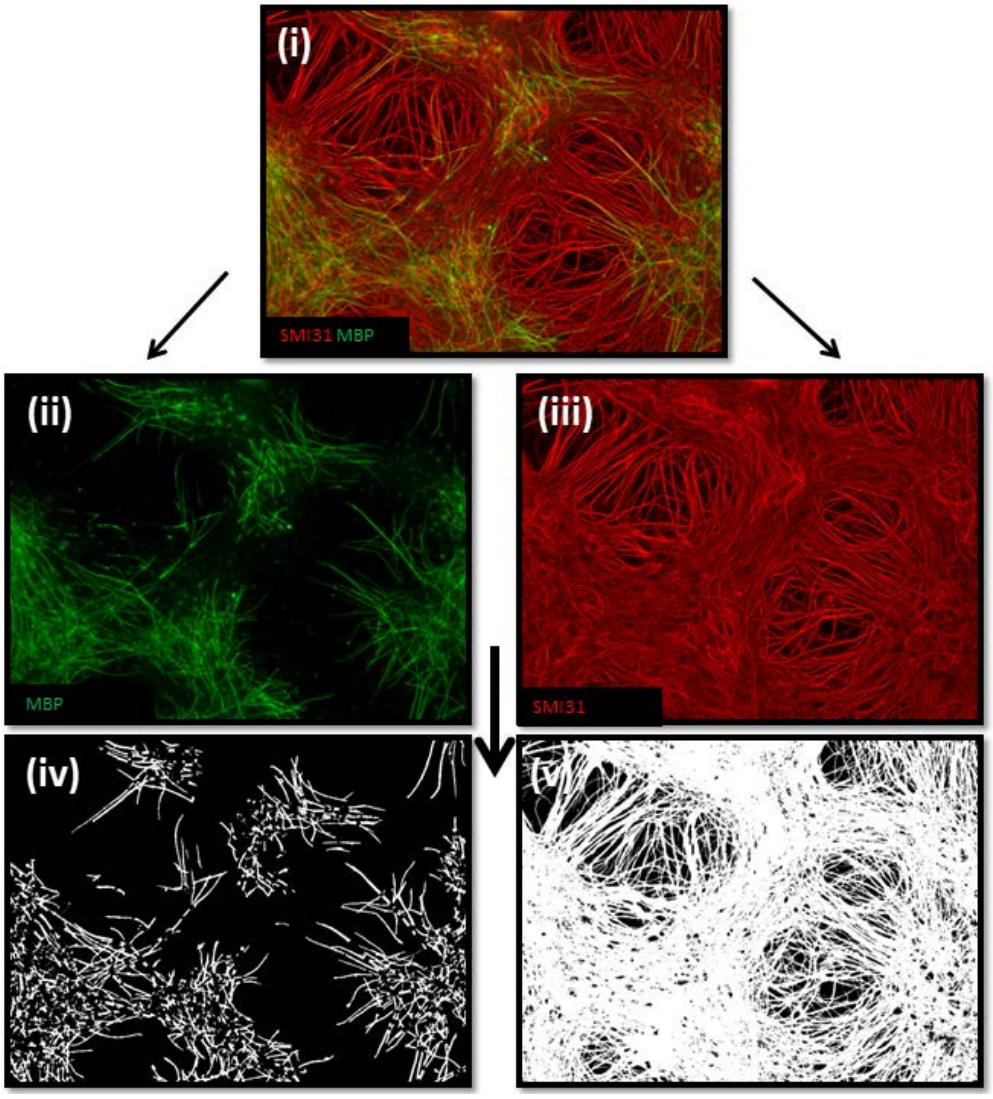


Figure 2.1. Quantification of myelinated axons using CellProfiler.
This figure illustrates the process adopted by CellProfiler to quantify myelinated axons in images. This representative image shows myelin stained with anti-MBP (green) and axons with anti-SMI31 (red) (i). Image is first split into its different colour channels green (ii) and red (iii). The threshold is applied to both images (iv), (v), which gives the total number of myelin and axon pixels per field.

2.4.4.2 Microglial cell quantification

To assess microglial cells, 10 randomly acquired images were taken from each of the three coverslips, resulting in 30 images per treatment (20X magnification; Olympus BX51 fluorescent microscope). Microglial cells were counted using ImageJ cell counter function (Version 1.41o, National Institute of Health, USA). Cells positive for IBA1 with a $DAPI^+$ nucleus at the same plane of focus were counted to measure total number of microglial cells. Cells which were $IBA1^+$, $DAPI^+$ and $ED1^+$ were counted as activated microglial cells.

$$\% \, ED1^+ \, Cells = ED1^+ / IBA1^+DAPI^+ \, X \, 100$$

2.4.5 Analysis of supernatant from myelinating cultures

2.4.5.1 Detection of cytokines from myelinating culture supernatants

Supernatants were harvested at end point of experiment and centrifuged at 300g for 5 minutes, then aliquoted and stored a -20°C. Thawed supernatant cytokine levels were measured using a Proteome Profiler Rat Cytokine Array (#ARY008, R&D systems) according to manufacturer's guidelines and quantified using ImageQuant Total Lab software.

2.4.5.2 Cell migration assay

Supernatants from myelinating cultures were harvested and centrifuged at 300g for 5 minutes, then aliquoted and stored a -20°C. Transwell® plate (Corning Life Sciences) were first incubated for 10 minutes at 37°C with chemotaxis buffer (DMEM, 0.5% BSA). This was then replaced with 600 µL of thawed supernatants. 3.0 µM membrane insert were then lowered into the well and incubated for a further 10 minutes at 37°C to allow chemokine binding to filter. 100 µL of MOG-specific T cells, which had been thawed and incubated O/N at 37°C in DMEM, were added into the upper well. The plate was incubated for 4 hours at 37°C, after

which media from the lower well was transferred into a FACS tube. Full sample was acquired and migrated cells recorded on a MACSQuant and data analysed using FlowJo software.

2.5 ANTIBODY ASSAYS

2.5.1 Flow cytometry of transfectant cell lines

2.5.1.1 Mouse serum MOG reactivity

Detached cells were resuspended in FACS buffer at a density of 2 x10^6/mL, confirmed by counting viable cells with trypan blue staining on a microscope slide with a 0.0025mm^2 graticule. A total of 100,000 cells were incubated with thawed serum at a 1/30 dilution for 30 minutes at 4°C. Cells were washed twice with 300 µL FACS buffer and stained with FITC-labelled anti-mouse IgG (Southern Biotech, Birmingham, AL, USA) antibody at a 1/200 dilution for 30 minutes at 4°C. Cells were washed twice, and stained with Viaprobe (BD Biosciences). A total of 25,000 events per sample were recorded on the FACS Calibur (BD Biosciences) and data were analysed using FlowJo software and GraphPad Prism.

2.5.1.2 Patient serum MOG reactivity

Experiment was performed as described in section 2.5.1.1 with only difference being use of FITC-labelled anti-human IgG (Southern Biotech) to detect anti-MOG binding.

2.5.2 Enzyme Linked Immunosorbent Assay (ELISA)

2.5.2.1 MOG protein reactivity

Anti-MOG antibody titres were determined by ELISA. Round bottomed 96-well plates (Corning Life Sciences) were coated with 10 µg/mL rMOG O/N at 4°C. Plates were washed three times with 0.05% Tween PBS (PBST) in between each step. Wells were blocked with 1% BSA and incubated at 37°C for 1 hour. Mouse sera

was used at 1/1000, 1/3000 and 1/9000 and incubated for 1 hour at 37°C. Horseradish peroxidase (HRP)-labelled IgG and IgM secondary antibodies (Southern Biotech) were used at 1/25000 and incubated for 1 hour at 37°C. Antibody binding was detected using developing solution and incubated in the dark. Reaction was stopped with 4 M sulphuric acid (H_2SO_4). Optical density (O.D.) was measured at 492 nm at the end point of reaction using a plate reader (Magellan Tecan, Männedorf, Switzerland). Each sample was analysed in quadruplicate.

2.6 MOLECULAR BIOLOGY

2.6.1 RNA extraction

2.6.1.1 RNA extraction from myelinating cultures using silica-gel membrane technology

Cells were first lysed using lysis buffer provided by supplier and then homogenised using QIAshedders (Qiagen, UK). RNA was extracted using the RNeasy Plus Micro Kit (#74034, Qiagen) as per manufacturer's standard protocol. RNA was stored at -80°C or used immediately.

2.6.1.2 RNA extraction from whole brains using Trizol

Whole brain samples from C57BL/6 mice were provided by Trevor Owens from University of Southern Denmark, Odense. Each brain was lysed by vortexing with two 5mm TissueLyser metal beads (Qiagen) in 1 mL Trizol (Ambion, UK) for 10 minutes at 50 oscillations; this vortex stage was repeated. Then 250 µL brain homogenate was mixed with 750 µL Trizol and incubated for 5 minutes at RT. To this, 200 µL chloroform was added and shaken vigorously, by hand, for 15 seconds and incubated at RT for a further 3 minutes. Mixture was then centrifuged for 15 minutes at 12, 000g at 4°C. 700 µL of the interface was removed and added to 700 µL 70% EtOH and mixed by pipetting. RNA was then extracted using PureLink® RNA

Mini Kit (Ambion) as per manufacturer's standard protocol. RNA was stored at -80°C.

2.6.2 cDNA synthesis

RNA was first quantified using a Nanodrop (Thermo Fisher Scientific, Northumberland, UK) and diluted to a concentration of 500 ng/mL using RNase-free water. RNA was then converted into cDNA in PCR thermo tubes using QuantiTect Reverse Transcription Kit (#205311, Qiagen) as per manufacturer's standard protocol. cDNA was used immediately or stored at -20°C.

2.6.3 qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using 7500 Fast Real-Time PCR System (Applied Biosystems) in accordance with manufacturer's guidelines. qRT-PCR amplifications were performed in triplicate set up in MicroAmp® Fast Optical 96-well Reaction Plates (Applied Biosystems). Each reaction contained the following reagents in a 15 µL volume:

SYBR® Green master mix	7.5 µL
Forward and reverse primer mix	100 pmol/µL
cDNA	500 ng/mL
RNase-free water	X µL

Primers (Sigma Aldrich) were designed using primer3 software. Sequences were found at National Centre for Biotechnology Information (NCBI) (National Institute for Health, USA) and primers were confirmed using BLAST (National Institute for Health, USA). Upon arrival they were resuspended in RNase-free water and stored in a 1:1 mix of forward and reverse primers. Sequences are detailed in Table 2.6. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control gene for all experiments.

Table 2.6. Primer sequences used in qRT-PCR studies

CCL5	Forward	5' CTT TGC CTA CCT CTC CCT CG 3'
	Reverse	5' TCC CCA GCT GGT TAG GAC T 3'
GAPDH	Forward	5' ATG ACT CTA CCC ACG GCA AG 3'
	Reverse	5' TAC TCA GCA CCA GCA TCA CC 3'

2.6.3.1 Cycling conditions

An initial denaturation of 95°C for 10 minutes, (95°C for 15 seconds, then a combined annealing and extension step at 65°C for 1 minute) x 40 cycles, and a final extension of 72°C for 5 minutes.

2.6.3.2 Quantification

The gene of interest (GOI) expression was quantified by the cycle threshold value (C_T value), which is the number of cycles required for the fluorescence to cross the threshold level. Samples of interest can then be compared to controls using the comparative C_T method (Livak and Schmittgen, 2001). The median of the triplicate wells for each sample was taken and any wells with values of 35 C_T or above were considered to have little or no mRNA expression. C_T values were first normalised to the housekeeping gene, GAPDH, using the equation shown;

$$\Delta C_T = C_T \text{ of GOI} - C_T \text{ of internal control (GAPDH)}$$

From this the fold change expression of the GOI could be calculated by comparing the sample of interest to the control or baseline sample;

$$\Delta\Delta C_T = \Delta C_T \text{ of sample of interest} - \Delta C_T \text{ of control sample}$$

$$\text{Fold change} = 2^{(-\Delta\Delta CT)}$$

2.6.4 qRT-PCR array

RNA was extracted from cultures as in section 2.6.1.1. cDNA was synthesised using RT^2 First Strand Kit (Qiagen) and analysed using cytokine and chemokine PCR arrays (#PARN-150Z, Qiagen), both performed as per manufacturer's guidelines. cDNA was used at 1 µg/mL consistently. qRT-PCR was run using 7500 Fast Real-Time PCR System in accordance with the manufacturer's guidelines. Quantification of results was performed as described in 2.6.3.2.

2.7 BIOCHEMICAL METHODS

2.7.1 Purification of recombinant MOG protein from bacteria

2.7.1.1 Expression of recombinant MOG in bacteria

Rat recombinant MOG protein (rMOG) was expressed in *Escherichia coli* - strain DH5α Fiq, which had been previously transformed with pQE12 (Qiagen) containing cDNA coding for rat MOG_{1-125}, the extracellular domain of MOG. Gene expression was under the regulation of the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lac promoter. The plasmid contained codes for six C-terminal histidine residues to allow purification under denaturing conditions by nickel-chelate chromatography (Amor et al., 1994). Successfully transformed bacteria were selected using ampicillin.

Bacteria were grown in seven starter cultures where 15 mL of L. Broth medium (LB) (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast in dH_2O) was inoculated with 15 µL transformed *E. coli* glycerol stocks, kanamycin and ampicillin and incubated for 5 hours at 37°C on a shaker. This was then transferred into 1 L of LB supplemented with more kanamycin and ampicillin, and grown O/N in same conditions. The 1 L was then divided into four 2 L volumes of LB. When bacterial growth was in exponential phase, identified by measuring O.D. at 600 nm, 20 mL of 0.1 M IPTG (BioTech Trade) was added to each flask to induce expression of rMOG. These flasks were incubated for a further 5 hours, and the bacteria harvested at 4500 g

(F10BCI, Beckman Coulter) for 15 minutes. This step was repeated until all the bacterial culture was pelleted. Next, the pellet was stored at -20°C O/N.

2.7.1.2 Lysis of bacteria

The bacterial pellet was resuspended in 5 mL/g PBS and homogenised on ice. To lyse cells DNase (5 µg/mL) (Sigma Aldrich) and lysozyme (1mg/mL) (Sigma Aldrich) were added and shaken on ice for 30 minutes. Then 0.5% lauryldimethylamine-oxide (LDAO) (Fluka Biochemika) was added and the mixture sonicated on ice (Soniprep 150, MSE) for 10 minutes with the amplitude set at 10-15. To retrieve rMOG from inclusion bodies, the mixture was centrifuged at 27,000 g (JA25.50, Beckman Coulter) for 20 minutes, supernatant discarded and pellet resuspended in 10 mL PBS, then homogenised on ice and resuspended in 20 mL PBS. This process was repeated three times. After final centrifugation the pellet was resuspended in 10 mL 8 M Urea buffer containing 4 mM β-mercaptoethanol and shaken for 30 minutes at RT. Solution was centrifuged at 27,000 g for 20 minutes and supernatant collected and filtered (0.45 µm, Nalgene).

2.7.1.3 Nickel-chelate affinity chromatography using AKTAprime

His-tagged rMOG was purified from supernatant using an AKTAprime (GE Healthcare) chromatography system where supernatant was loaded on to a HisTrap column (GE Healthcare) and eluted using an increasing gradient of imidazole. Eluted fractions were analysed using sodium dodecylsulfate polyacrylamide gel electrophoresis (15% SDS-PAGE) stained with Coomassie blue.

2.7.1.4 Dialysis

Protein containing fractions were pooled and then dialysed against 4 L 20 mM acetate buffer (pH 3.5) in dialysis tubing (12-14 kDA, Spectra/ Por Biotech). Protein dialysed for 48 hours at 4°C. Protein was then concentrated up using Centricon Plus 70 centrifugal filter units (Millipore) as per manufacturer's instructions.

2.7.1.5 BCA protein determination to measure concentration of MOG

Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturer's guidelines. Round bottomed 96-well plate (Corning Life Sciences) were used and measured with an ELISA plate reader (Magellan Tecan) with light absorbance set at 562 nm. Protein concentration was determined using a standard curve of light absorption against protein concentration using known BSA concentration standards and blank references. Final rMOG preparations were stored at -20°C at 2 mg/mL concentration.

2.7.2 Purification of hybridoma derived antibodies

2.7.2.1 Purification of IgG Antibodies

Z2 was purified from supernatants, section 2.3.1, using Hi-Trap Protein G HP (#17-0404-01, Amersham Biosciences) columns in accordance with the manufacturer's protocol. Supernatants were first filtered (0.45 µm, Minisart, Sigma Aldrich) to remove any particles. Eluted fractions were checked on Nanodrop, and antibody containing fractions pooled and dialysed in dialysis tubing (12-14 kDa) against PBS for 48 hours at 4°C. Final antibody concentration was determined by Nanodrop.

2.7.2.2 Purification of IgM Antibodies

O4 and O10 was purified from supernatants, section 2.3.1, using Hi-Trap IgM purification HP (#71-5004-37, GE Healthcare) columns as per manufacturer's instructions. Next, supernatants were filtered (0.45 µm) and eluted fractions were checked and dialysed as above, 2.7.2.1.

2.8 Statistical analysis

GraphPad Prism was used for all statistical analyses: t-tests, one and two-way ANOVA with repeated measures were used as appropriate. Data are expressed as the mean ± SEM or mean ± SD. P values < 0.05 were considered significant, and all tests were 2-sided.

CHAPTER THREE

MOG-SPECIFIC AUTOANTIBODIES IN MS AND ADEM PATIENTS

3 MOG-SPECIFIC AUTOANTIBODIES IN MS AND ADEM PATIENTS

3.1 INTRODUCTION

The concept that MS is a purely T cell mediated disease developed from studies demonstrating the adoptive transfer of myelin-specific $CD4^+$ T cells was sufficient to induce EAE in syngeneic recipients (Ben-Nun et al., 1981). This is now seen to have been an oversimplification as EAE can also be induced by autoreactive CD8+ T cells (Sun et al., 2001). In addition from the outset it was recognised these T cell mediated disease models fail to induce extensive primary demyelination, the pathological hallmark of the human disease. Subsequent studies revealed the development of a demyelinating "MS-like" pathology in these animal models was dependent on the availability of autoantibodies targeting the surface of the myelin sheath and/or oligodendrocytes (Linington et al., 1988, Svensson et al., 2002).

The recent discovery that B cell depletion suppresses the development of new inflammatory foci and reduces relapse frequency in patients with early RRMS (Hauser et al., 2008, Hawker et al., 2009, Naismith et al., 2010, Bar-Or et al., 2010) reignited interest in the potential role of autoantibody dependent mechanisms in the pathogenesis of MS, in particular in paediatric inflammatory demyelinating diseases that are often associated with an autoantibody response directed against the extracellular domain of MOG (reviewed in Reindl et al., 2013a).

This response is seen in around 20% of paediatric MS and ADEM cases, but rarely in adult onset MS (McLaughlin et al., 2009, Brilot et al., 2009, Probstel et al., 2011). These autoantibodies recognising surface exposed epitopes of MOG are predominantly of the IgG1 subclass indicating they have the potential to mediate complement-dependent demyelination in these young patients, but as yet no correlations have been identified between the presence or absence of these antibodies and disease severity (Kuhle et al., 2007). A longitudinal study of 77 anti-

MOG seropositive patients revealed this response was transient in ADEM but persisted, albeit at reduced titres, in the majority of patients who subsequently developed clinically definite MS (Probstel et al., 2011). These observations suggest the pathological significance of MOG-specific autoantibody responses in paediatric MS may be more subtle than in animal models, in which high doses of MOG-specific antibodies are used to induce an acute exacerbation of disease activity associated with widespread antibody-mediated demyelination (Linington et al., 1988).

To address this question the ability of anti-MOG seropositive sera to mediate demyelination *in vitro* using myelinated cultures derived from embryonic spinal cord was investigated. This strategy was previously successfully used to identify demyelinating, and more rarely axopathic, autoantibodies in a small subset of adult patients with severe, steroid resistant exacerbations (Elliott et al., 2012).

3.2 RESULTS

3.2.1 Z2 treatment induces myelin loss in myelinated cultures in a dose dependent manner

To investigate the bioassays sensitivity for a dose response experiment was performed using a complement fixing mouse monoclonal anti-MOG antibody Z2 (Brehm et al., 1999, Piddlesden et al., 1993). Myelinating cultures (24 DIV) were incubated with a range of different concentrations of Z2 in the presence of 2% fresh rat serum as an exogenous source of complement and demyelination was assessed 16 hours later by immunofluorescence microscopy. The remaining myelin was detected using a monoclonal antibody against myelin basic protein (MBP) and axons were stained using an antibody against phosphorylated neurofilament (SMI31). This revealed significant demyelination was mediated by antibody concentrations as low as 50 ng/mL ($p < 0.05$, 1way ANOVA) (Figure 3.1). This concentration (approximately 0.3 nM) is lower than the range reported for AChR-specific autoantibodies in myasthenia gravis (0.5 nM to 8 nM; (Leite et al., 2008)).

However, the myelin analysis programme continues to detect some residual myelin even in those cultures treated with high concentrations of Z2 (5 µg/mL; Figure 3.1). This could be due to the program being unable to differentiate between intact myelin sheaths and residual debris, some of which remains associated with $SMI31^+$ neuritis which can be observed by eye (Figure 3.2). This can also be corrected for by manual analysis (Nash et al., 2011). Nonetheless, in agreement with previous studies this experiment indicates these myelinated cultures can be used to detect demyelinating autoantibodies at concentrations of $\geq$ 0.3 nM.

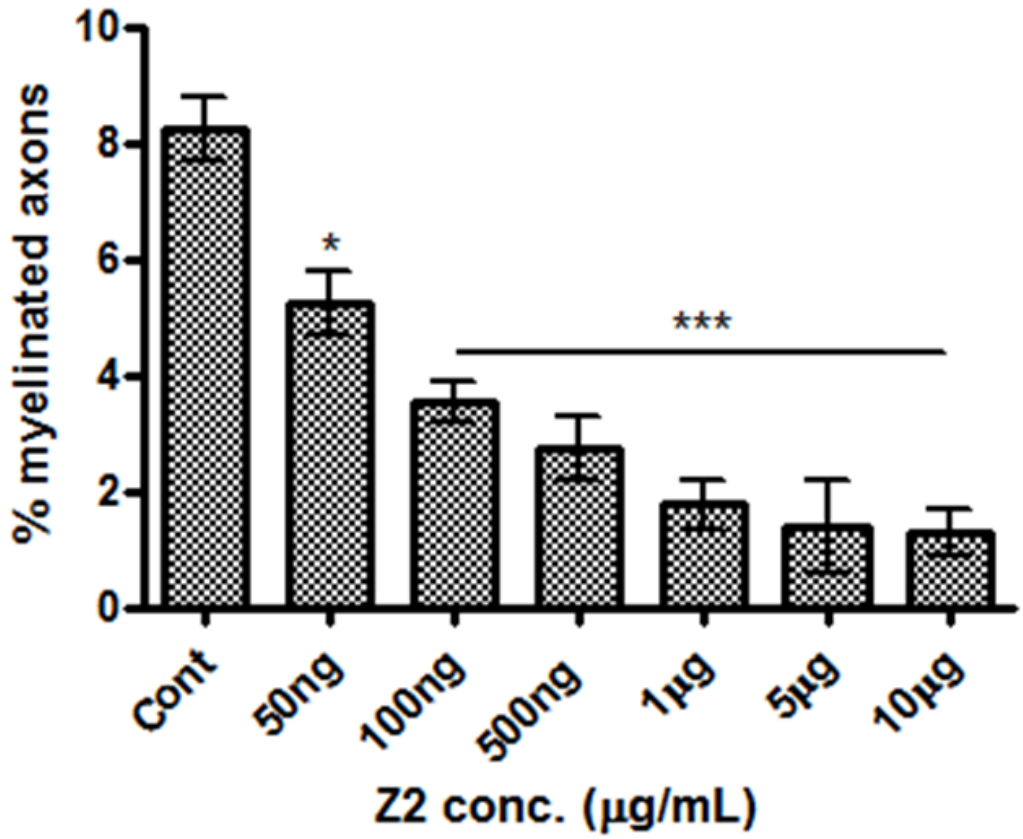


Figure 3.1 Z2 plus complement mediates demyelination in a dose dependent manner.
Myelinating cultures (24 DIV) were incubated with a range of concentrations of Z2 in the presence of 2% rat serum as a source of complement at 37°C for 16 hours. Controls included 2% normal rat serum alone (used as control in graph) and media alone, neither of which caused significant demyelination. Cultures were stained with anti-MBP and anti-SMI31 to detect the myelin and axons, respectively. These were then identified using secondary fluorescent-labelled antibodies. Myelin loss was quantified using CellProfiler. Full methodology is described in materials and methods (Section 2.4.2 and 2.4.3). Data presented as percent remaining myelin (± SEM) relative to untreated control cultures from two independent experiments each of which was performed in triplicate. *, $p < 0.05$, ***. $P < 0.001$ (1way ANOVA).

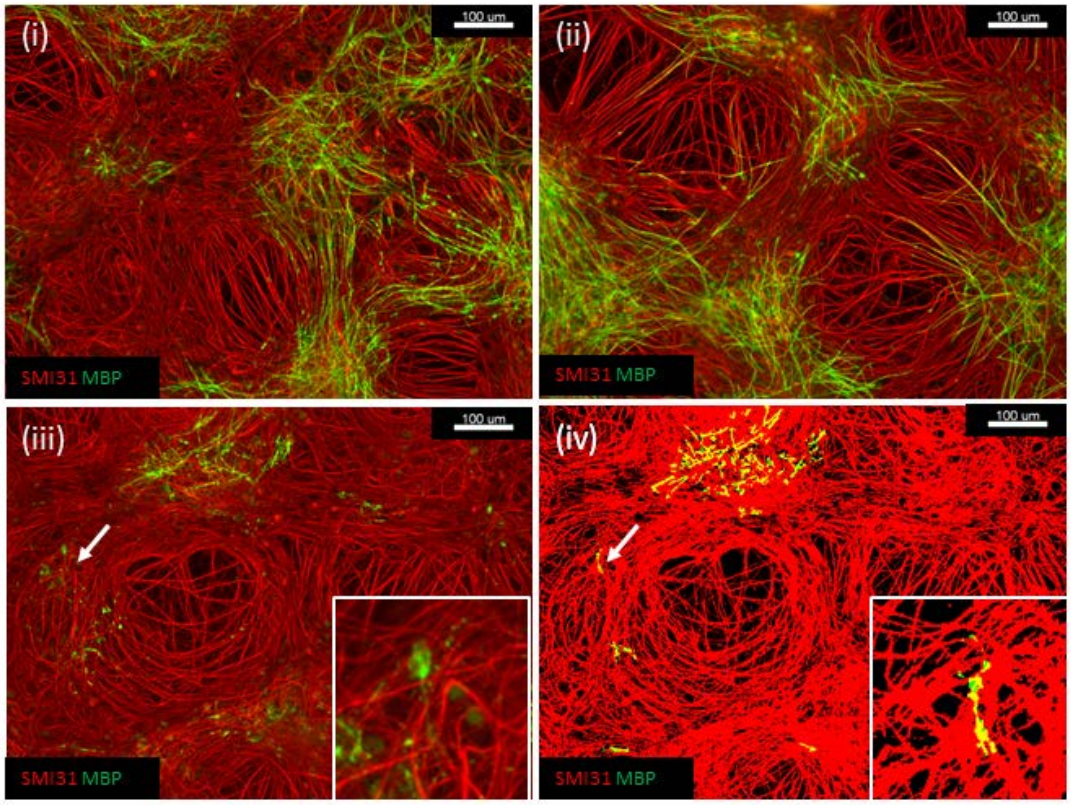


Figure 3.2. Representative images of Z2 demyelination.
Myelinated cultures (24 DIV) were incubated with a range of concentrations of Z2 in the presence of 2% rat serum as a source of complement at 37°C for 16 hours. Representative images compare myelination of controls, including 2% normal rat serum alone (i), and normal feeding media alone (ii), neither of which caused significant demyelination. Representative image showing myelin loss of myelinating culture incubated with 5 µg Z2 (iii), and image after being processed by CellProfiler (iv). Programme occasionally recognises myelin debris as myelinated axons. Inserts in (iii) and (iv) show area highlighted by arrows magnified. They identify an example where the programme has falsely calculated debris as myelin sheath (SMI31: red, MBP: green, 10X magnification). Scale bar representative of 100µm.

3.2.2 Demyelinating activity associated with MOG seropositive sera

For this experiment a cohort of patient sera was kindly donated by Prof. Reindl Markus from Innsbruck Medical University, Austria (demographic and clinical diagnosis of patient along with ethical approval is provided in section 2.4.1.1). These samples had previously been identified as being MOG-seropositive or seronegative (Figure 3.3). In addition to this they provided a small number, MOG-seronegative, healthy control donor serum. These samples were all screened for demyelinating activity using myelinated cultures.

With the exception of one case, ADEM4, none of the samples induced significant demyelination, irrespective of their anti-MOG antibody status (Figure 3.3). Notably ADEM3, which had higher titres of anti-MOG antibodies, had no detectable effect on demyelination. Z2 was used as a positive control, which showed robust demyelination indicating that there was no problem with the model. There was variation in myelin loss with most of the patient serum samples. Comparing the healthy control serum samples, taking all five together, there was a mean % myelin loss of 10.34 ± 2.82%. This suggests that when using this model to screen patient serum samples 10-13% myelin loss should be regarded with caution.

Analysis of the effects of ADEM4 on myelination confirmed this serum contained antibodies capable of mediating complement-dependent demyelination *in vitro* ($p < 0.05$; Figure 3.4), but the magnitude of this effect was small (control; 11.49 ± 1.01, ADEM4; 8.61 ± 0.68). Also it was not significant at the 95% level when compared to healthy control sera ($p > 0.05$; range 5.82 to 12.92 % myelin loss) (Figure 3.3).

Several different reasons may explain why this small study failed to identify an obvious correlation between serum *in vitro* demyelinating activity and the presence of MOG-specific autoantibodies:

(1) The concentration of MOG-specific antibodies is below the detection threshold of the assay

(2) The disease associated antibody response to human MOG does not cross-react with its rat homologue

(3) The response is simply not pathogenic

Patient ID	Diagnosis	Antibody Titre	MOG IgG	Murine MOG Reactive	% myelin loss
MS1	Adult MS	0.0	-	-	4.76 ± 6.65
MS2	Adult MS	1 in 40	-	low	0
MS3	Adult MS	0.0	-	-	0
MS4	Adult MS	0.0	-	-	0
MS5	Adult MS	0.0	-	-	23.21 ± 32.81
MS6	Adult MS	0.0	-	-	17.19 ± 24.31
ADEM1	Adult ADEM	1 in 640	+	no	0.07 ± 0.13
ADEM2	Pediatric ADEM	1 in 2560	+	-	7.88 ± 10.34
ADEM3	Pediatric ADEM	1 in 5210	+	high	3.46 ± 5.99
ADEM4	Pediatric ADEM	1 in 640	+	high	20.01 ± 7.13
OND1	Pediatric ON	1 in 640	+	no	21.91 ± 30.00
OND2	Adult Myelitis	1 in 640	+	no	9.70 ± 8.41
HC1	-	0.0	-	-	10.61 ± 18.37
HC2	-	0.0	-	-	5.82 ± 6.39
HC3	-	0.0	-	-	12.47 ± 21.10
HC4	-	0.0	-	-	12.92 ± 15.85
HC5	-	0.0	-	-	9.88 ± 17.11

Figure 3.3. Myelin loss is induced by incubation with patient serum samples *in vitro*.
Figure shows results for 6 MS, 4 ADEM and 2 other neurological disorder (OND) patients and 5 healthy controls. Patient sera were previously tested for antibody titre, presence of MOG IgG and reactivity to murine MOG. Sera were then tested on myelinated cultures (24 DIV) for demyelinating activity. They were incubated at 37°C for 16 hours with heat inactivated patient serum at a 1/30 dilution with or without 2% rat sera. Cultures treated with patient sera alone were used as controls. Z2 was used as a positive control. Data was from three independent experiments performed in triplicate, except MS5, 6 and ADEM 4 due to sample size constraints, (mean % myelin loss ± SD).

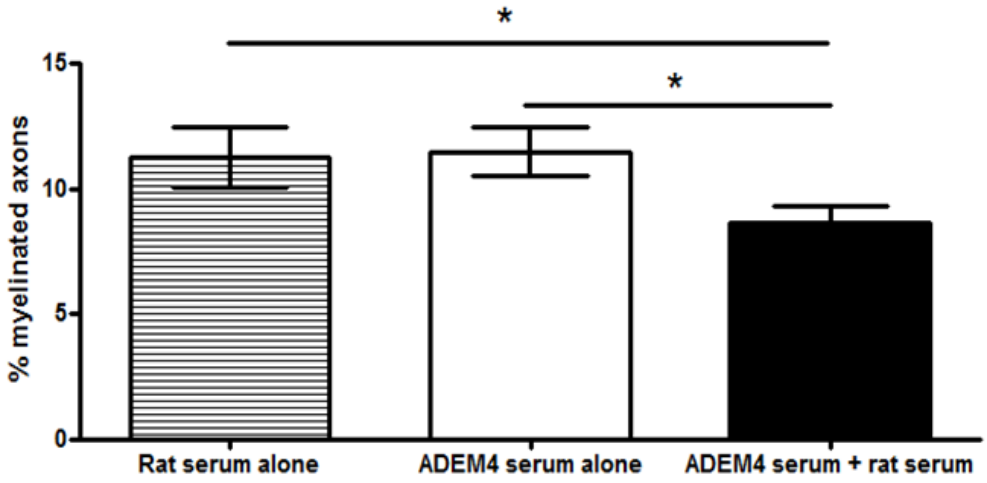


Figure 3.4. ADEM4 patient serum induces demyelination *in vitro*.
Myelinated cultures (24 DIV) were incubated at 37°C for 16 hours with heat inactivated patient serum at a 1/30 dilution with or without 2% rat serum as an exogenous complement source. Cultures were also treated with rat serum alone as a control. Bar graph shows representative results from two independent experiments performed in triplicate, mean (± SD). Significance determined by a t-test, *, $p < 0.05$. Rat serum alone and ADEM4 serum alone had the same % myelinated axons, $p = 0.846$.

In an attempt to resolve these points another cohort of patient sera was attained. These were kindly provided by Prof. Amit Bar-Or from the Montreal Neurological Institute (ethical approval and patient data is provided in section 2.4.1.1). These samples had been previously analysed by flow cytometry to determine: (1) the anti-human MOG antibody titre; (2) its cross-reactivity with murine MOG; and (3) its MOG-reactive IgG subclass profile. The experiment was blinded as to sample identity, but the collaborators ensured the sample cohort included at least one sample with a very high titre of cross reacting IgG1 MOG-reactive antibodies (samples 4 and 5) predicted to mediate complement-dependent demyelination (Mayer et al., 2013) (Figure 3.5). In both this experiment and the previous one serum was used instead of purifying the IgG, as although this would have been a preferable methodology there was a very limited volume of sera. Nonetheless screening these samples failed to identify any patients that mediated a significant level of complement-dependent demyelination when assayed at a dilution of 1 in 30, which was the highest concentration possible with the serum volume available (Figure 3.5).

Patient ID	Diagnosis	Murine MOG reactive	Human MOG reactive	% myelin loss
1	Mono ADS	+	-	0.20 ± 0.28
2	Mono ADS	+	-	0
3	Mono ADS	+	-	7.47 ± 10.56
4	ADEM	+	+	0
5	ADEM	+	+	1.27 ± 1.80

Figure 3.5. Paediatric MS and ADEM sera do not cause demyelination *in vitro*.
Shown are the % myelin loss results from 5 paediatric patients. Patient sera from patients with mono ADS (one acquired demyelinating event) and ADEM were previously tested for antibody binding to murine and human MOG. Myelinated cultures (24 DIV) were incubated at 37°C for 16 hours with heat inactivated patient serum at a 1/30 dilution with or without 2% rat sera. Cultures treated with patient sera alone were used as controls. Z2 was used as a positive control. Data was from two independent experiments performed in triplicate, (mean % myelin loss ± SD).

3.3 DISCUSSION

The adoptive transfer of antibodies recognising the native extracellular domain of MOG will induce a severe exacerbation of clinical disease in animals with EAE associated with extensive complement-dependent demyelination (Linington et al., 1988, Svensson et al., 2002). The identification of MOG-specific autoantibodies with a similar specificity profile in paediatric cases of MS and ADEM prompted speculation these would also act to induce demyelination and exacerbate disease severity (Reindl et al., 2013b). This hypothesis was tested in the current study by investigating whether we could identify the expected correlation between the presence of this MOG-specific autoantibody response in patient sera and its ability to mediate demyelination *in vitro*.

The use of myelinated cultures to detect demyelinating autoantibodies *ex vivo* was described first in a series of studies carried out in the 1960's that reported some, but not all sera from MS patients demyelinated rodent cerebella explant cultures (Bornstein, 1963, Bornstein and Appel, 1965). The inability of other groups to reproduce these findings however led to this experimental approach being neglected for over 30 years until it was resurrected following advances in myelinating culture techniques, immunofluorescence microscopy, data acquisition and analysis. These technical advances led to the development of a bioassay in which myelinated cultures were derived from embryonic rat spinal cord (Sorensen et al., 2008). They have been used to identify demyelinating, and more rarely axopathic autoantibodies in a subset of patients with severe steroid resistant relapses (Elliott et al., 2012).

The high degree of sequence homology between human and murine MOG together with the cross-species reactivity of murine MOG-specific monoclonal antibodies led us to anticipate the response in patients would also cross-react with rat MOG in the bioassay; a pre-requisite if we were to use it to explore its ability to mediate demyelination *in vitro*. Unfortunately during the course of this study it

became apparent that although the extracellular domains of human and rat MOG exhibit a sequence identity of >90 % this does not necessarily support a cross-reactive response (Mayer et al., 2013).

A recently published study of the epitope specificity of MOG-specific autoantibodies in 111 seropositive patients reported a large proportion of the MOG-reactive repertoire does not recognise murine MOG (Mayer et al., 2013) indicating rodent derived myelinated cultures are not an appropriate tool to screen for demyelinating autoantibodies in clinical samples. In rodents >80% of the MOG-specific response recognises a single motif exposed at the tip of the FG loop of MOG (Breithaupt et al., 2008), but in man epitope recognition is far more heterogeneous and involves at least 7 different patterns (Mayer et al., 2013). These all involve residues exposed on loops connecting the β strands of the extracellular IgV-like domain of MOG, including in a minority of cases the FG' loop motif recognised by many demyelinating MOG-specific murine mAbs (Breithaupt et al., 2008). Crucially only 19 of the 111 samples analysed exhibited comparable binding to human and murine MOG expressed at the surface of transfected cell lines. In the remaining cases recognition of mouse MOG was either absent or significantly lower than for the autologous human antigen. This observation stresses the importance of developing human culture systems to screen for pathogenic autoantibodies in MS and other neurological diseases. This is not possible at present due to a variety of technical and ethical issues, but will hopefully be resolved in the foreseeable future following developments in the use of stem cell based techniques to generate complex organotypic cultures.

Lack of cross-species reactivity provides a simple explanation as to why sera shown to contain MOG-specific autoantibodies were unable to mediate demyelination *in vitro*. However, this does not explain why sera 4 and 5 in the second small cohort failed to demyelinate the cultures, as this was selected on the basis of their high titre of cross-reactive IgG1 autoantibodies as determined by flow cytometry of transfected cell lines. The inability of these sera to mediate demyelination may be

attributed to two potential causes: (1) the titre of potentially pathogenic MOG-specific autoantibodies is below the detection threshold of the assay. (2) The assay itself detects responses that are not able to mediate demyelination *in vitro*.

The first is considered most likely as the bioassay will detect complement-dependent demyelination mediated by mAb Z2 down to concentrations <300 pM (Elliott et al, 2012; Figure 3.1), which is within the concentration range reported for autoantibody responses associated with other human autoimmune diseases e.g. in MG (Leite et al., 2008). Sensitivity could be increased by assaying sera at concentrations higher than the 1/30 dilution used in this study, but this could not be investigated due to the limited availability of sera from these anti-MOG seropositive donors.

Another possible reason why these antibodies appear to play a minor role may be due to the antibody target. MOG is a structural protein of the myelin sheath but its function remains unknown (Chekhonin et al., 2003). Studies using MOG-deficient mice failed to identify a phenotype associated with this knock-out (Delarasse et al., 2003). Therefore antibody-mediated damage relies solely on complement or ADCC targeting. In contrast the majority of antibodies in MG target and eliminate AChR receptors, an important, functional receptor (Cavalcante et al., 2012). Therefore it is possible that a higher titre of anti-MOG antibody than anti-AChR antibody is required to see a clinical effect.

Possibly more worrying are concerns around the suitability of using transfected cell lines as a strategy to identify potentially pathogenic autoantibody responses to membrane bound antigens in clinical samples. This is now viewed as the gold standard approach to identify MOG-specific autoantibodies, although a number of other techniques are available to explore this response in childhood cases of inflammatory demyelinating disease (reviewed in Mayer and Meinl, 2012). It is reasoned that the conformation of MOG as it is displayed at the surface of transfected target cells is identical to that *in vivo* in which MOG is exposed at the

surface of the myelin sheath/oligodendrocyte continuum. However, there may be flaws in this argument as the molecular environment in which MOG is expressed is very different in these two situations. In particular, interactions with other molecules in the plane of the membrane may result in changes in epitope accessibility, or even the conformation of the extracellular domain of MOG itself; a problem recently highlighted during a detailed analysis of the axopathic autoantibody response induced by different isoforms of neurofascin (Lindner et al., 2013). To elucidate the pathological relevance of these FACS detected antibodies adoptive transfer experiments using EAE models would need to be performed. This is not possible due to the volume of serum that would be required to get sufficient immune-purified anti-MOG antibodies and of the low occurrence of donors with high, cross reactive antibody titres.

The identification of low levels of demyelinating autoantibodies is supportive of the growing consensus that antibody dependent mechanisms, irrespective of their specificity, play some role in the pathogenesis of MS (Wilson, 2012). In the case of MOG-specific autoantibodies in paediatric MS and ADEM patients, the tacit assumption was that this autoantibody response will induce extensive myelin loss, and exacerbate clinical disease. This study does not support that assumption. Further, in most seropositive patients the antibody titre is never particularly high and declines over time (Probstel et al., 2011). Moreover, no correlation exists between disease severity and the presence or absence of a MOG-specific antibody response in either MS or ADEM (Di Pauli et al., 2011).

These observations lead us to ask two simple questions:

1) How much antibody is required to induce significant exacerbation of disease severity in EAE?

2) How does antibody concentration relate to the levels of demyelinating activity identified in patients?

Published EAE studies show that large doses of intravenously injected Z2 are required to induce a severe but survivable exacerbation of EAE in T cell mediated models of EAE in the rat (Piddlesden et al., 1993, Lindner et al., 2013). Assuming a plasma volume of 4 mL/100 g and a body weight of 200 g this equates to an IgG plasma concentration of 82.5 µg/mL (IgG plasma concentration being approximately 0.5 nM) (Fowler et al., 1986). This is approximately four orders of magnitude higher than that required to induce complete demyelination using monoclonal anti-MOG antibodies in the bioassay, as shown in Figure 3.1. This observation suggests that higher concentrations of demyelinating autoantibodies are required to cause clinically significant deficits than what is required to elicit effects in the *in vitro* assay; although the situation in patients is complicated by factors such as lesion load, whether the lesions involve clinically eloquent tracts and blood brain barrier leakiness.

This hypothesis can be tested by calculating the concentration of mAb Z2 required to mediate a similar level of demyelination *in vitro* since a previous published study acquired IgG preparations from patients with severe steroid non-responsive relapses, which benefited clinically from plasma exchange (Elliott et al., 2012). Assuming a serum IgG concentration of 12 mg/mL and taking values from Elliott et al. (2012), this value for the concentration of "mAb Z2 equivalents" is approximately 75 nM. In contrast, applying the same calculation to ADEM4's serum (Figure 3.4), the only sample that consistently mediated a low level of complement-dependent demyelination in the current study gave a value of < 1 nM.

Taken together these observations suggest that in functional terms the concentration of MOG-specific autoantibodies detected in childhood cases of MS and ADEM is at least three orders of magnitude lower than that required to induce a clinically significant response in EAE (Lindner et al., 2013), and at least two orders of magnitude lower than the demyelinating IgG response associated with acute clinical relapses in some adult patients with MS (Elliott et al., 2012). Admittedly

these calculations are not exact but nonetheless they suggest that low concentrations of MOG-specific autoantibodies present in cases of paediatric MS and ADEM may not have a major impact on disease severity. This interpretation is supported by the lack of any correlation in these patients between disease severity and the presence or absence of a MOG-specific antibody response. Therefore the question remains, what is the functional relevance of these low titres of MOG-specific antibodies in MS and ADEM?

CHAPTER FOUR

CHARACTERISATION OF MYELIN-SPECIFIC ANTIBODY FUNCTION

4 CHARACTERISATION OF MYELIN-SPECIFIC ANTIBODY FUNCTION

4.1 INTRODUCTION

As stated in the previous chapter there is an increasing body of evidence identifying anti-MOG autoantibodies in patients with demyelinating disorders, particularly in patients with paediatric MS (Reindl et al., 2013b, Mayer and Meinl, 2012). The primary pathogenic role of these MOG-specific antibodies is thought to be through complement or cell-mediated pathways, which is a well-established mechanism for demyelination and axonal damage and has been identified in many neurological disorders (Gasque et al., 2000). This mechanism has also been studied in multiple EAE models (Terenyi et al., 2006). In a study by Linington and colleagues it was demonstrated that depletion of serum complement markedly decreased clinical symptoms of EAE (Linington et al., 1989). In addition complement-dependant demyelination can be seen in post mortem lesions of MS patients (Lucchinetti et al., 2000). However, the previous chapter illustrated the fact that the presence of MOG autoantibodies do not always correlate with antibody-mediated demyelination and to date no one has identified a functional role for MOG-specific antibodies.

The lack of evidence supporting MOG-specific autoantibody mediated complement-dependent demyelination, possibly due to low antibody titres, raises the possibility that these antibodies may be playing a sub-lytic role in disease pathogenesis. Less is known about how these antibodies might contribute to disease in the absence of complement but there are several established complement-independent effector pathways: -

1. *Antibody-dependent cell-mediated cytotoxicity (ADCC)*

   This process results in the lysis of a targeted cell, which has been labelled with an antibody. Effector immune cells, mainly natural killer (NK) cells, bind

to the Fc region of the antibody via their Fc receptors, which triggers the release of perforin and granzyme granules, resulting in targeted cell death (Shi and Van Kaer, 2006). Studies have shown EAE is ameliorated in FcRγ-deficient mice due to inhibition of ADCC (Abdul-Majid et al., 2002). Perforin^{+} NK cell numbers were shown to be increased in PPMS and SPMS patients compared to healthy controls suggesting a role in MS (Plantone et al., 2013). Human microglia cells have also been shown to lyse opsonised antigen via their Fc receptor *in vitro* (Ulvestad et al., 1994). However, it is unknown what titre of antibodies is required for this mechanism to occur.

2. *Opsonisation for phagocytosis*

In this pathway antibodies attach to their antigens and act as a binding enhancer to immune cells expressing Fc receptors (Ravetch and Bolland, 2001). This interaction leads to phagocytosis of the flagged cell (Underhill and Goodridge, 2012). It has been previously shown that autoantibody binding is responsible for destruction of the myelin sheath (Genain et al., 1999). This publication demonstrated that Ig and myelin debris could be found in phagocytes in patient MS lesions.

3. *Direct effects of antibody binding*

Studies have shown that anti-MOG antibodies can have a direct effect on the myelin sheath upon binding. The investigation reported a novel pathogenic antibody mechanism by which crosslinking of MOG on the surface of oligodendrocytes led to repartitioning of MOG into insoluble microdomains (Marta et al., 2003). Furthermore, they showed MOG crosslinking mediated a physiological change in oligodendrocytes; an increase in calcium influx and activation of MAPK and Akt, and morphological changes; e.g. cell process retraction (Marta et al., 2005b, Marta et al., 2005a). They also presented microglial cells as the likely endogenous cells mediating this cross-linking (Marta et al., 2008).

Collectively, Marta's published data demonstrate a novel demyelinating pathway induced by anti-MOG autoantibodies, which could be disease relevant.

As a result of the previous patient cohort results in chapter 3, this chapter explored a new role for these autoantibodies; could prolonged exposure to antibody in the absence of exogenous complement have another sub-lytic effect in disease? This would be relevant in many autoimmune diseases where there are low titres of autoantibodies. Previously a similar approach was carried out *in vivo* by implanting an O10 hybridoma into rat spinal cords, which resulted in focal demyelination, remyelination and disruption of newly forming myelin (Rosenbluth and Schiff, 2009). Rosenbluth et al' have also explored the effects of implanting different hybridoma cells secreting IgM antibodies specific to other CNS antigens; O1 and O4, which bind to GalC and sulphatide, respectively (Rosenbluth et al., 1999, Rosenbluth et al., 2003). These studies showed similar myelin disruption. However, this model is problematical for investigating complement independent effects due to the complex relationship between the systemic immune system and CNS, and because of the local synthesis of complement (Veerhuis et al., 2011, Morgan and Gasque, 1996). Therefore myelinating cultures were used for these studies as they could be considered an "immunologically sterile" culture system with no systemic immune response, even though there are microglial cells present in the cultures.

The aim of this chapter was to investigate the role of autoantibodies in the absence of complement. Using the myelinating cultures this study found that autoantibodies contributed to myelin damage and inflammation in three main ways (discussed fully in this chapter):-

- Disruption and inhibition of myelin formation

- Activation of microglial cells

- Production of pro-inflammatory chemokines

Therefore, this chapter presents a novel pathway in which anti-myelin autoantibodies may potentiate CNS inflammation during MS, independent of complement.

4.2 RESULTS

4.2.1 Z2 and the O4 antibody inhibit myelination in a complement-independent manner

To test the effects of antibodies on CNS myelination in the absence of the peripheral immune system, myelinating cultures were treated for 10 days with 20 µg/mL of Z2, the O4 antibody or their respective isotype control in the absence of an exogenous source of complement (antibody details in Table 2.3). Cultures were assessed 10 days later by immunofluorescence microscopy to quantify myelination. Myelination was significantly inhibited when the anti-myelin/oligodendrocyte antibodies were present compared to their isotype controls (Figure 4.1). The O4 antibody is an IgM antibody which binds to several antigens including sulphatides and sulphated glycosphingolipids. These are found on pre-oligodendrocytes, mature oligodendrocytes and a constituent of myelin sheath (Sommer and Schachner, 1981). The O4 antibody treatment abolished the myelination that should have occurred between day 18 and 28, as there was no significant difference between % myelinated axons in the non-treated cultures on 18 DIV and the O4 antibody treated cultures at 28 DIV. Myelination increased by 7.4% in the untreated control between 18 DIV and 28 DIV, which was significantly reduced with the O4 antibody treatment to 2.2%, which was approximately a third of the control level.

Z2 also inhibited myelination but it was not as marked as the O4 antibody, with myelination levels being approximately half the control (Figure 4.1). Z2 is a monoclonal IgG2a antibody, which binds to the extracellular domain of MOG.

Results with Z2 were more varied than with the O4 antibody, most likely due to the changeable amounts of MOG available in the cultures, as maturation of oligodendrocytes and myelination in the cultures will differ between preparations. The O4 antibody had a more pronounced effect on myelination compared to Z2, possibly because there was more antigen availability on the cells in the cultures. This observation showed that the O4 antibody and Z2 in the absence of any peripheral immune system could affect the levels of myelination.

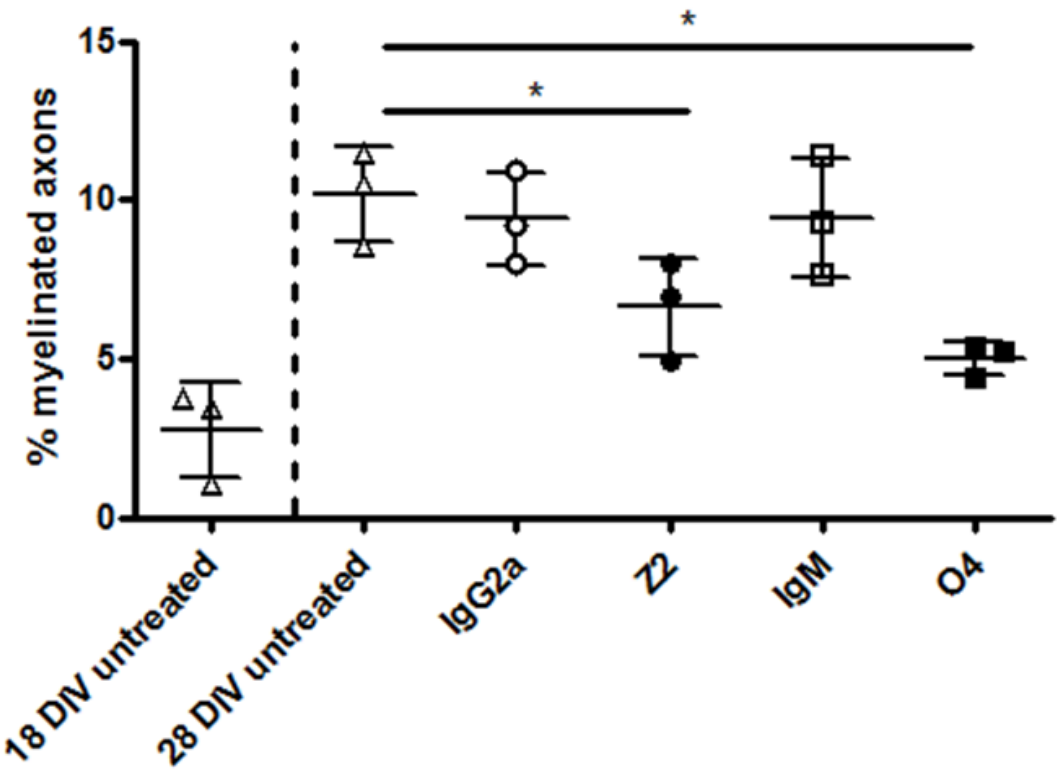


Figure 4.1. Antibody treatment in the absence of complement inhibits myelination.
To investigate the effects of treating the myelinating cultures with antibodies in the absence of complement, myelinating cultures (18 DIV) were treated every 48 hours for 10 days with 20 µg/mL Z2, the O4 antibody or their respective isotype control. These were supplemented into the normal feeding media. The control cultures were incubated with normal feeding media alone. Myelination was quantified at 18 DIV and 28 DIV using immunocytochemistry as described in materials and methods (Section 2.4.2 and 2.4.3). Cultures were stained with anti-SMI31 and AA3 to detect axons and myelin, respectively. Experiment was performed in triplicate from three independent experiments. Data points represent mean (± SD). Significance was determined by student t-test. *, $p < 0.05$.

4.2.2 Antibody treatment without complement leads to microglial activation

The previous graph showed that treatment of cultures with antibodies for 10 days led to significant inhibition of myelination. Next to be established was what mechanisms were involved in this effect. Microglial cells were investigated as they are important immune cells of the CNS and have Fc receptors that could be interacting with the antibodies O4 and Z2 (Giunti et al., 2013). Activated microglial cells were identified using IBA1 as a microglial cell marker and ED1 as an indication of cellular activation. ED1 antibody binds to a cytoplasmic antigen found in lysosomal membranes, which increases with phagocytic activity, a property seen in activated microglial cells (Bauer et al., 1994, Damoiseaux et al., 1994, Dijkstra et al., 1985). This revealed that the number of $IBA1^+$ cells were approximately two-fold more after cultures were treated for 10 days with the antibodies compared to the isotype controls (Z2 and the O4 antibody , $p < 0.01$) (Figure 4.2A). As this experiment was performed in a closed *in vitro* bioassay it indicated that the microglial cells were proliferating in response to the antibody treatment. Staining for activated $IBA1^+$ cells using ED1 showed a significant increase in the number of activated microglial cells (Z2, $p < 0.05$; O4, $p < 0.001$) (Figure 4.2B). Z2 treatment led to a 2-fold increase in microglial activation and the O4 antibody treatment led to a 3-fold increase. In both cases the O4 antibody had a greater effect on the microglial cells compared to Z2 treatment, mirroring their effect on myelination.

There were also morphological changes in the microglia after treatment with Z2 and the O4 antibody (Figure 4.3). Most pronounced effects were observed the O4 antibody treatment, where cells exhibited a more rounded and ‘amoeboid’ shape, which is traditionally associated with cellular activation. This was in comparison to the ramified appearing microglial cells in the isotype control treated cultures. Consequently, these results suggest that the effect on myelination levels seen in Figure 4.1 was associated with the activation of microglial cells, with the O4 antibody being more potent than Z2.

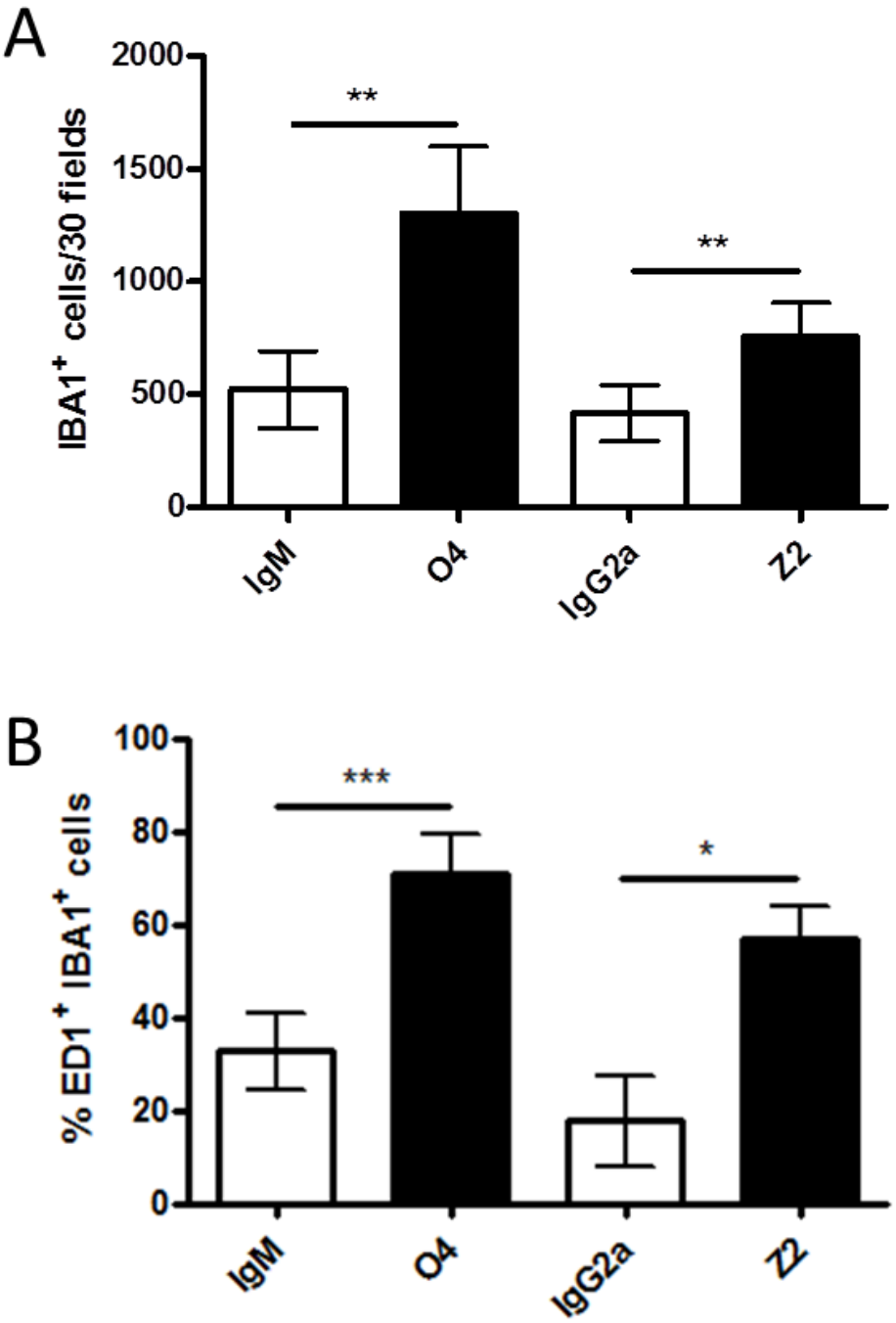


Figure 4.2. Antibody treatment increased the number of microglia and microglial activation. Myelinating cultures (18 DIV) were treated every 48 hours for a 10 day period with 20 µg/mL Z2, the O4 antibody or their respective isotype control. This was to observe the effects these antibodies had on microglial in the absence of complement. The antibodies were supplemented into the normal feeding media. The control cultures were incubated with normal feeding media alone. After 10 days of antibody treatment the cultures were fixed and stained to identify the microglia using IBA1 and ED1 for microglial cells and cellular activation, respectively. Cell numbers were counted using ImageJ. (A) Values shown are total $IBA1^+DAP1^+$ cells for total microglial cells counted in 30 fields. (B) % $ED1^+$ cells was the number of $IBA1^+DAPI^+$ cells that co-localised with ED1 in 30 fields. Experiment was carried out in triplicate from three independent experiments (n=3). Bar graph represents mean (± SD). Significance was determined by student t-test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

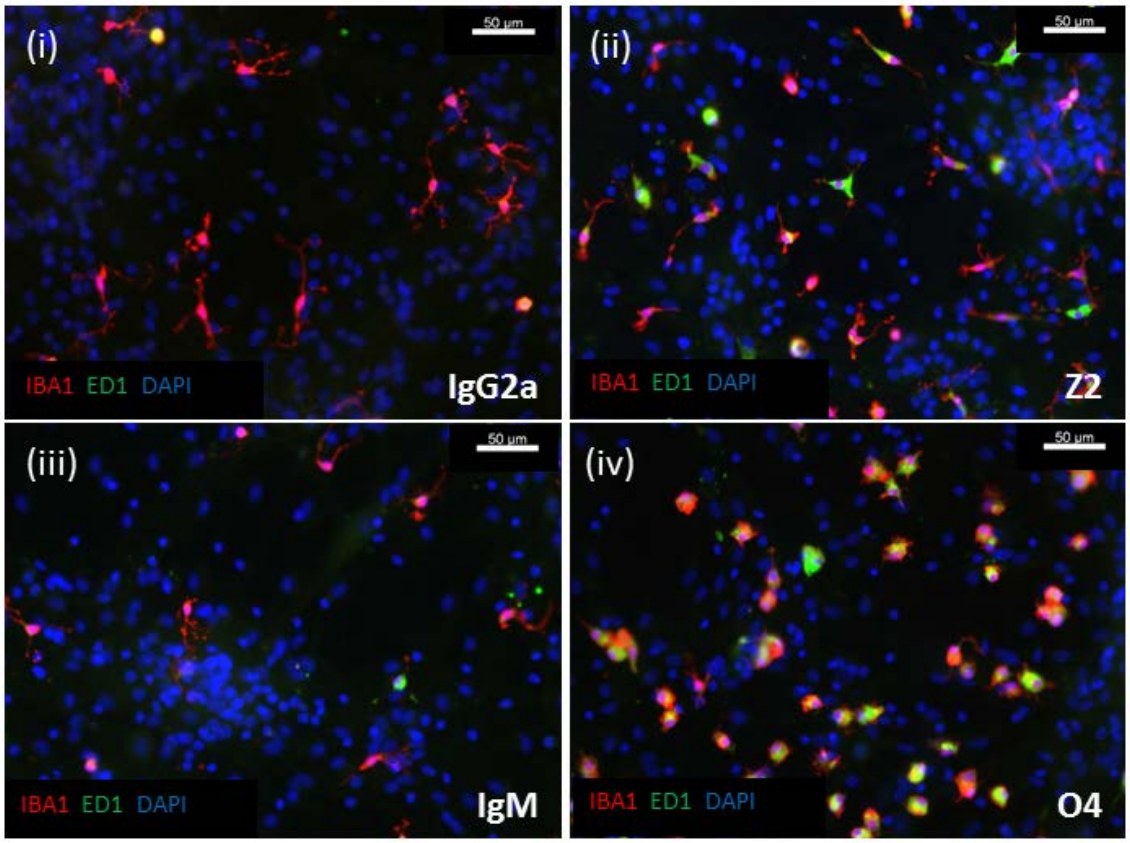


Figure 4.3. Representative images of microglial cells after antibody treatment.
Myelinating cultures were treated as in Figure 4.2, and the microglial cells analysed at 28 DIV. Representative images of staining with IgG2a (i) Z2 (ii), IgM (iii) and the O4 antibody (iv); showing the striking heterogeneity of morphology between the antibody treatment and their isotype controls (IBA1: red, ED1: green, DAP1: blue, 20X magnification). Scale bar representative of 50µm.

4.2.3 Antibody treatment induces a chemokine signature

4.2.3.1 Z2 and O4 treatment induces a chemokine and cytokine protein response

As changes indicative of microglial activation were observed in response to antibody treatment, chemokine and cytokine production, another hallmark of microglial activation was next investigated (Lehnardt, 2010, Kim and de Vellis, 2005). Using a proteome array, supernatants from myelinating cultures treated with Z2 and the O4 antibody for 10 days were assayed (Figure 4.4A and B). The data showed that there was a clear difference in cytokine and chemokine production between the antibody treated cultures and the isotype controls. Z2 treatment led to mostly chemokines and some cytokines being produced (Figure 4.5A). Notably CCL5, CCL20, CXCL1, CXCL5 and CXCL10, all of which are key pro-inflammatory chemokines associated with innate and adaptive immune cell migration (Szczucinski and Losy, 2007). The O4 antibody treatment showed a similar chemokine gene expression pattern to Z2 (Figure 4.5B). O4 antibody produced a larger response as most values were increased approximately two fold or more than that seen for Z2 treatment; for example CCL5: Z2, 22,989 ± 812; O4, 41,966 ± 1415. The highly produced chemokines were the same with both antibody treatments, except CXCL9 which was only produced in response to the O4 antibody treatment. Z2 also induced secretion of some other chemokines and cytokines which were visible by eye on the proteome arrays but were produced at low levels (<800) when compared to the others (Figure 4.4B). It is hard to determine whether they would have any biological effect at such low concentrations. TIMP-1 and VEGF were very pronounced on the array in both the isotype treated controls and antibody treated cultures, so they appear to be constitutively expressed by the myelinating cultures (Figure 4.4). From these data it can be deduced that the antibody treatment was eliciting a strong chemokine response. Which cells are producing these chemokines was not investigated in this study but it is probably the microglial cells, although, emerging evidence has shown that astrocytes can also produce some of these chemokines and cytokines (Miljkovic et al., 2011).

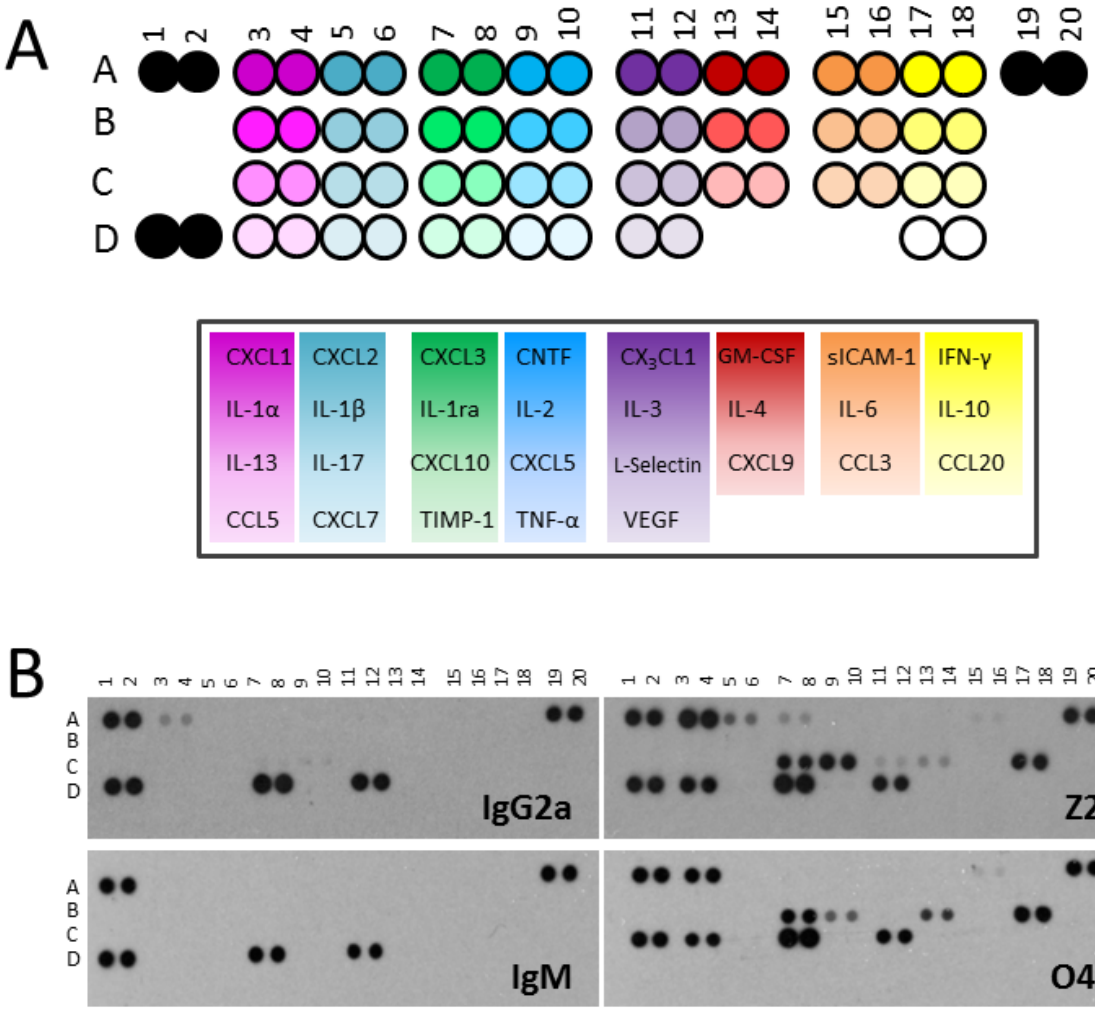


Figure 4.4. Representative rat cytokine array after incubation with supernatants from myelinating cultures were treated with Z2 and O4.
Myelinating cultures (18 DIV) were treated every 48 hours for 10 days with 20 µg/mL Z2, the O4 antibody or their respective isotype control. The antibodies were supplemented into the normal feeding media. After 10 days the supernatants were harvested to analyse the chemokines and cytokines produced. Protein levels were measured using Rat Cytokine Arrays as per manufacturer's instructions. (A) Rat cytokine array coordinates. Corresponding cytokines and chemokines, which are printed in duplicate, are colour coded below. Black circles represent positive controls and clear circle represents the negative control. Not to scale. (B) Representative scans of proteome array.

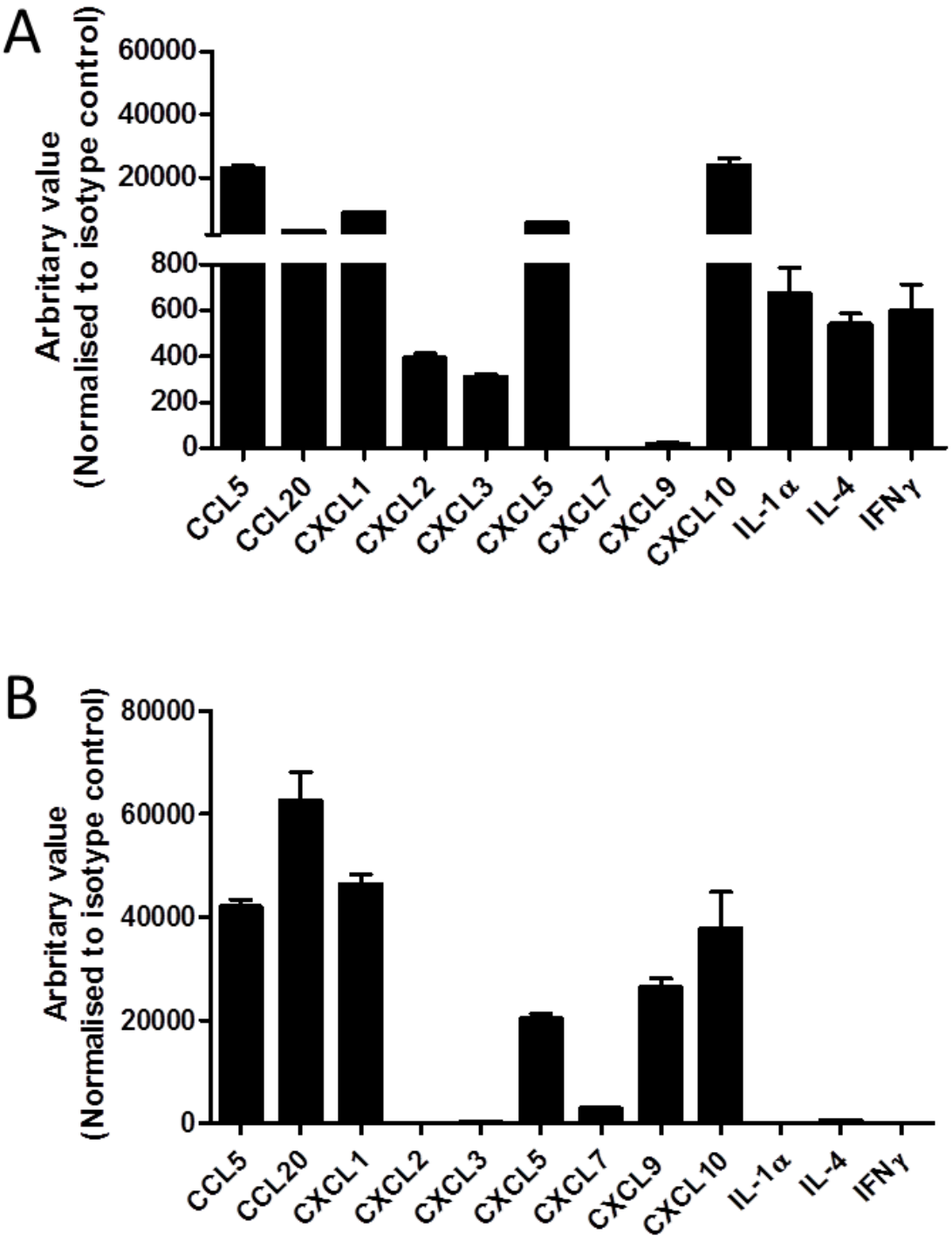


Figure 4.5. Chemokine and cytokine signatures identified with rat cytokine assay in response to antibody treatment of myelinating cultures.
Myelinating cultures were treated as in Figure 4.4 then supernatants were harvested at 28 DIV to detect chemokines and cytokines using Rat Cytokine Arrays as per manufacture instruction (representative proteome scan in Figure 4.4). (A) Chemokine and cytokine production from Z2 treated supernatants. (B) Chemokine production from the O4 antibody treated supernatants. Results were from one experiment performed in duplicate. Bar graph represents data normalised to isotype control, mean (± SEM).

4.2.3.2 Antibody treatment of myelinating cultures induces a functional chemotactic signal

The next step was to investigate if these secreted chemokines were functionally active. This was assessed using a migration assay in which supernatant was taken from the cultures after 10 days treatment and incubated with T cells. T cells were chosen as a target as the chemokines elevated in response to the antibody treatment have been shown to function as effector T cell chemoattractants. Notably CCL20 has been shown to play a role in recruiting T_H17 cells via their CCR6 receptor into the CNS during MS (Sallusto et al., 2012, Reboldi et al., 2009). In addition to this, T cells found in the MS lesion express CXCR3, which binds to CXCL9 and CXCL10 (Simpson et al., 2000). Also a high proportion of the T cells in the CSF of MS patients were found to express CCR5, which binds to CCL5 (Sorensen et al., 1999).

The results showed that there was a clear increase in cell migration with the addition of supernatants from the treated cultures as opposed to the isotype control treated cultures (Figure 4.6A and B). The experiment was not performed in triplicate so no statistical tests were performed. No conclusions could be drawn as to which chemokines were actively inducing cell migration as specific chemokines were not assessed in this experiment. This data offers evidence that the chemokines detected in these supernatants are functionally active and capable of attracting T cells. This would be interesting *in vivo* where these chemokines could attract peripheral circulating immune cells into the CNS, where they would possibly contribute to disease.

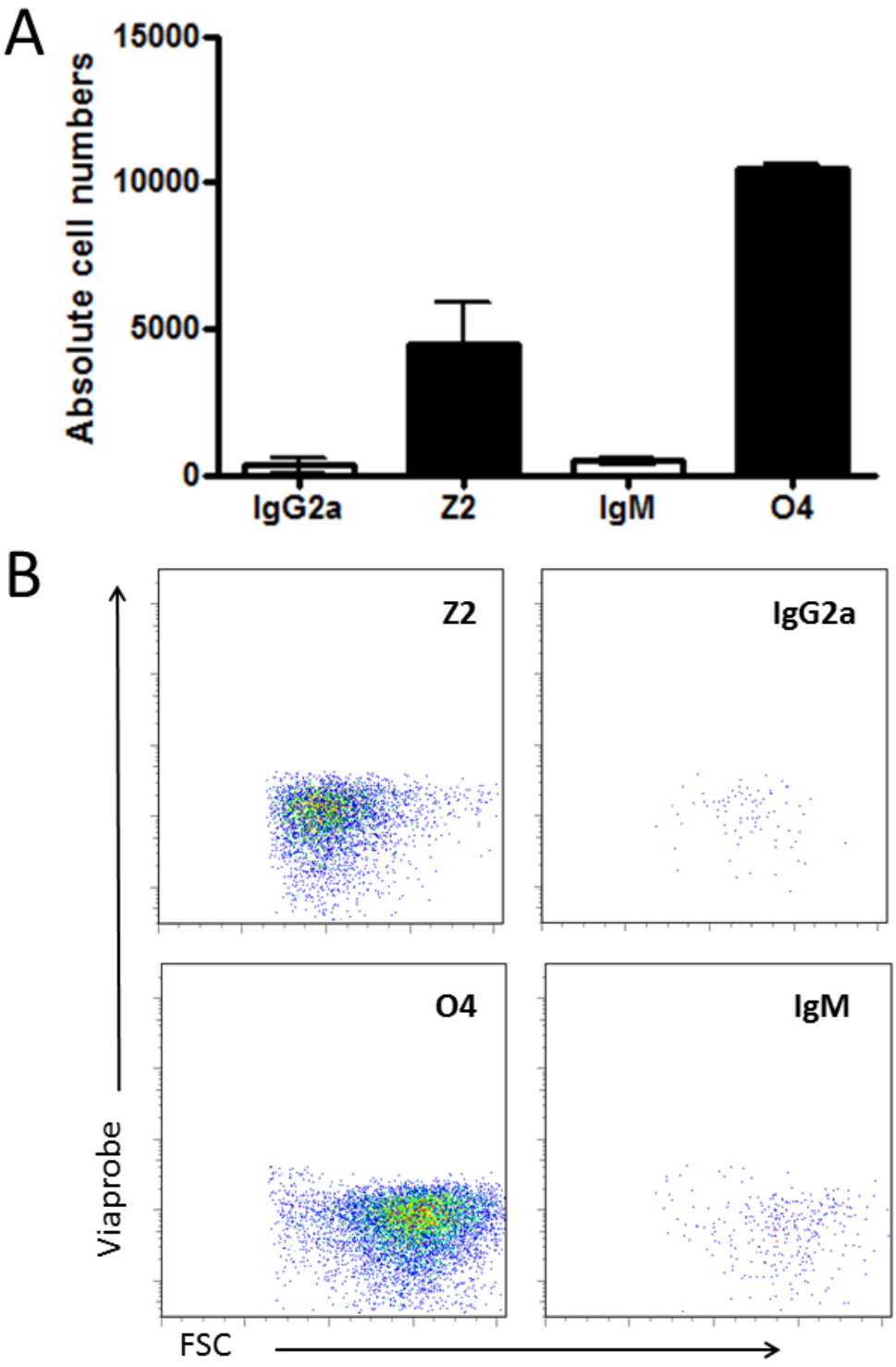


Figure 4.6. Antibody treatment induces chemotactic signal which induces T cell migration. To test whether chemokines detected in the supernatants after 10 days treatment with Z2 and the O4 antibody were functional, supernatants were incubated with unstimulated T cells in a Transwell® plate and migrated cells recorded by FACS. (A) Bar graph indicates mean values (± SD) of Viaprobe- cells from one experiment performed in dublicate. (B) Representative FACS plots show absolute numbers of Viaprobe- cells within total single cells.

4.2.3.3 Chemokine and cytokine response also observed at mRNA level after antibody treatment

To investigate if the protein array results were mirrored at a transcriptional level, mRNA was extracted from the cultures treated for 10 days with 20 µg/mL of Z2, the O4 antibody or their respective isotype control and analysed using a qPCR array. Fold change results showed a similar pattern to what was observed with the protein array as there was a comparable increase in chemokine and cytokine mRNA upregulation in response to the antibody treatment compared to the isotype controls. After Z2 treatment CCL5, CXCL3, IL-1α and IL-4 were detected at protein and mRNA levels (Figure 4.7A). O4 antibody treatment only induced an increase in CCL5 and CXCL9 protein and transcriptional levels (Figure 4.7B). The O4 antibody upregulated the gene expression of other chemokines which were not on the proteome array, e.g. CXCL13 and CCL22. Again, the O4 antibody treatment had a much more pronounced response than that detected by the Z2 treatment. For example, Z2 induced a 5.17 fold change in CCL5 mRNA expression, whereas the O4 antibody treatment produced a 17.1 fold change, which is over a three-fold increase compared to the Z2. The differences between protein and mRNA expression of certain chemokines and cytokines may be due to differential expression at this time point. For instance in the case of CCL20 protein, which was detected at high levels in Z2 and the O4 antibody treated supernatants at day 10 may be down regulated at the transcriptional level at this time point. Also the PCR array covered more candidates than the protein array, e.g. IL-22, IL-24, CCL12 and CXCL13. Therefore, this outcome supports previous results, again suggesting that pro-inflammatory chemokines and cytokines are induced after 10 days treatment with the O4 and Z2 antibodies.

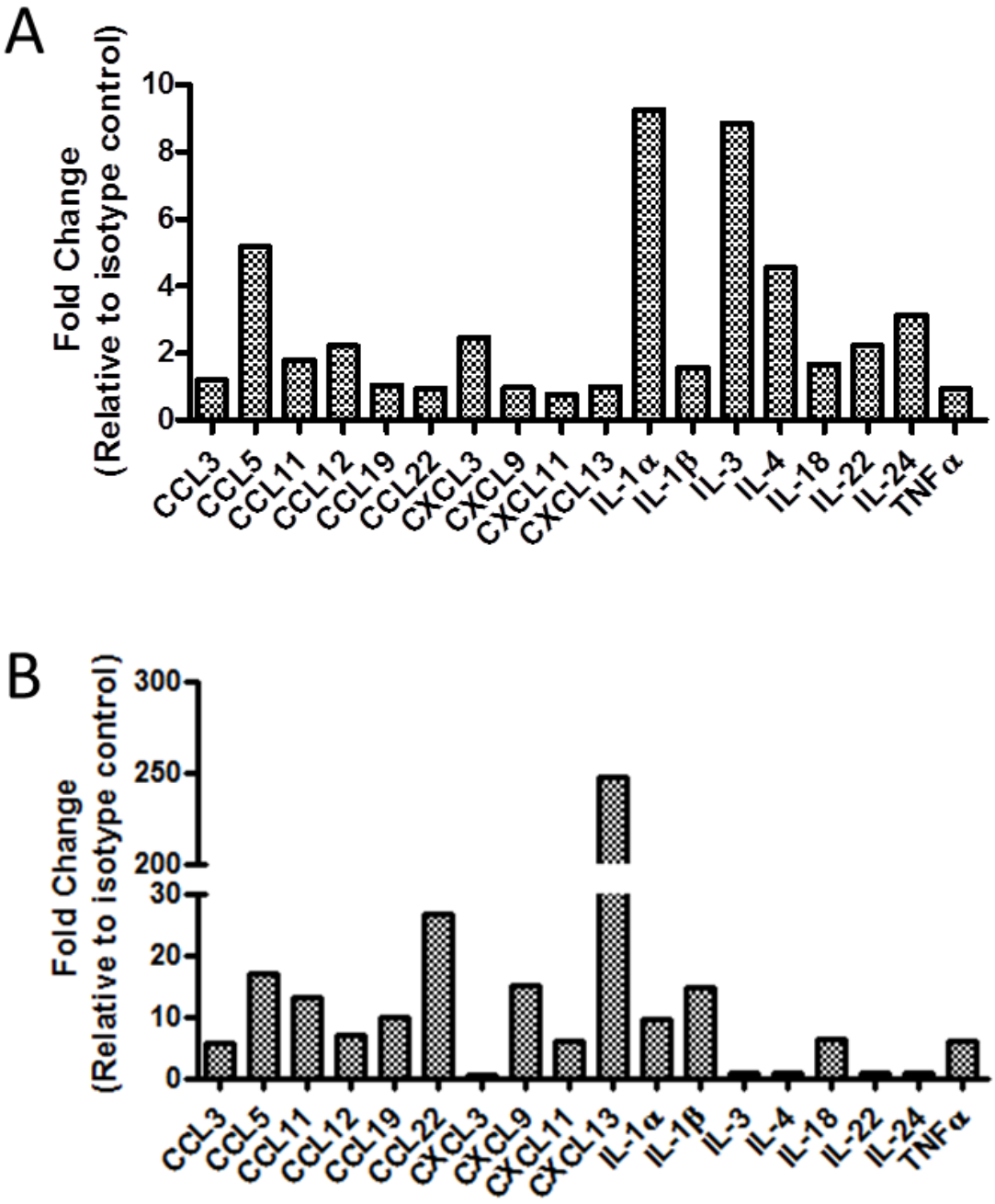


Figure 4.7. Chemokine and cytokine signature is also expressed at mRNA level after ten days of antibody treatment.

Myelinating cultures (18 DIV) were treated every 48 hours for 10 days with 20 µg/mL Z2, the O4 antibody or their respective isotype control. The antibodies were supplemented into the normal feeding media. mRNA was extracted from cultures at 28 DIV. cDNA was synthesised and analysed using RT^2 Profiler PCR Arrays as per manufacturers instruction. Bar graphs illustrate fold changes from upregulated genes highlighted in Figure 4.6. Bar graphs show genes that were upregulated by Z2 treatment (A), and the O4 antibody treatment (B). Data normalised by housekeeping genes and relative to isotype control. Bar graphs are representative of one experiment.

4.2.4 Characterising the chemokine response using CCL5 production

Temporal dynamics and the dosage required to elicit this chemokine response were subsequently investigated. CCL5 was selected as a target gene to analyse chemokine upregulation because it was increased at the protein and mRNA level with both antibody treatments relative to isotype controls, therefore making it the ideal candidate (Figure 4.8).

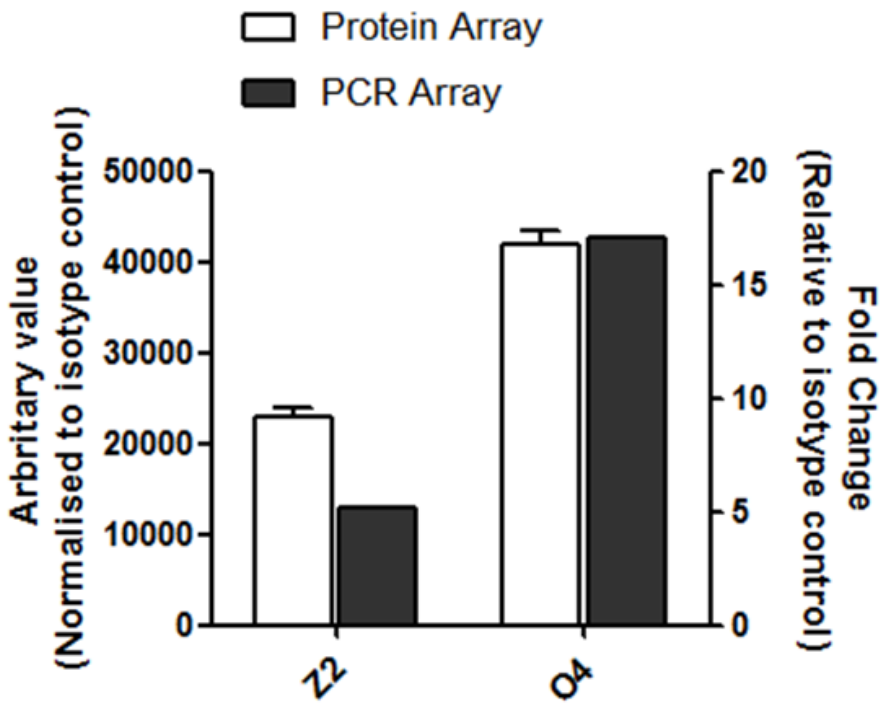


Figure 4.8. CCL5 is expressed at protein and mRNA level with both Z2 and the O4 antibody treatments.
This figure illustrates that CCL5 is upregulated at the transcriptional level in response to both antibody treatments. CCL5 data collated from the previous proteome and PCR array experiment, Figure 4.5 and Figure 4.7, respectively. This comparative data showed CCL5 is upregulated at protein and mRNA level after 10 days of treatment, highlighting CCL5 as a candidate marker to explore the dynamics of the chemokine response.

4.2.4.1 Dynamics of CCL5 gene expression after treatment with anti-myelin antibodies

Firstly a time course experiment was performed to investigate how long it took for CCL5 to be observed at a transcriptional level. mRNA was extracted at day 1, 2 and 10 from cultures treated with Z2 and O4. The data showed that the CCL5 response occurred very quickly, before 24 hours, and then decreased with time (Figure 4.9). As seen previously CCL5 fold change was greater with the O4 antibody treatment, even at early time points, but did not reach significance (fold change at 24 hours; Z2, 820.54 ± 494.75; O4, 1452.32 ± 497.47).

Next the concentration of antibody required to induce CCL5 gene expression was investigated to examine if low concentrations of antibody were enough to elicit a chemokine response from the cultures. Both antibodies were used at 2 ng, 200 ng and 20 μg and mRNA extracted after 24 hours, as this was when CCL5 mRNA expression was at its greatest. Z2 induced a slight upregulation of gene expression (fold change = 1.63) at 200 ng of antibody but had no effect at 2 ng (Figure 4.10A). The O4 antibody treatment resulted in a robust dose dependent response, where there was still a 2-fold upregulation of CCL5 at a concentration of 2 ng (2.4 ± 0.57), showing that very low doses of the O4 antibody were sufficient (Figure 4.10B).

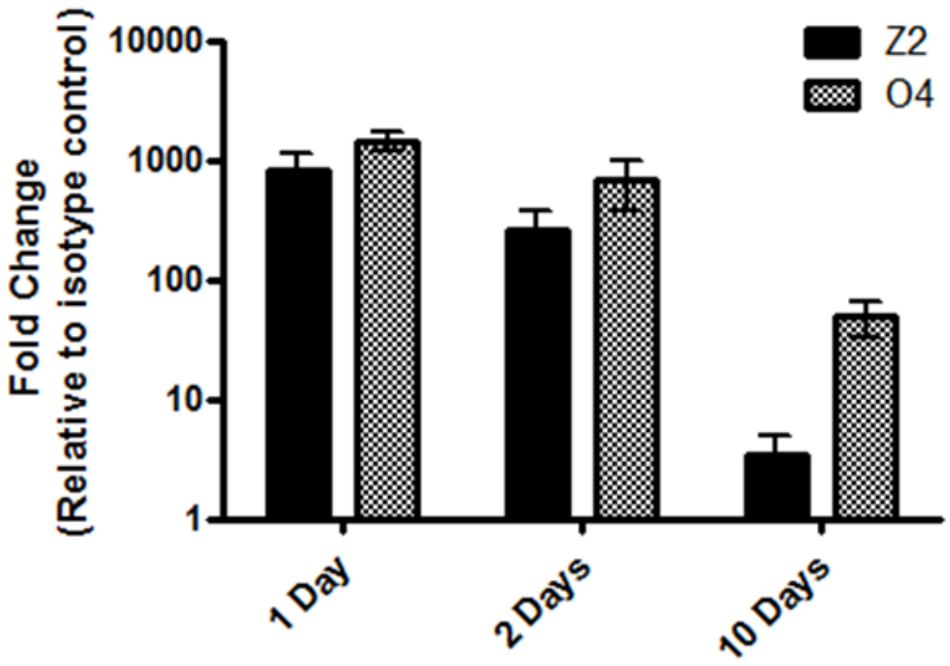


Figure 4.9. CCL5 gene expression decreases over time after antibody treatment.
This figure illustrates the temporal dynamics of the CCL5 response in reaction to antibody treatment. Cultures were treated for 10 days as per Figure 4.1. To investigate when CCL5 was upregulated after Z2 and the O4 antibody treatment, mRNA was extracted at day 1, 2 and 10 and CCL5 levels were assessed using qRT-PCR (detailed in materials and methods Section 2.6). Bar graph shows fold changes from three independent experiments, mean (± SEM). CCL5 expression was normalised to GAPDH and relative to isotype control.

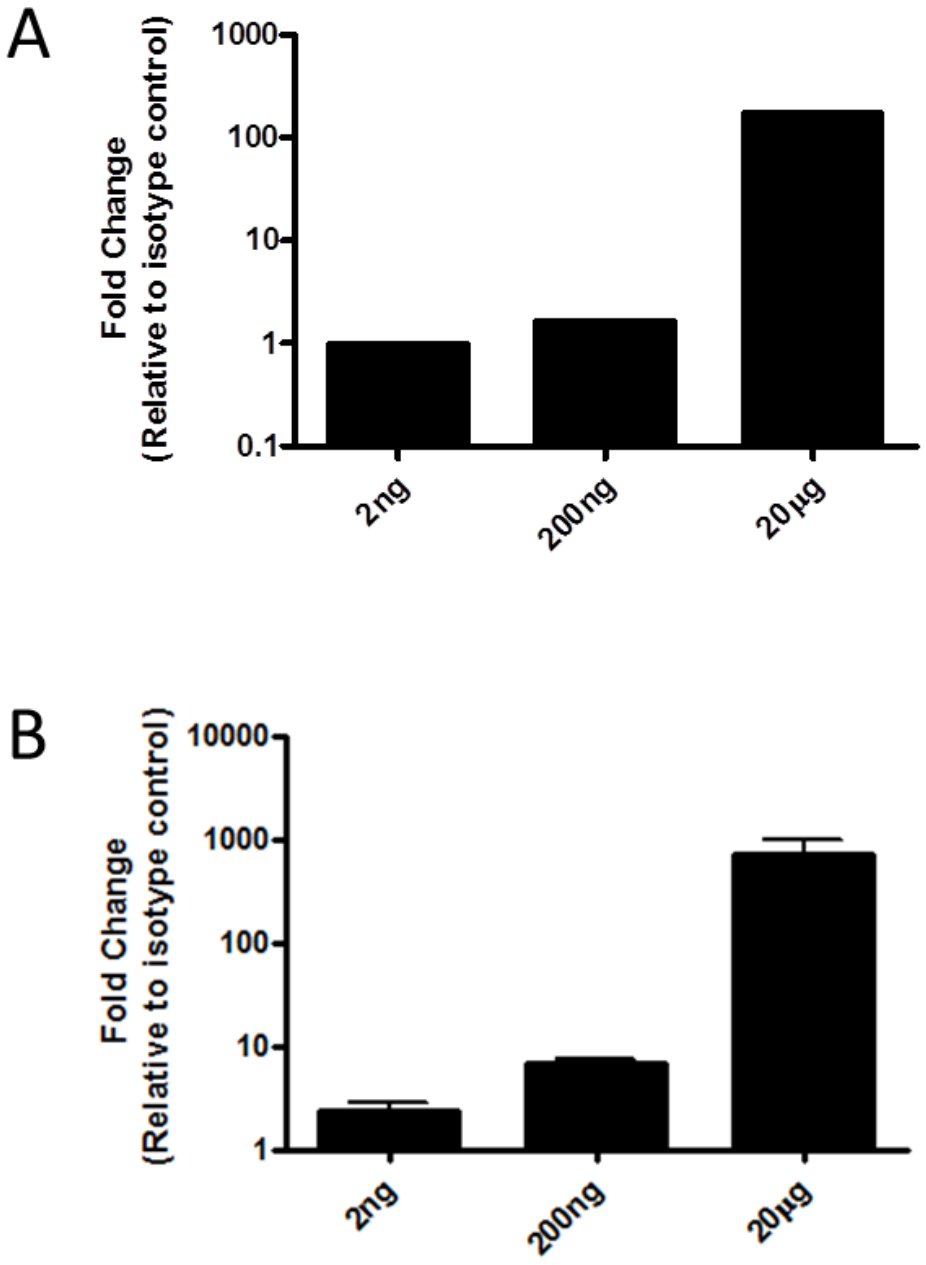


Figure 4.10. CCL5 mRNA is upregulated in a dose dependent manner with Z2 and the O4 antibody treatment.
To investigate what concentrations of antibody could induce CCL5 mRNA upregulation, cultures (18 DIV) were incubated with 2, 200 ng/mL and 20 µg/mL Z2, the O4 antibody or their isotype control for 24 hours prior to mRNA extraction. CCL5 gene expression was then assessed using qRT-PCR. (A) Bar graph shows fold change in CCL5 mRNA levels from one independent experiment. (B) Bar graph shows fold change in CCL5 mRNA levels from two independent experiments, mean (± SEM). CCL5 expression was normalised to GAPDH and relative to isotype control.

4.2.4.2 CCL5 mRNA response independent of source of antibody

To investigate if the chemokine response was an artefact from the antibody purification methodology, myelinating cultures were treated with 20 µg/mL purified O4 antibody, commercially brought O4 antibody and IgM for 24 hours and the mRNA extracted. When comparing CCL5 mRNA upregulation the results showed that there was no difference between the two O4 antibody treatments. This indicated that CCL5 induction was irrelevant of the source of antibody and importantly, that the purification method wasn't introducing any contaminating elements (Figure 4.11).

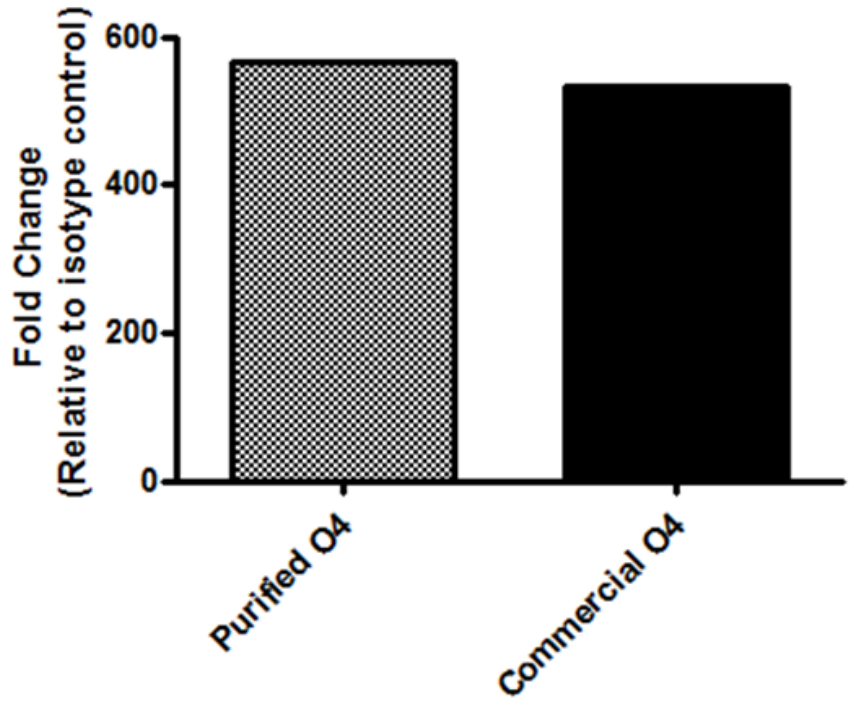


Figure 4.11. CCL5 gene expression is independent of source of antibody.
Myelinating cultures (18 DIV) were incubated with 20 µg/mL O4 antibody (Linington laboratory), O4 (R&D systems) or their isotype control IgM for 24 hours then mRNA was extracted. CCL5 gene upregulation was assessed by qRT-PCR. Expression of CCL5 was normalised to GAPDH and expressed relative to isotype control. Bar graph is representative of two independent experiments.

4.2.5 Investigating mechanisms behind CCL5 upregulation

4.2.5.1 CCL5 gene expression requires antigen recognition of target antigen

The O4 antibody and Z2 both recognise exposed antigens on the myelin sheath and oligodendrocyte cell bodies therefore it was proposed that the CCL5 upregulation may be associated with recognition of cell surface membrane epitopes by the antibody. To decipher this, a panel of antibodies against PLP were used including; the O10 antibody which binds to the exposed, extracellular portion of PLP (Jung et al., 1996), and AA3 which binds to the sequestered, cytoplasmic C-terminus of PLP (Kramer-Albers et al., 2006). Therefore, by comparing the CCL5 gene transcription results from both antibodies it could be possible to decipher the importance of antigen recognition. The results from incubating the myelination cultures (24 DIV) 24 hours with these antibodies showed that the O10 antibody upregulated CCL5 gene expression, whereas AA3 did not (Figure 4.12A). This suggested that antigen recognition by the antibody was required for gene expression of CCL5. To illustrate that AA3's binding target is normally sequestered the cultures were live stained with AA3 and the results revealed very little positive staining (Figure 4.12B). Fixed staining revealed that AA3 could bind after the cultures had been permeabilised confirming that its binding epitope is hidden.

CCL5 transcription levels with the O10 antibody treatment were almost equal to that seen with the O4 antibody treatment, where both induce approximately a thousand fold increase in CCL5 gene expression. They may both elicit such a strong CCL5 response due to their IgM isotype, where its pentamic structure and high avidity could play a role, or because they both bind to the same target cells despite having different antigens. Overall, this data suggests that CCL5 induction is a downstream effect that may be dependent on antibody recognition and binding to exposed antigen.

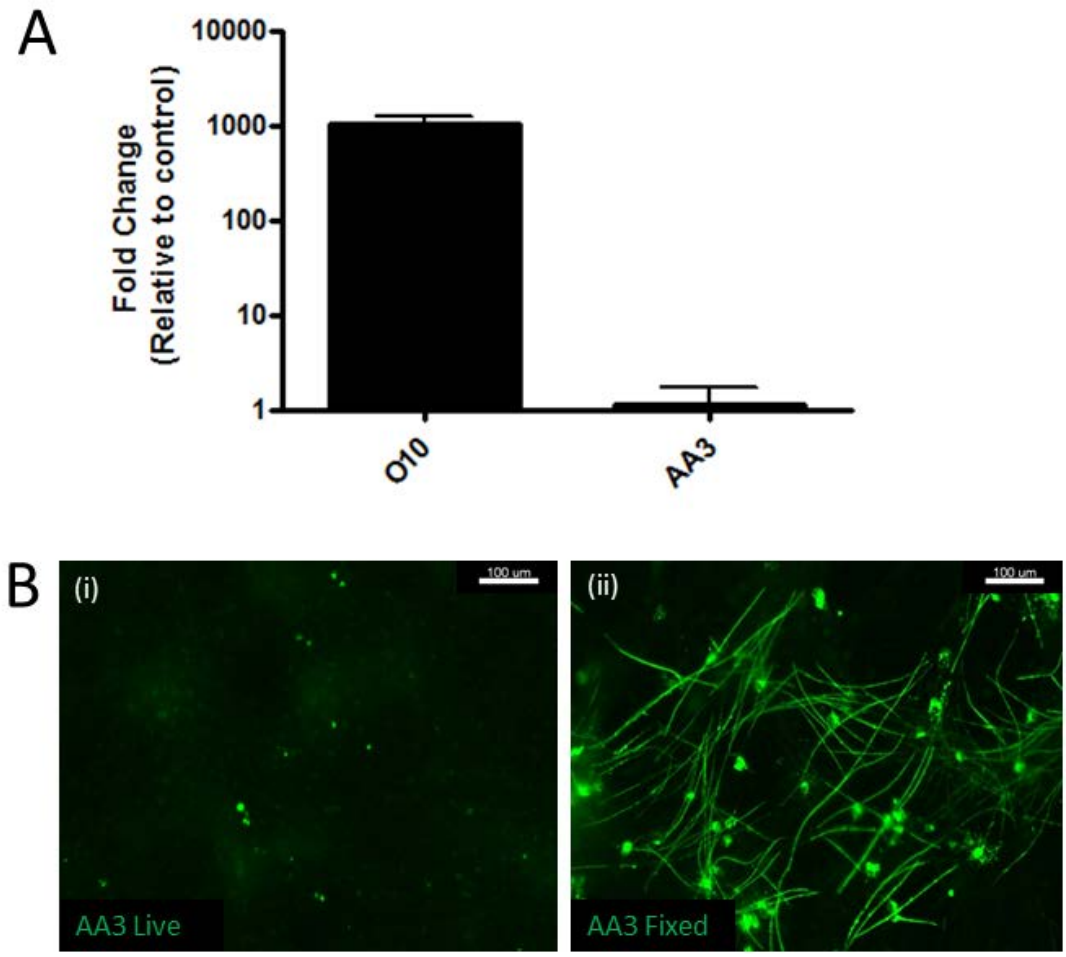


Figure 4.12. CCL5 mRNA upregulation requires antibody recognition and binding to target antigen.
To explore if antibody binding was required for this chemokine response we used the O10 antibody and AA3 which bind to the extracellular and intracellular portions of PLP, respectively. (A) Myelinating cultures (24 DIV) were incubated with the O10 antibody and AA3 at 20 µg/mL for 24 hours then mRNA was extracted and qRT-PCR performed. Expression of CCL5 was normalised to GAPDH and expressed relative to control. Bar graph represents two independent experiments, mean (± SD). (B) To visualise antibody binding myelinating cultures (24 DIV) were stained live or fixed with the O10 antibody and AA3, and visualised with fluorescent-conjugate secondary antibodies (staining protocols detailed in section 2.4.2). Representative images of myelin cultures stained with AA3 live (i) and AA3 fixed (ii) (20X magnification). Scale bar representative of 100µm.

4.2.5.2 CCL5 mRNA is not upregulated in the presence of exogenous complement

To investigate what would happen to the chemokine expression in the presence of an exogenous source of complement, cultures were incubated for 24 hours with 20 μg/mL Z2 or the O4 antibody in the presence or absence of 2% rat sera. It was anticipated that there would be a similar or even larger elevation in CCL5 gene expression due to the complement-mediated damage leading to increased pro-inflammatory chemokine signalling. Intriguingly the opposite was seen, in this experiment the CCL5 transcriptional levels were substantially decreased, irrespective of antibody specificity in the presence of exogenous complement (Figure 4.13A). Z2 treatment led to 33 times greater CCL5 mRNA induction when rat serum was absent compared to when it was present. A similar pattern was observed with the O4 antibody treatment where there was 25 times more CCL5 gene activation in the absence of rat serum, compared to when it was added. .

To investigate if this inhibition of chemokine response was only seen with CCL5 or if it was a more general effect a quantitative PCR array was performed. The myelinating cultures were treated with 20 μg/mL Z2 in the absence or presence of 2% rat sera for 24 hours, followed by mRNA extraction. These results mirrored the same pattern as seen for CCL5 in the RT-PCR experiments, as the presence of an exogenous source of complement globally down regulated chemokine expression (Figure 4.13B). These results were surprising and particularly interesting as it suggested that the mechanisms leading to chemokine induction when complement is present differs to when complement is absent.

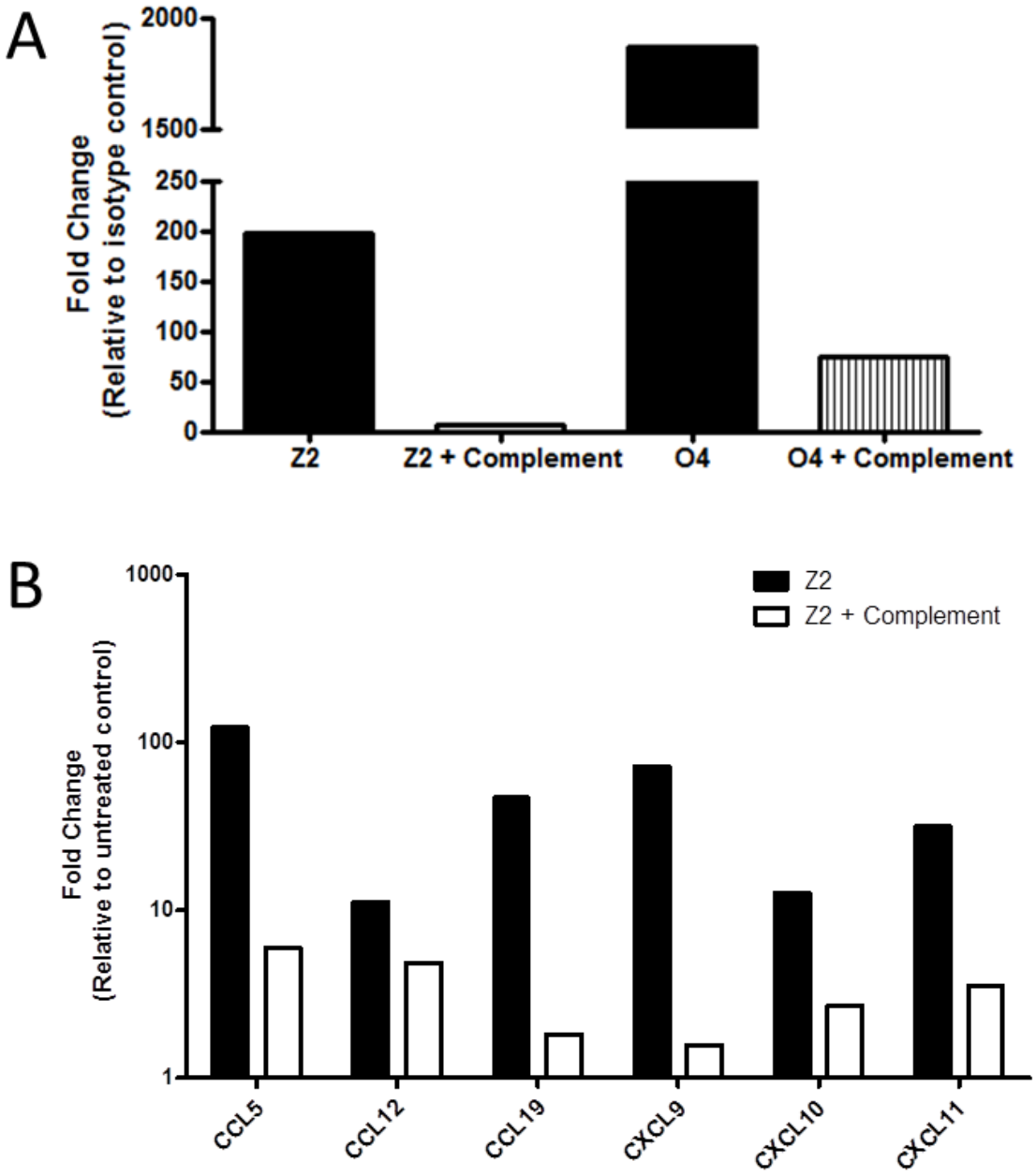


Figure 4.13. Antibody treatment together with complement greatly reduces CCL5 upregulation.

CCL5 upregulation was investigated in the presence of complement. (A) Myelinating cultures (18 DIV) were incubated with 20 µg/mL Z2, O4 or their isotype controls, in the absence or presence of 2% rat sera for 24 hours, prior to mRNA extraction. qRT-PCR was used to analyse upregulation of CCL5 gene. Bar graph demonstrated CCL5 mRNA fold change, normalised to GAPDH and relative to isotype control. Data obtained from one experiement, which was performed in triplicate. (B) Cultures (18 DIV) were treated with 20 µg/mL Z2 in the absence or presence of 2% rat serum for 24 hours prior to mRNA extraction. RT^2 Profiler PCR Array was used as per manufactures instructions to analyse overall chemokine response. Values shown are fold change normalised by housekeeping genes and relative to untreated control. Bar graph shows genes that were highly upregulated by Z2 treatment in the absence of an exogenous source of complement.

4.2.5.3 Oligodendrocytes do not upregulate CCL5 mRNA when incubated with the O4 antibody

The previous experiment showed that the presence of complement substantially reduced CCL5 induction by Z2 and the O4 antibody, suggesting that CCL5 upregulation may have been lost due to antibody-mediated lysis of the target cell, i.e. oligodendrocytes. Therefore, to test this theory oligodendrocyte only cultures (which were isolated from astrocyte monolayers and donated by my colleague Dr. Debbie Allan) were incubated with the O4 antibody and the isotype control for 24 hours prior to mRNA extraction. The O4 antibody was chosen as it induced the most pronounced CCL5 mRNA upregulation in previous experiments and because the O4 antigen is expressed on OPCs and mature oligodendrocytes. The data showed that there was no significant difference between the O4 antibody and IgM on CCL5 mRNA expression (Figure 4.14), suggesting oligodendrocytes were not producing CCL5 in response to antibody binding.

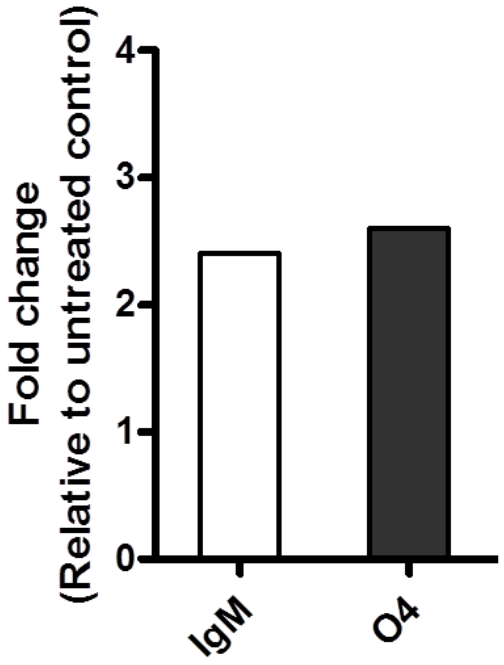


Figure 4.14. The O4 antibody treatment does not induce CCL5 mRNA upregulation in oligodendrocyte cultures.
Oligodendrocyte cultures were incubated with the O4 antibody or IgM at 20 µg/mL for 24 hours and then mRNA was extracted for analysis using qRT-PCR. Data normalised to GAPDH and relative to untreated control. Values shown are from one experiment.

4.2.5.4 OVA immune complexes induce expression of CCL5 mRNA in the myelinating cultures

If the antibodies weren't acting directly upon oligodendrocytes to induce CCL5 gene expression, then it is possible that antibody binding to the cell surface must prompt a response in another cell type. It is feasible that the immune complexes shed from the cell surface could be responsible for the effects observed. Immune complexes have previously been shown to exist in the CSF and serum of MS patients (Jans et al., 1984). They can cause damage and inflammation through complement and macrophage/microglial activation. To test this hypothesis a new model was developed using preformed ovalbumin (OVA) immune complexes (OIC) where OVA antibody and OVA antigen were incubated together for 30 minutes at room temperature. They were then used in a serial dilution to treat the myelinating cultures for 24 hour incubation prior to mRNA extraction. CCL5 gene expression increased in a dose-dependent manner (Figure 4.15). This data indicates that immune complexes could also induce a similar complement-independent response as seen with the monoclonal antibody treatment.

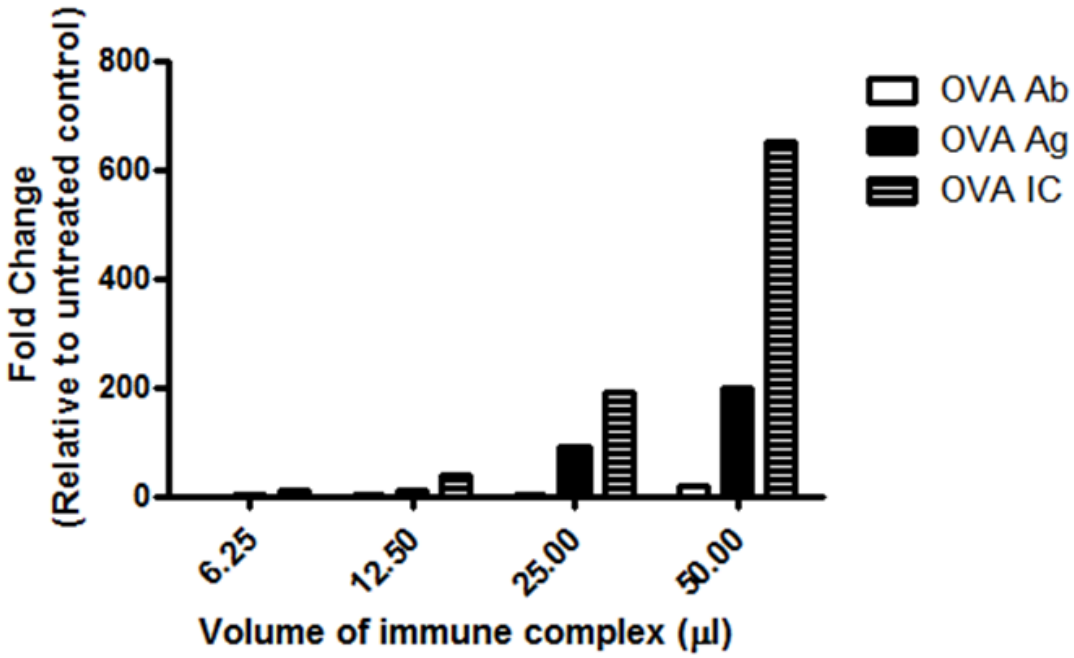


Figure 4.15. OVA immune complex can induce CCL5 mRNA upregulation.
Myelinating cultures (18 DIV) were incubated with a series dilution of OVA immune complexes or equivalent volumes of anti-OVA or OVA for 24 hours prior to mRNA extraction Expression of CCL5 mRNA was normalised to GAPDH and expressed relative to untreated controls. Bar graph representative of two independent experiments, performed in triplicate.

4.2.6 Z2 and the O4 antibody induction of responses are not due to a general inflammatory signal

4.2.6.1 LPS treatment induces global upregulation of chemokines and cytokines

Overall, the above studies indicated an association between the antibody treatment and inhibition of myelination, along with microglial activation and chemokine production. To investigate if the induction of chemokines observed was a general response occurring due to a pro-inflammatory signal, the cultures were incubated with a strong pro-inflammatory inducer, LPS, with the expectation of similar, if not more pronounced results. To compare differences in chemokine and cytokine upregulation between Z2 antibody and LPS treatment, mRNA was extracted from myelinating cultures after 24 hours incubation. The resulting plots showed huge upregulation of chemokines, as well as some cytokines, in response to LPS treatment, all of which were approximately an order of magnitude higher than seen with Z2 treatment (Figure 4.16).

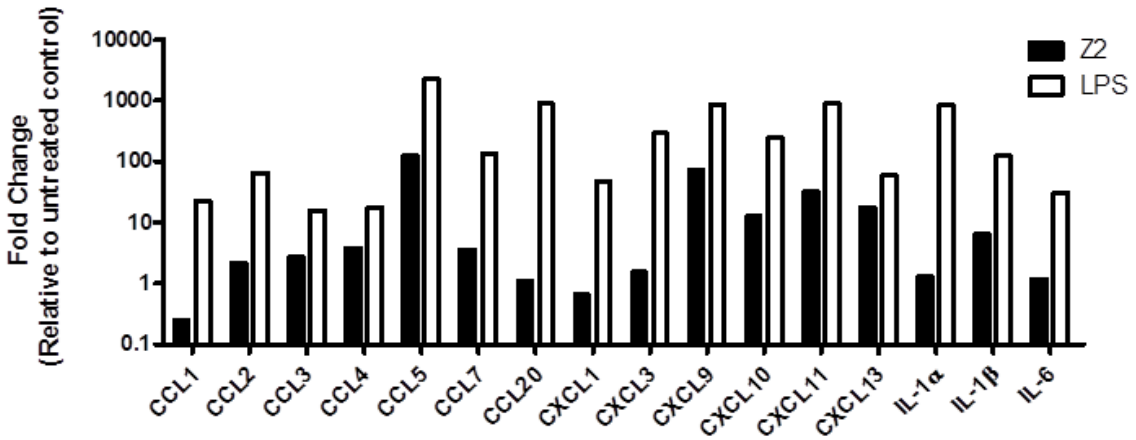


Figure 4.16. LPS treatment induces a strong mRNA upregulation of chemokines and cytokines.
Myelinating cultures (18 DIV) were incubated for 24 hours with 100 ng/mL LPS or 20 Z2 µg/mL then mRNA extracted. cDNA was synthesised and analysed using RT^2 Profiler PCR Array as per manufactures instruction to analyse overall chemokine response. Values shown are fold change normalised by housekeeping genes and relative to untreated control from one experiment. Bar graph compares gene expression of LPS and Z2 treated cultures.

4.2.6.2 LPS treatment does not inhibit myelination

To elucidate if the induction of chemokines was responsible for the effects on myelination observed with the 10 day antibody treatments the cultures were treated with 100 ng/mL LPS, 20 μg/mL the O4 antibody and IgM and myelination was analysed after 10 days. It was expected that LPS treatment would have a similar or greater effect on myelination. In contrast to this hypothesis, the data clearly showed that myelination was unaffected by LPS, as the % of myelinated axons were equal to that seen in the untreated control and IgM isotype control (LPS; 8.33 ± 0.91, control; 8.95 ± 1.43, IgM; 8.42 ± 0.21), whereas O4 still significantly inhibited myelination compared to its isotype control, $p < 0.05$ (Figure 4.17). This was of great interest as it revealed that the chemokine production itself was not directly responsible for the inhibition of myelination. This was apparent as LPS treatment led to a massive upregulation in chemokine mRNA expression after only 24 hours, yet had no observable effect on myelination when the cultures were treated for 10 days. This highlighted that the chemokine response was most likely the by-product of an initial mechanism that affects myelination.

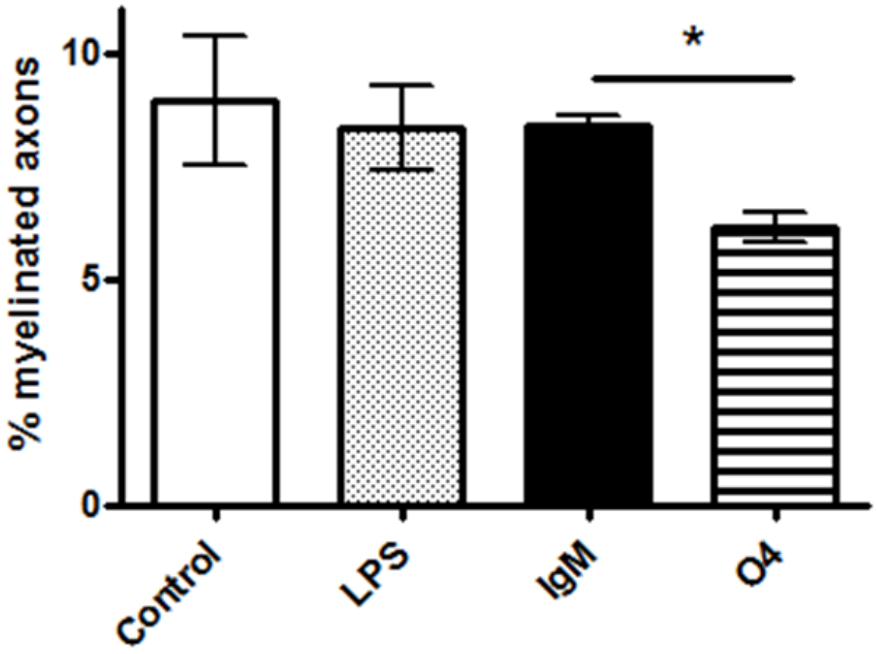
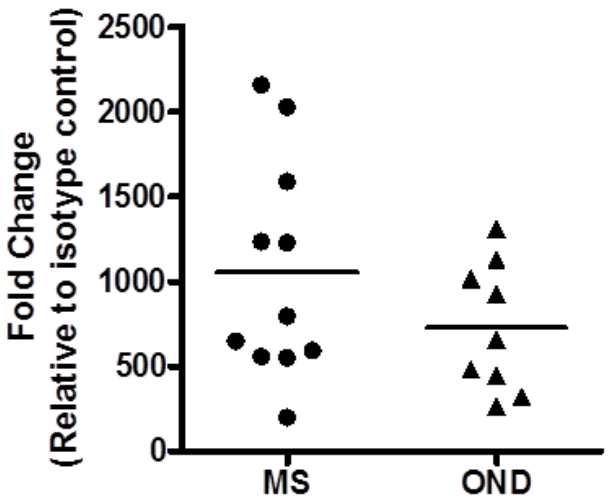


Figure 4.17. LPS has no significant effect on myelination.
Myelinating cultures (18 DIV) were treated every-other-day for 10 days with 20 µg/mL O4 and IgM and 100 ng/mL LPS, which was supplemented into the normal feeding media. Immunochemistry was used to quantify demyelination. Data from one experiment performed in triplicate. Bar graph represents mean (± SD). Significance was determined by student t-test. *, $p < 0.05$.

4.2.7 Patient derived IgG induces CCL5 upregulation in myelinating cultures

Next it was established whether patient derived IgG would elicit the same CCL5 response in the cultures. To address this question, a small cohort of patient IgG preparations was collated from 11 MS and 9 OND patients (ethical approval, patient consent and details can be found in section 2.4.2.2 and Table 2.4). Myelinating cultures (24 DIV) were incubated for 24 hours with 500 μg/mL patient derived IgG then mRNA extracted. The results were interesting as they showed that all patient IgG samples elicited a CCL5 response but to varying degrees (Figure 4.18). The highest fold changes seen were 2157.23, 1587.66 and 2027.62, which were all from RRMS patients, MS2, MS5 and MS6 respectively. The MS cohort had a wider range of data points and their mean CCL5 fold change in mRNA was slightly higher than the OND group (MS, 1052.92 ± 646.99; OND, 725.67 ± 378.82). This patient cohort had previously been analysed for demyelinating activity (Elliott et al., 2012). Patients that showed demyelinating activity in the Elliott et al., study did not correlate with higher fold changes of CCL5 mRNA in this study. This outcome implies that patient derived IgG could also induce the same effects on myelination and microglia observed with the Z2 and O4 antibodies, but long term incubations with the patient IgG to investigate this were not performed due to patient sample availability and time constraints.

A



B

Patient ID	Diagnosis	CCL5 fold change
MS1	SPMS	550.00
MS2	RRMS	199.71
MS3	RRMS	2157.23
MS4	RRMS	1235.16
MS5	RRMS	1587.66
MS6	RRMS	2027.62
MS7	RRMS	1228.87
MS8	RRMS	649.87
MS9	RRMS	593.52
MS10	RRMS	557.61
MS11	RRMS	794.84
OND1	GBS	1015.17
OND2	GBS	321.02
OND3	CIDP	1305.08
OND4	CIDP	1130.05
OND5	CIDP	922.49
OND6	NMO	443.11
OND7	Tranverse Myelitis	259.22
OND8	CIS	480.75
OND9	MG	654.12

Figure 4.18. Patient derived IgG induces CCL5 mRNA upregulation.
Myelinating cultures (24 DIV) were incubated with patient derived IgG at 500 µg/mL for 24 hours prior to mRNA extraction. (A) Expression of CCL5 gene was normalised to GAPDH and relative to human IgG treated control. (B) Summary of patient's diagnosis and CCL5 fold change induced by IgG purified from patient. All patient IgG samples induced CCL5 mRNA upregulation. Further patient demographic details are in Table 2.4.

4.2.8 Z2 treatment induces CCL5 gene expression *in vivo*

It was further investigated if CCL5 gene expression could be observed with antibody treatment *in vivo*. This experiment was performed in collaboration with Trevor Owens at the University of Southern Denmark, Odense. Trevor Owens laboratory intrathecally injected 5 C57BL/6 mice with either 4.5 mg/mL Z2 or IgG2a or 100 ng/mL LPS as a positive control. After 24 hours the whole brain was harvested and snap-frozen for shipment. These tissue samples were then processed as described in section 2.6.1.2. After mRNA extraction the CCL5 signal was measured using qRT-PCR. The results showed that CCL5 was upregulated in all the Z2 treated mice (fold change > 2) but didn't reach significance compared to control, $p = 0.066$, t-test (Figure 4.19). This was because of the variance in CCL5 induction across the Z2 treated mice, mean = 8.39 ± 7.64. However this produced an interesting observation because it reveals that CCL5 mRNA expression induction could be measured from a whole brain sample and verifies that this chemokine induction can be translated from the *in vitro* model to an *in vivo* model.

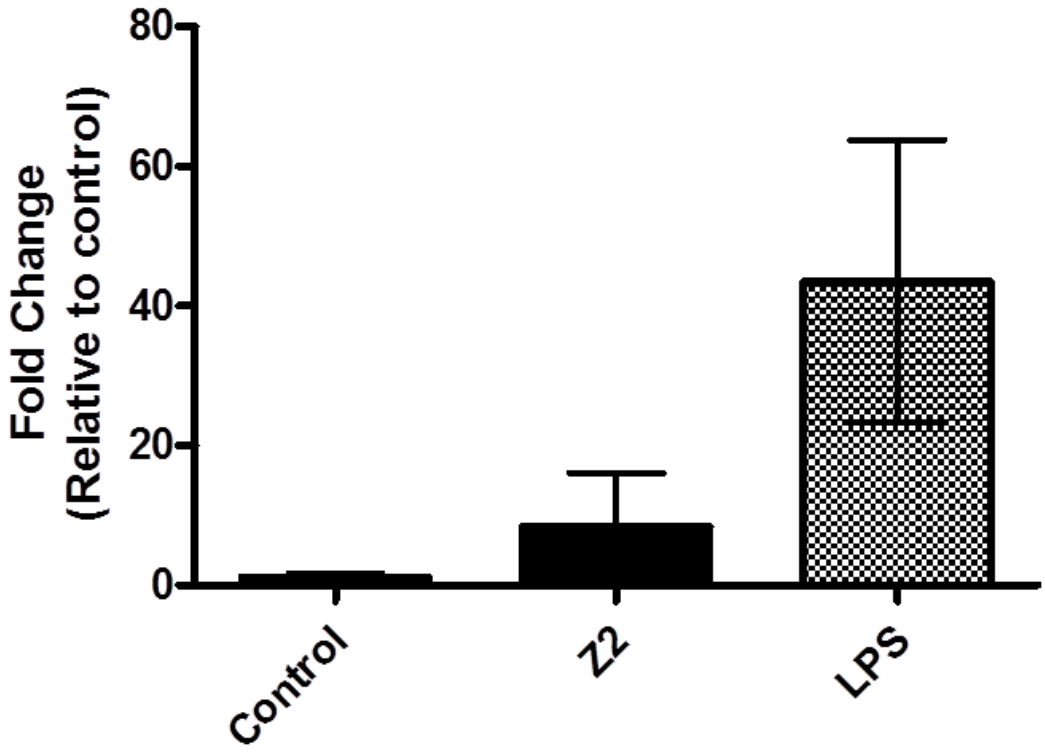


Figure 4.19. CCL5 mRNA level is upregulated in whole mice brains after intrathecal injection of Z2.
C57BL/6 mice were intrathecally injected with 4.5 mg/mL IgG2a (control), Z2 or 100 ng/mL LPS (n=5). After 24 hours mice were perfused, their brains snap frozen, followed by mRNA extraction for CCL5 gene expression quantification by qRT-PCR. Data was normalised to GAPDH and expressed relative to isotype control. Values shown are mean (± SEM). Results did not reach significance; student t-test, control vs. LPS, p=0.069, control vs. Z2, p=0.066.

4.3 DISCUSSION

This chapter established a novel pathway by which autoantibodies in the absence of exogenous effector mechanisms might contribute to MS pathogenesis. Z2 and O4 treatment of myelinating cultures without any exogenous source of complement or peripheral immune cells led to three distinct effects:-

1. Inhibition of myelination
2. Activation of microglial cells
3. Rapid induction of chemokine production

Firstly, the data presented in this chapter showed that cultures treated with anti-myelin antibodies Z2 and O4 alone led to inhibition of myelination (Figure 4.1). Blocking of further myelination was seen irrespective of the antibody target on the myelin sheath. As not all myelin was lost it was hypothesised that further myelination of the cultures is inhibited as opposed to destruction of myelin already present. These results are the first description of antibody independent effects on myelination *in vitro* in this myelinating culture system but its mechanism of action is as yet unknown.

It is likely this effect was due to antibody recognition of their surface antigens. Previous published data revealed that there are a number of ways in which these antibodies could have led to disruption of myelin ensheathment after antibody binding. A relevant study in which a group implanted the O10 hybridoma into the spinal cord of rats, led to production of an IgM antibody against PLP (Rosenbluth and Schiff, 2009). This led to focal demyelination and remyelination when implanted into adult spinal cords while in contrast, when transplanted into juvenile rats led to dysmyelination, and IgM was seen to be incorporated into newly forming myelin. This appeared only to arise when the adjacent processes were both of oligodendrocyte origin, therefore suggesting IgM binding to PLP on both

sides was necessary for the formation of expanded myelin. In addition, this effect was not seen with control hybridomas which lacked specificity to exposed myelin epitopes, indicating antigen recognition was required. In both scenarios these effects were not associated with axon degeneration, which was also observed in antibody treatment of the myelinating cultures (data not shown). This suggests that antibody-mediated inhibition of myelination presented in this chapter could be a result of antibody incorporation into forming myelin sheaths. This process would be disease relevant as it could play a role in inhibiting attempts at remyelination that occurs during MS.

As discussed in the introduction, anti-MOG antibodies have also been shown to lead to repartitioning of the myelin sheath and physiological changes of oligodendrocytes (Marta et al., 2003, Marta et al., 2005b). These studies showed crosslinking of antibodies bound to MOG led to the formation of insoluble rafts on the surface of oligodendrocytes, which subsequently triggered a cascade of cellular events including withdrawal of oligodendrocyte processes. This phenomenon does not appear to be specific to MOG as studies using antibodies against MAG on oligodendrocytes also results in repartitioning of MAG into insoluble complexes (Marta et al., 2004). Therefore, this process could be proposed as a possible initial event of myelin/oligodendrocyte-specific antibody mediated demyelination in the absence of complement.

Blocking of myelination could also be due to steric hindrance, where antibody binding leads to loss of function. The O4 antibody binds to sulfatides which are thought to play a major role in function and stability of the myelin sheath. This is evident in mice deficient of the enzyme required for sulfatide synthesis during myelin formation, which display severe tremoring and ataxia, thought to be a result of the development of thinner myelin sheaths (Coetzee et al., 1996). Therefore, binding of the O4 antibody in the myelinating cultures could be having a direct effect on sulfatide function, which could in turn be responsible for the inhibition of further myelination. Moreover, the binding of the O4 antibody to the

sulfatide on the surface of oligodendrocytes could be inhibiting their function, or cell development into mature, myelinating oligodendrocytes, as it has been shown that direct binding of antibody to this cell can trigger cellular changes (Marta et al., 2005a). An interesting point here is that the function of MOG is still to be uncovered so direct effects on MOGs function due to antibody binding should not be discounted.

The second distinct effect observed in association with the inhibition of myelination, was activation of microglial cells. Microglia are resident CNS macrophages and activation of these cells are a hallmark of MS pathology (Goldmann and Prinz, 2013). Microglial cells, along with macrophages from the blood, are quickly recruited to active MS lesions where they interact with T cells (Jack et al., 2005). The results presented here clearly show that the microglia cells were significantly activated in response to the antibody treatment (Figure 4.2). There were also significantly higher numbers of $IBA1^+$ cells in the treated cultures. Microglia cells are derived from mesodermal progenitors, distinct from monocytes, and migrate to the CNS during embryonic and foetal development (Chan et al., 2007, Schulz et al., 2012, Gomez Perdiguero et al., 2013). It is also evident that these CNS resident cells after injury/disease can renew their population intrinsically (Lassmann and Hickey, 1993). With this in mind, and as the *in vitro* model is a closed system, it is most likely the $IBA1^+$ cells were self-dividing. Future experiments would use BrdU staining to confirm this hypothesis.

Microglial cells were most likely being activated via their Fc receptors, but whether this effect was via a Fc mediated effector pathway is yet to be determined. Future experiment could be carried out by treating the myelinating cultures with Fab and F(ab)2' regions and effects on microglia assessed. Microglial cells express Fcγ receptors I, II and III (Ulvestad et al., 1994). Antibody-mediated activation of microglial cells via their Fc receptors leads to chemokine secretion and phagocytosis (Song et al., 2004). The study did not investigate if the microglial cells were phagocytosing the myelin; although it is unlikely to be the main effector

function occurring, as complete myelin loss did not occur even after 10 days of treatment, when residual myelin could be observed. However, the microglial cells did show a significant increase in $ED1^{+}$ staining and ED1 is a marker of phagocytic activity, indicating that antibody treatment is increasing this cell function (Bauer et al., 1994). To explore this, future experiments would be to stain the microglia for myelin debris to determine if phagocytosis is occurring. It has also been shown that incubation of microglial cells with antibody coated antigen led to ADCC and oxidative bursts (Ulvestad et al., 1994). Oxygen radicals have been shown to cause oligodendrocyte cell death *in vitro*, suggesting Fc mediated ADCC and oxidative bursts could be killing oligodendrocytes, inhibiting further myelination (Kim and Kim, 1991). Therefore microglial activation in response to the O4 and Z2 treatment could have affected myelination through the mechanisms discussed above.

Microglial cells could have also been activated by immune complexes through their Fc receptors, as the data also showed changes in CCL5 gene expression when the myelinating cultures were incubated with OVA immune complexes (Figure 4.15). This would correspond to a recent study which showed that immune complexes in the brain parenchyma led to persistent local inflammation and microglial recruitment and activation (Teeling et al., 2012). Interestingly, this inflammation was blocked in Fcγ-deficient mice but not in C1q-deficient mice, showing that it was Fcγ receptor dependent but not complement dependent, which is relevant to the novel mechanism discussed here.

This study went on to investigate expression of markers of microglial activation which led to the final phenomenon which was observed in association with the antibody treatment; secretion of chemokines. The results showed that pro-inflammatory chemokines were upregulated at the transcriptional level after only 24 hours and that after 10 days treatment they could be identified in the supernatant using a proteome array (Figure 4.5 and Figure 4.7). There was some production of cytokine but it was predominantly a chemokine response. In addition to this, these chemokines appeared to be biologically active as they could

induce T cell migration (Figure 4.6). The cell responsible for chemokine production was not elucidated in this study, however reports in the literature suggest it is likely to be microglial cells as they produce many cytokines and chemokines in response to pro-inflammatory stimuli (Aravalli et al., 2005, Cheeran et al., 2001, Cheeran et al., 2003), and after activation Fc mediated signalling. It has been known for many years that microglia play a crucial role as an immune cell in the CNS and produce many soluble immune factors (Gehrmann et al., 1995). Astrocytes also have an emerging role as secretors of chemokines and cytokines, so they could also be contributing to the production of chemokines (Dong and Benveniste, 2001). Astrocytes also express an Fc receptor, FcγRI, but it has not been linked with any pro-inflammatory downstream signalling (Okun et al., 2010), making them less likely to be binding the antibodies directly but could be interacting with the microglia cells. The results showed that the oligodendrocyte cultures did not upregulate any CCL5 mRNA in response to O4 treatment, therefore suggesting they are not responsible (Figure 4.14). However a recent review discussed oligodendrocytes role in inflammation as it has been shown that these cells can also secrete chemokines in response to pro-inflammatory stimuli (Peferoen et al., 2013).

As chemokine production occurred in association with inhibition of myelination, it was important to determine whether these chemokines were responsible for this effect using treatment with LPS a known chemokine stimulator. The results from treating the cultures with LPS revealed a large upregulation in chemokine transcription levels, yet it did not have any effect on myelination, indicating that the chemokines secreted were not responsible for the blocking of myelination (Figure 4.16 and Figure 4.17). However, in an *in vivo* setting the release of these chemokines would play an important function in potentiating disease by attracting peripheral immune cells into the CNS. Chemokines are known to play an important role in MS, as they are essential in recruiting peripheral immune cells across the blood brain barrier into the CNS (Szczucinski and Losy, 2007). Many of the chemokines highlighted in these results have previously been identified in the

literature as playing possible roles in MS pathogenesis. CCL5 and CXCL10 production were both found in the myelinating cultures and these chemokines were also detected in increased levels in the CSF of MS patients (Szczucinski and Losy, 2011). In addition to this, it has previously been shown in this myelinating culture system that CXCL10 could inhibit myelination (Nash et al., 2011). CCL5 is a potent inflammatory chemokine involved in the recruitment of lymphocytes and leukocytes due to its promiscuous binding to CCR1, 3 and 5, which are found on many immune cells (Mueller and Strange, 2004, Trebst et al., 2001). Another chemokine of interest was CCL20, which O4 and Z2 both induced. CCL20 binds constitutively to CCR6 and this interaction has been shown necessary to attract T_H17 cells into the CNS during EAE (Reboldi et al., 2009). This is of interest as T_H17 cells are known to play a role in MS (Romme Christensen et al., 2013). These observations taken together suggest that low titres of antibodies could potentiate MS by triggering the release of chemoattractants, drawing effector T cells and other immune cells across the blood brain barrier.

The patient data alludes to chemokine induction, so therefore possibly inhibition of myelination if these autoantibodies act in the same way as the anti-myelin antibodies Z2 and O4 have. A larger MS patient cohort with healthy controls would be required to verify this however (Figure 4.18). Unpublished data Dr. Christina Elliott in the Linington laboratory showed that when myelinating cultures were incubated with IgG derived from MS patient sera at a concentration of 50 μg/mL for 12 days it resulted in inhibition of myelination in 1 out of the 7 patients tested. This was a similar response to what is presented in this chapter using anti-myelin monoclonal antibodies. Furthermore, CCL5 induction was seen in whole mouse brain samples *in vivo* after only 24 hours (Figure 4.19) thereby reinforcing that long-term exposure to antigen-specific antibodies could be a disease relevant mechanism.

Future investigations will address the mechanisms involved in this novel finding, and ask whether it is a cell-cell interaction responsible for the inhibition of

myelination or if it is a secreted soluble factor. Future experiments to resolve this will use supernatant transfer from treated myelinating cultures to un-treated cultures to explore if that is enough to inhibit myelination. Moreover, depletion of specific cell populations before antibody treatment could elucidate the cell responsible for the effects on myelination. Another aspect of this response to be further studied is which cell is producing the chemokine signature? To dissect this out single-cell cultures of astrocytes or microglial cells will be set up and conditioned medium collected to test in myelinating cultures. In addition in-situ hybridisation experiments of mixed cultures could be carried out for chemokine mRNA expression which would be the most effective way to detect the cell responsible and allow for cross talk between cells. The technical issue with this is finding the appropriate rat antibodies. Figure 4.12 suggests that for this response to occur the antibody must have a target that is accessible, as the data shows CCL5 is only induced when the antibody can bind to PLP. This experiment needs to be repeated with the 10 day treatment to see if this is also true for inhibition of myelination. Therefore antigen recognition is required but experiments using Fab fragments need to be done to assess whether the Fc region is also required.

Most importantly these data collectively present a novel pathway in which these autoantibodies could contribute to disease. There is data highlighting many antigenic targets for autoantibodies in MS (Somers et al., 2009), but there is still no identified role for them. This chapter presents a new hypothesis where sub-lytic levels of myelin-specific antibodies could inhibit remyelination and potentiate disease by microglia activation and inducing chemokine production leading to immune cell recruitment.

Chapter Five

Selective depletion of autoantigen-specific B cells: A strategy to treat multiple sclerosis

5 SELECTIVE DEPLETION OF AUTOANTIGEN-SPECIFIC B CELLS: A STRATEGY TO TREAT MULTIPLE SCLEROSIS

5.1 INTRODUCTION

Early studies demonstrated that autoantibody responses to surface exposed MOG epitopes could induce primary demyelination and exacerbate disease severity in EAE (Linnington et al., 1984, Lebar et al., 1986, Linington et al., 1988). The pathology of autoantibody-mediated demyelination in EAE reproduces many immunopathological features associated with demyelination in MS lesions (Genain et al., 1995, Genain et al., 1999). In particular, replicating the lesion subtype pattern II, co-deposition of immunoglobulins and complement activation proteins, found in active lesions of MS patient brains (Merkler et al., 2006, Lucchinetti et al., 2000).

These observations from EAE studies stimulated interest in the role of B cell-dependent pathogenic mechanisms in MS that resulted in the first clinical trial exploring the effects of B cell depletion in patients with MS. However, research into the presence of B cells in MS dates back several decades to preliminary studies performed by Kabat et al., 1942, in which antibodies were first discovered in patient CSF. Further studies went on to identify OCBs as a pathological hallmark of MS as they can be found in around 90% of MS patients (Ebers and Paty, 1980). Since then it has been identified that there is intrathecal synthesis of antibodies and clonal expansion of B cell populations within the CNS (Obermeier et al., 2008). Patient studies have revealed beneficial clinical effects for some patient's treated with therapeutic plasma exchange, particularly in patients with pattern II lesions (Keegan et al., 2005, Linker and Gold, 2008). More recent studies showed functional evidence of MS patient-derived antibodies demyelinating and axopathic capabilities *in vitro* (Elliott et al., 2012). In conclusion, there is now a substantial

body of evidence supporting a role for B cells and autoantibodies in MS, as discussed fully in the general introduction (Section 1.4.2).

Compelling evidence of B cell involvement in MS comes from current B cell therapies which are based on systemic depletion of the whole B cell repertoire using anti-$CD20^+$ antibodies (Deiss et al., 2013). Rituximab is a monoclonal anti-CD20 chimeric antibody that selectively targets and depletes $CD20^+$ B cells. First phase II clinical trials in RRMS patients were successful in rapidly reducing inflammation in the CNS (Hauser et al., 2008). Patients showed substantial reductions in total numbers of lesions and in the number of newly occurring gadolinium-enhancing lesions, and the treatment was also associated with a decrease in the relapse rate (Hauser et al., 2008). Treatment was in conjunction with almost complete depletion of peripheral B cells after 2 weeks. This was mirrored in the CSF where the B cell population was markedly decreased after 24 weeks and in addition CSF T cells were also found to be reduced by up to 55% in this time frame (Cross et al., 2006, Piccio et al., 2010). These results suggest that B cells are required to maintain inflammation in the CNS but the clinical role of autoantibodies is doubtful, since the therapeutic benefits of rituximab occurred before significant decreases in serum immunoglobulin levels and also antibody-producing plasma cells are CD20 negative. This treatment has also been trialled in PPMS patients, which resulted in a reduced volume of T2 lesions, although time until confirmed disease progression was not significant between groups (Hawker et al., 2009). Rituximab has also been efficacious in treatment of NMO, a demyelinating disorder of the CNS with a B cell involvement (Cree et al., 2005, Kim et al., 2013a). The second generation of anti-CD20 therapeutics are currently in development using humanised monoclonal antibodies (ocrelizumab) or fully human monoclonal antibodies (ofatumumab), both of which have successfully reduced disease activity in clinical trials (Kappos et al., 2011, Sorensen et al., 2014).

B cells contribute to MS inflammation through a variety of mechanisms, which are discussed in the general introduction, but the definitive mode of action by which B cell depletion is beneficial in inflammatory demyelinating diseases is still poorly understood. Experimental studies suggest B cell-mediated presentation of antigen to encephalitogenic T cells plays a key role in potentiating disease (Weber et al., 2010). B cells have been highlighted as being extremely effective at presenting antigen to their cognate T cells, as their B cell receptors are much more efficient at antigen recognition and better at antigen uptake, processing and presentation than other antigen presenting cells (Lanzavecchia, 1985, Yan et al., 2006). Another possibility is the depletion of inflammatory cytokine-producing B cells (Barr et al., 2012). This study showed that B cells from EAE and MS patients produce more IL-6 and that ablation of B cells in their EAE model ameliorated disease as a result of decreased IL-6 secretion. B cells have also been shown to produce other pro-inflammatory cytokines and cytokines involved in lymphoneogenesis (Duddy et al., 2004, Duddy et al., 2007).

Although B cell depletion is an effective therapy, this approach is limited by a range of adverse side effects such as hypogammaglobulinemia (Gottenberg et al., 2010) and neutropenia (Tesfa et al., 2011), which are associated with long-term complications such as opportunistic infections, including the life-threatening viral infection progressive multifocal leukoencephalopathy (PML) (Carson et al., 2009). Early EAE studies in mice which were genetically B cell deficient had a higher disease burden, suggesting that B cells can also have a regulatory role (Wolf et al., 1996). These studies and others brought about the emergence of a new B cell subset, regulatory B cells, which are yet to be fully characterised (Gray and Gray, 2010). Their main suppressive function is thought to be via the secretion of anti-inflammatory cytokine IL-10, and more recently identified IL-35 (Shen et al., 2014). Further EAE experiments have shown that depletion of this regulatory B cell phenotype using anti-CD20 therapies increased disease severity (Ray et al., 2011). Contrary to this, a recent study by Shen et al. (2014), showed that

immunosuppressing cytokines IL-10 and IL-35 were being secreted from plasma cells, which are $CD20^-$.

Taken together, this evidence raises the question; could selective depletion of autoantigen-specific components of the B cell repertoire have a similar level of clinical effectiveness as global elimination of B cells but without loss of immunoregulation? Immunotoxins are an effective way to target and eliminate an antigen-specific population and this therapeutic approach has been studied in depth (Shapira and Benhar, 2010, Madhumathi and Verma, 2012), particularly in the treatment of cancer (Kreitman, 2006, Pastan et al., 2007). They have also been shown to be a promising therapeutic approach in the context of other autoimmune diseases like rheumatoid arthritis, where *in vitro* and *in vivo* studies led to effective depletion of targeted cells (van Roon et al., 2003, Nagai et al., 2006). Previous immunotoxin studies in EAE observed a delayed onset of disease and reduced disease activity by eliminating activated T cells (Jia et al., 2006, Chen et al., 2007). This antigen-specific approach requires the identification of appropriate antigen candidates. Recent studies in paediatric MS patients have revealed significant evidence of increased B cell reactivity to MOG in a subgroup of around 20% (Brilot et al., 2009, McLaughlin et al., 2009, Probstel et al., 2011). This highlighted MOG as a candidate target for the development of an antigen-specific therapy.

This study investigated the efficacy of a recombinant immunotoxin (MOG-ETA'), which was developed by Professor Stefan Barth's group in Aachen, Germany (Barth et al., 2000, Nachreiner et al., 2008). *Pseudomanas aeruginosa* exotoxin A (ETA) is a potent toxin commonly used in immunotoxins (Bruell et al., 2003, Stocker et al., 2005). Once internalised the catalytic domain of the toxin is processed and transported to the cytosol where the toxin causes cell apoptosis by ADP-ribosylation of elongation factor 2 (EF-2), an essential factor for protein synthesis (Weldon and Pastan, 2011). The original binding domain of the toxin was replaced

with the extracellular domain of MOG (amino residues 1-125) to specifically target and eliminate MOG-specific B cells in a receptor-mediated fashion (Figure 5.1). MOG-ETA's specificity and selective cytotoxicity was demonstrated using 8-18C5 (anti-MOG mouse monoclonal antibody) hybridoma cells *in vitro* and B cells from transgenic IgH_{MOG} mice *ex vivo* (Nachreiner et al., 2008). The results showed dose-dependent depletion of MOG-specific B cells. Previous attempts at depleting MOG-specific B cells used a recombinant fusion protein, composed of the extracellular domain of MOG and the Fc domain of human IgG1 (Zocher et al., 2003). Despite the fusion protein being capable of depleting MOG-specific B cells it required much higher concentrations to achieve any significant results, so was less efficient than the immunotoxin adopted in this study.

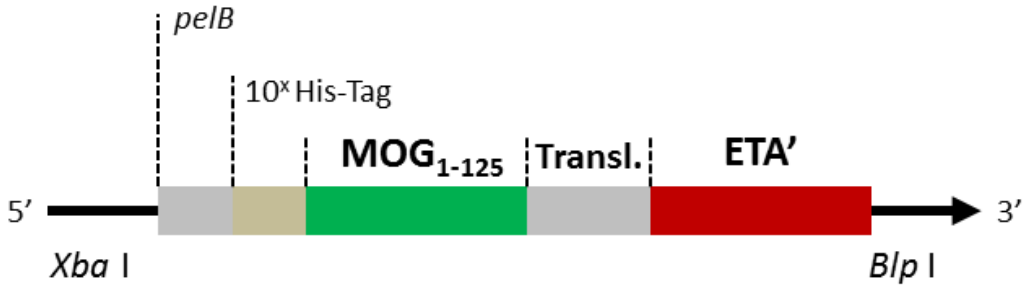


Figure 5.1. Diagram of the MOG-ETA' construct used in these MOG-ETA' studies.
The immunotoxin has a *pelB*-leader sequence at the N-terminus, which is followed by a 10^x polyhistidine tag (10^xHis-Tag) for protein purification by affinity chromatography. The extracellular domain of MOG is connected to the translocation domain (Transl.), which is next to the catalytic domain (ETA') of *Pseudomonas* Exotoxin A. Figure adapted from Nachreiner et al., 2008.

To assess MOG-ETA's efficacy in reducing disease, MOG-induced EAE in DBA/1j mice was adopted; a well-established mouse model which replicates many of the major immunological features associated with paediatric MS (Abdul-Majid et al., 2000). MOG is unique as it is the only protein which can induce both a T and B cell response in contrast to other myelin and non-myelin CNS antigens, which can only stimulate a T cell response (Iglesias et al., 2001). DBA/1j mice are highly susceptible to MOG-induced EAE and were chosen for this study as it was discovered that H2-b mice, such as C57BL/6, are unable to develop a demyelinating autoantibody response to MOG (Abdul-Majid et al., 2000, Bourquin et al., 2003), which is essential for this study as it is important for lesion formation, a pathological hallmark of MS (Genain et al., 1999).

Therefore DBA/1j mice were used as MOG-immunisation of this species induced an encephalitogenic T cell response as well as developed an autoantibody response directed against conformation-dependent determinants of the extracellular domain of MOG (Abdul-Majid et al., 2002, Svensson et al., 2002, Abdul-Majid et al., 2003). This two-hit model ensures disruption of the BBB due to the presence of anti-MOG encephalitogenic T cell response, which then allows pathogenic MOG-specific antibodies to enter the CNS. Studies have previously shown that without a compromised BBB, circulating antibodies cannot access the CNS to cause damage (Litzenburger et al., 1998, Bourquin et al., 2000). In summary, the aim of this chapter was to analyse MOG-ETA's therapeutic efficacy in MOG-induced EAE with the goal of effectively suppressing disease using this novel antigen-specific approach.

5.2 RESULTS

5.2.1 MOG-ETA' treatment ameliorates disease activity in MOG-induced EAE

5.2.1.1 Establishing MOG-induced EAE model in DBA/1j mice

The immunotoxins efficacy was tested using MOG-induced EAE in DBA/1j mice. As discussed in this chapters introduction, the model was chosen as previous studies revealed it incorporated both T and B cell dependent mechanisms, therefore recapitulating key immunological features of paediatric MS (Abdul-Majid et al., 2002, Abdul-Majid et al., 2003). Analysing all the MOG-induced EAE experiments together (mice, n=25) DBA/1j mice were highly susceptible to MOG-induced EAE, which was characterised by high lethality, with approximately only 20% surviving longer than 16 days post immunisation (Figure 5.2). Figure 5.3 shows again that this protocol leads to a severe disease phenotype, which in this experiment led to it being terminated 13 d.p.i. due to the animals being too sick. It also illustrates that increased weight loss is associated with an increase in clinical disease severity and appears to precede the onset of EAE symptoms.

For the planned MOG-ETA' investigations it was essential that the mice mounted an autoantibody response mimicking that observed in approximately one fifth of paediatric MS patients (McLaughlin et al., 2009). This was investigated by incubating pooled mouse serum (26 d.p.i) with murine MOG-transfected cell lines, which express correctly folded murine MOG on their surface. The results showed that the antibodies bound to MOG transfected cells in a dose dependent manner (Figure 5.4A). Control mouse serum from naïve mice and mice immunised with CFA alone did not bind to the MOG transfected cells (Figure 5.4B). This confirmed that EAE development was associated with the production of anti-MOG antibodies that recognise the native, extracellular domain of MOG, similar to results from paediatric MS patients (McLaughlin et al., 2009, Di Pauli et al., 2011, Probstel et al., 2011).

The antibodies were shown to be pathogenic when incubated with myelinating cultures (28 DIV). 26 d.p.i. pooled mice sera was incubated for 16 hours along with 2% fresh rat serum as an exogenous source of complement. The results showed that mice sera dilutions between 1:50 and 1:500 induced demyelination occurring in the range of 71-96% (Figure 5.5). No myelin loss was seen with rat serum alone or with serum from mice immunised with CFA only compared to untreated myelinating cultures (data not shown). Therefore, verifying that the MOG-induced EAE mouse model incorporated a demyelinating autoantibody response.

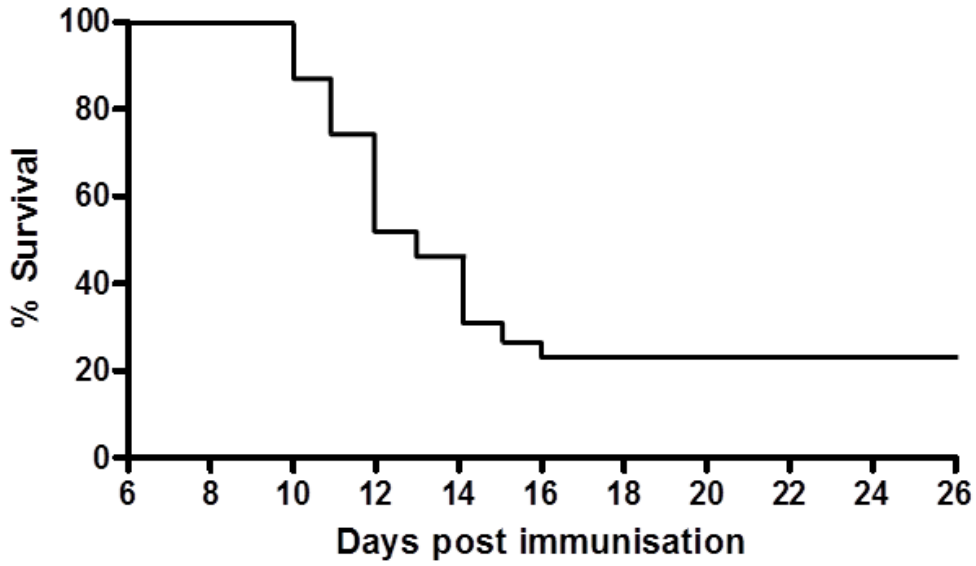


Figure 5.2. MOG_{1-125} induced EAE leads to a chronic disease phenotype.
7-8 week old DBA/1j mice were immunised with 100 µl complete Freund's adjuvant containing 50 µg MOG_{1-125} and 150 µg heat killed *M. Tuberculosis* to establish EAE. The graph illustrates survival curve of animals over time, with each point representing the percentage of animals alive (n=25).

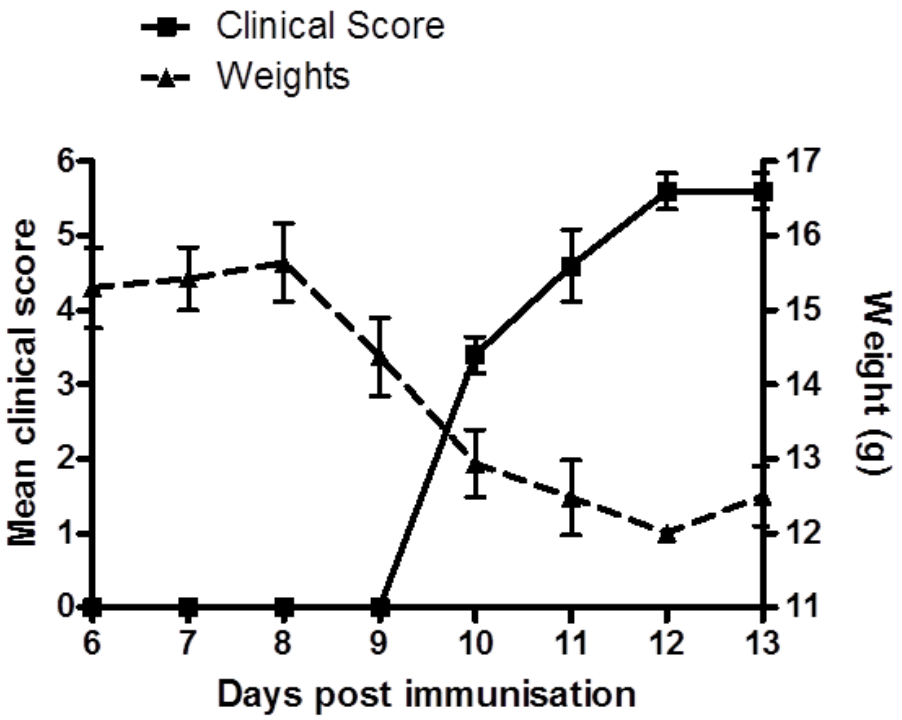


Figure 5.3. MOG_{1-125} induced EAE induces severe disease phenotype.
7-8 week old DBA/1j mice were immunised with 100 µl complete Freund's adjuvant containing 50 µg MOG_{1-125} and 150 µg heat killed *M. tuberculosis*. Data shows that mice develop a severe disease between 9 and 13 d.p.i. which is associated with loss of weight. Data points represent mean (± SEM), *n=5*.

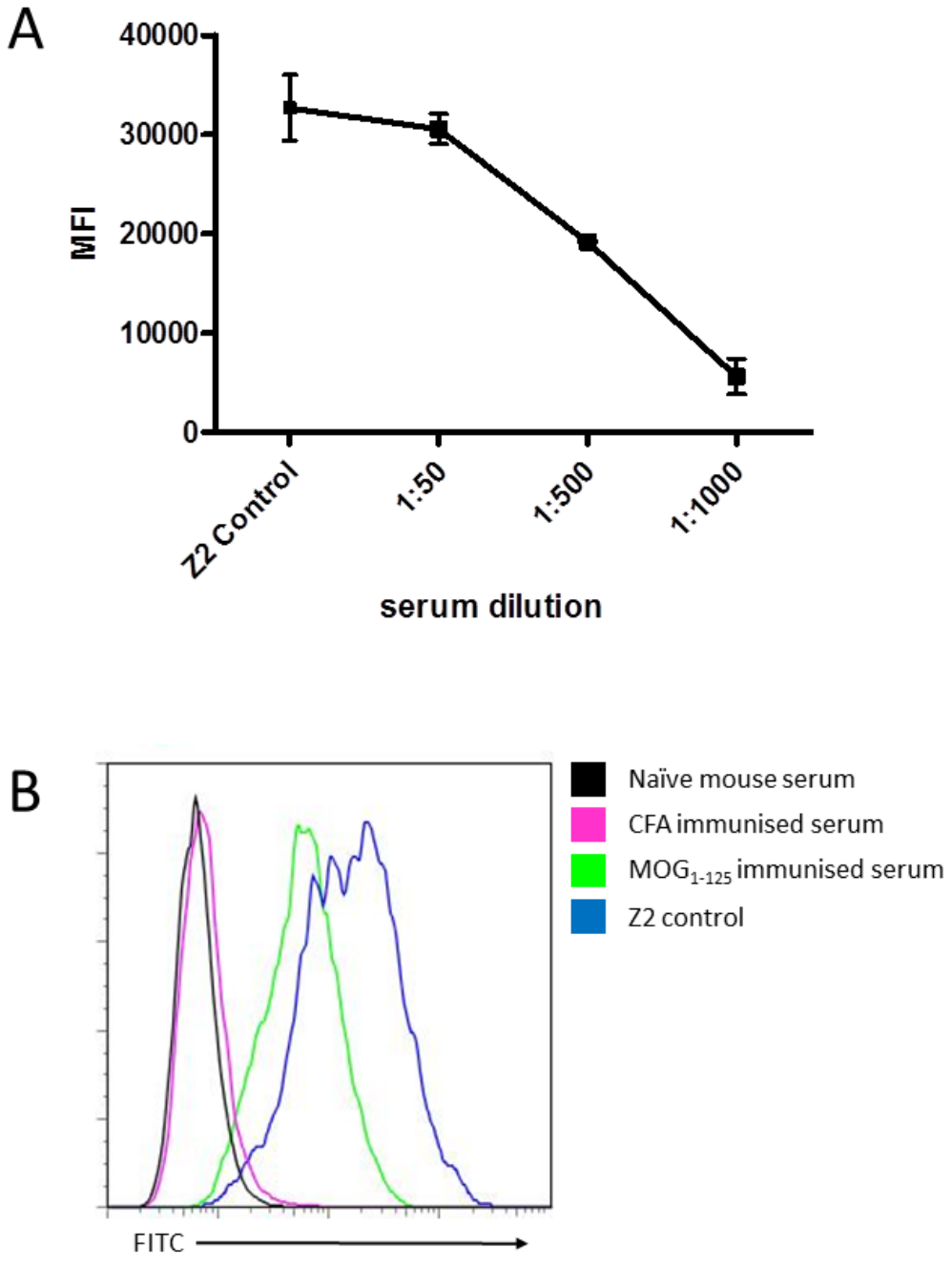


Figure 5.4. MOG_{1-125} induced EAE induces a conformation-dependent MOG-specific autoantibody response.
(A) 7-8 week old DBA/1j mice were immunised with 100 µL complete Freund's adjuvant (CFA) containing 50 µg MOG1-125 and 150 µg heat killed *M. tuberculosis*. MOG-specific antibodies were detected by incubating pooled mice sera (n=5, 26 d.p.i) with murine MOG-LTK transfected cells in a serial dilution. Z2 was used at a concentration of 10 µg/mL as a positive control. Antibody binding was detected using FITC-labelled anti-mouse IgG antibodies. Data points represent mean fluorescent intensity (MFI) (MOG+ LTK transfected cells MFI minus the control LTK cells MFI). Cells were pre-gated on live single cells. Data points from two biological repeats (mean ± SD). (B) MOG-LTK transfected cells were incubated with pooled serum (n=5) from naïve mice, mice immunised with 100 µL of 3 mg/mL CFA (26 d.p.i) and MOG1-125 immunised mice (as above). Pooled sera were used at a 1:50 dilution. Z2 was used at 10 µg/mL as a positive control. MOG-specific antibody binding was detected as before. Cells were pre-gated on live single cells. FACS histogram is representative of two biological repeats.

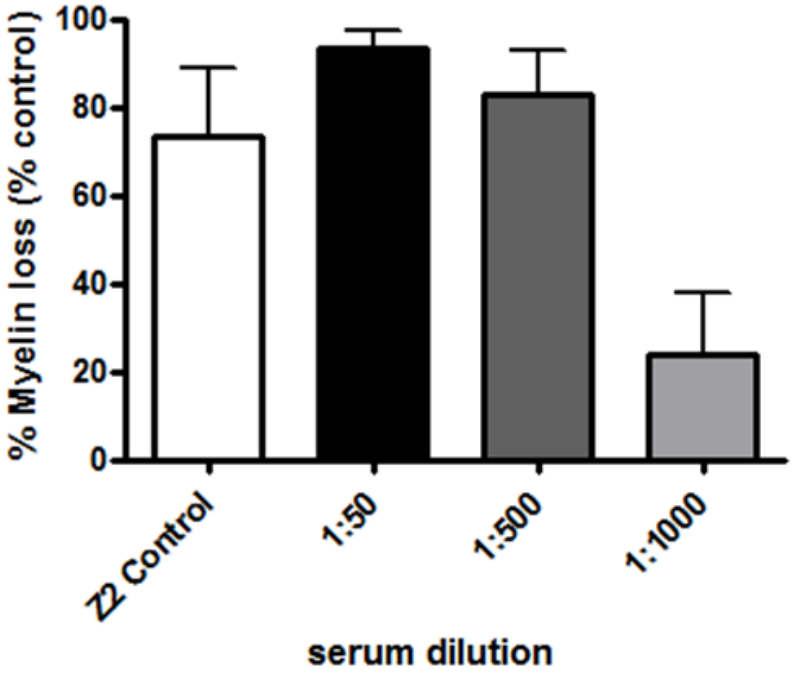


Figure 5.5. MOG_{1-125} induced EAE induces a pathogenic MOG-specific antibody response. 7-8 week old DBA/1j mice were immunised with 100 µL complete Freund's adjuvant (CFA) containing 50 µg MOG_{1-125} and 150 µg heat killed *M. tuberculosis*. 26 d.p.i. sera were collected and pooled from 5 mice. Myelinating cultures (28 DIV) were incubated for 16 hours with pooled serially diluted serum. 2% rat serum was used as an exogenous source of complement. Z2 was used at a concentration of 10 µg/mL as a positive control. Untreated cultures were used as a negative control. Result from one biological repeat performed in triplicate (mean ± SD).

5.2.1.2 MOG-ETA' treatment significantly reduced EAE severity

Adopting the MOG-induced EAE mouse model discussed above (5.2.1.1), the efficacy of MOG-ETA' to influence the development of EAE was assessed. Mice were treated from 2 d.p.i. and every 48 hours after with i.p. injections of 25 µg MOG-ETA' or the control immunotoxin Bo9-ETA', which had an irrelevant specificity to a pollen protein (Nachreiner et al., 2008). Treatment regime was selected to maximise probability of immunotoxin success. Injection schedule commenced 2 d.p.i. to deplete MOG-specific B cells as immune response occurs. Preliminary experiments indicated efficacy at 25 µg (data not shown). The clinical data revealed that the treatment did not affect the time of EAE onset (MOG-ETA', n = 30, 10.2 ± 0.6 d.p.i; Bo9-ETA', n = 30, 9.8 ± 0.5 d.p.i; $p > 0.05$), but MOG-ETA' significantly suppressed accumulation of disability (Figure 5.6A). Figure 5.6B shows that there was a trending decrease in the mean cumulative disease score with MOG-ETA' treatment but this did not reach significance. Unfortunately untreated MOG-induced EAE was not setup to run parallel to this experiment as a definitive negative control. Nevertheless, MOG-ETA' treatment is still associated with a decrease in disease severity when compared to the clinical scores depicted in the MOG-induced EAE experiment in Figure 5.3. However, to definitely compare these MOG-ETA', Bo9-ETA' and no treatment would have to be run at the same time under the same conditions. Due to disease severity, experiments had to be terminated 11 d.p.i. to ensure that there was a large enough pool of animals for further *ex vivo* analysis. Despite this, MOG-ETA' had a clear effect on slowing disease progression in this severe model of EAE.

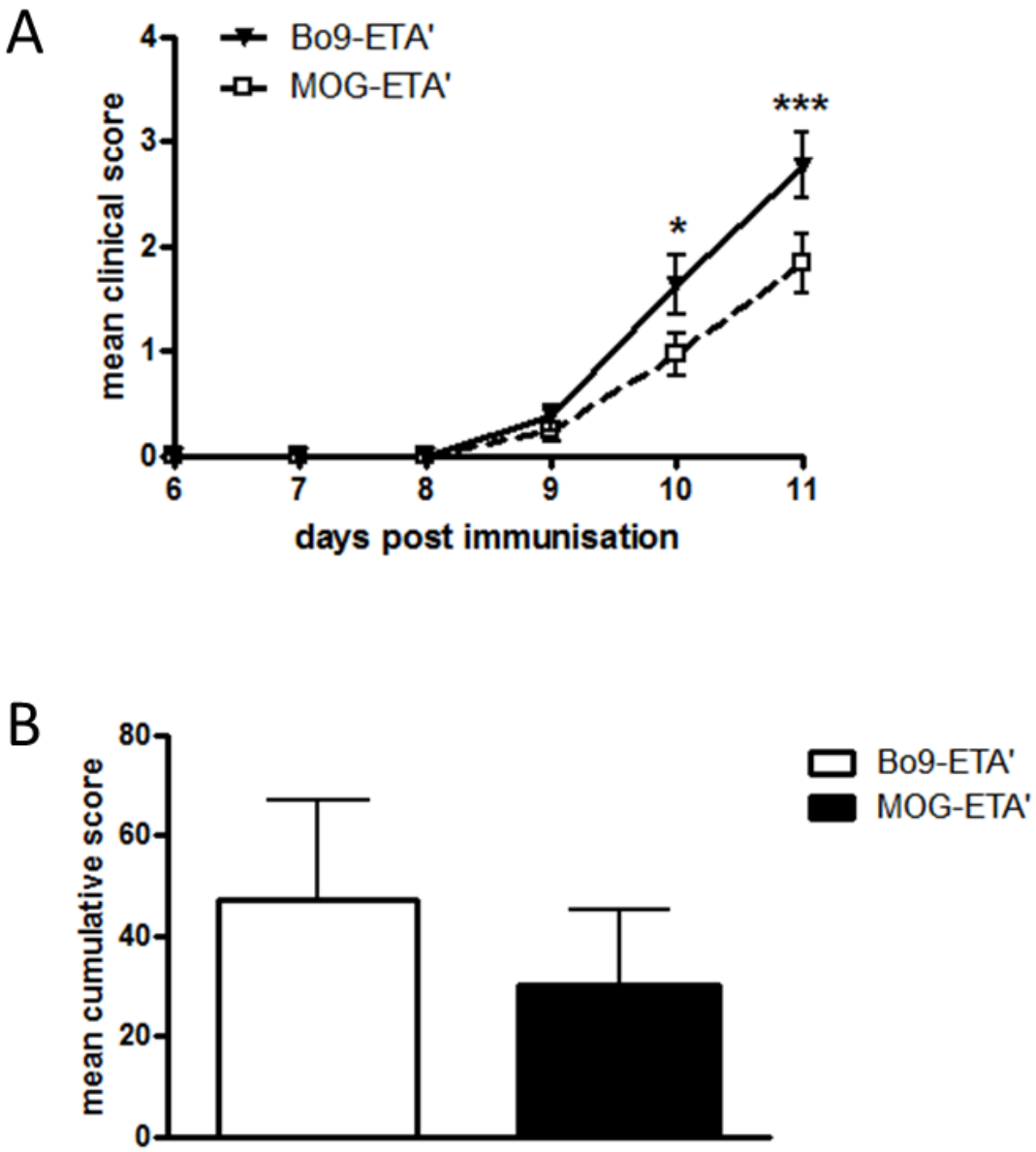


Figure 5.6. MOG-ETA' treatment modulates disease activity in EAE.
7-8 week old DBA/1j mice were immunised with 100 µL complete Freund's adjuvant (CFA) containing 50 µg MOG_{1-125} and 150 µg heat killed *M. tuberculosis*. Mice were then treated i.p. with 25 µg MOG-ETA' or Bo9-ETA' on day two and every 48 hours after immunisation. (A) Clinical scores represent pooled mean ± SEM (n=30). Overall clinical data from three independent experiments showed significant reduction of EAE. Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. *, $p < 0.05$, ***, $p < 0.001$. (B) Mean cumulative score was calculated by the addition of daily clinical scores of all mice. Bar graph represents mean ± SEM (n=30).

5.2.1.3 MOG-ETA' treatment reduces cellular infiltration into the spinal cord

To investigate if this reduction in EAE severity was associated with a decrease in CNS disease activity, the spinal cord from mice were harvested at 11 d.p.i. to investigate the cellular composition. Cells were isolated, washed, and then detected with fluorescently-labeled antibodies to identify infiltrating subsets. This revealed a significant reduction in $CD45^+$ leukocytes found in the spinal cord compared to the control Bo9-ETA' treated mice, approximately a 2-fold decrease (Bo9-ETA', 696,461 ± 184,933; MOG-ETA', 390,529 ± 138,400, $p < 0.05$).

Focusing on specific subsets, there was a significant decline in the $CD11b^+Ly6C^+$ monocyte population detected in the spinal cord; $p < 0.01$ (Figure 5.7A). This depletion was an important finding as macrophages are major effector cells in EAE (Huitinga et al., 1995). There were also reductions in $CD3^+CD4^+$ T cell, $B220^+$ B cell and $CD25^+FoxP3^+$ T_{Reg} cell recruitment into the spinal cord (Figure 5.7B). This suggested that MOG-ETA' was having a global effect on immune cell reduction, rather than a leukocyte subset-specific phenomenon. Using the same phenotypic markers the cellular composition of the peripheral LNs were also assessed but no differences in immune cell numbers were observed between the two treatment groups (data not shown), suggesting that this was a CNS specific effect. The B cells were additionally labelled with CD1d and CD5 to look for $CD1d^{high}CD5^+$ regulatory B cells, a subset phenotype previously defined by Yanaba et al. (2008). There was no difference in this population between immunotoxin treatments (data not shown). Overall this data shows that MOG-ETA' treatment reduced clinical disease severity and this was associated with a significant depletion of monocytes and decreased recruitment of lymphocytes into the CNS.

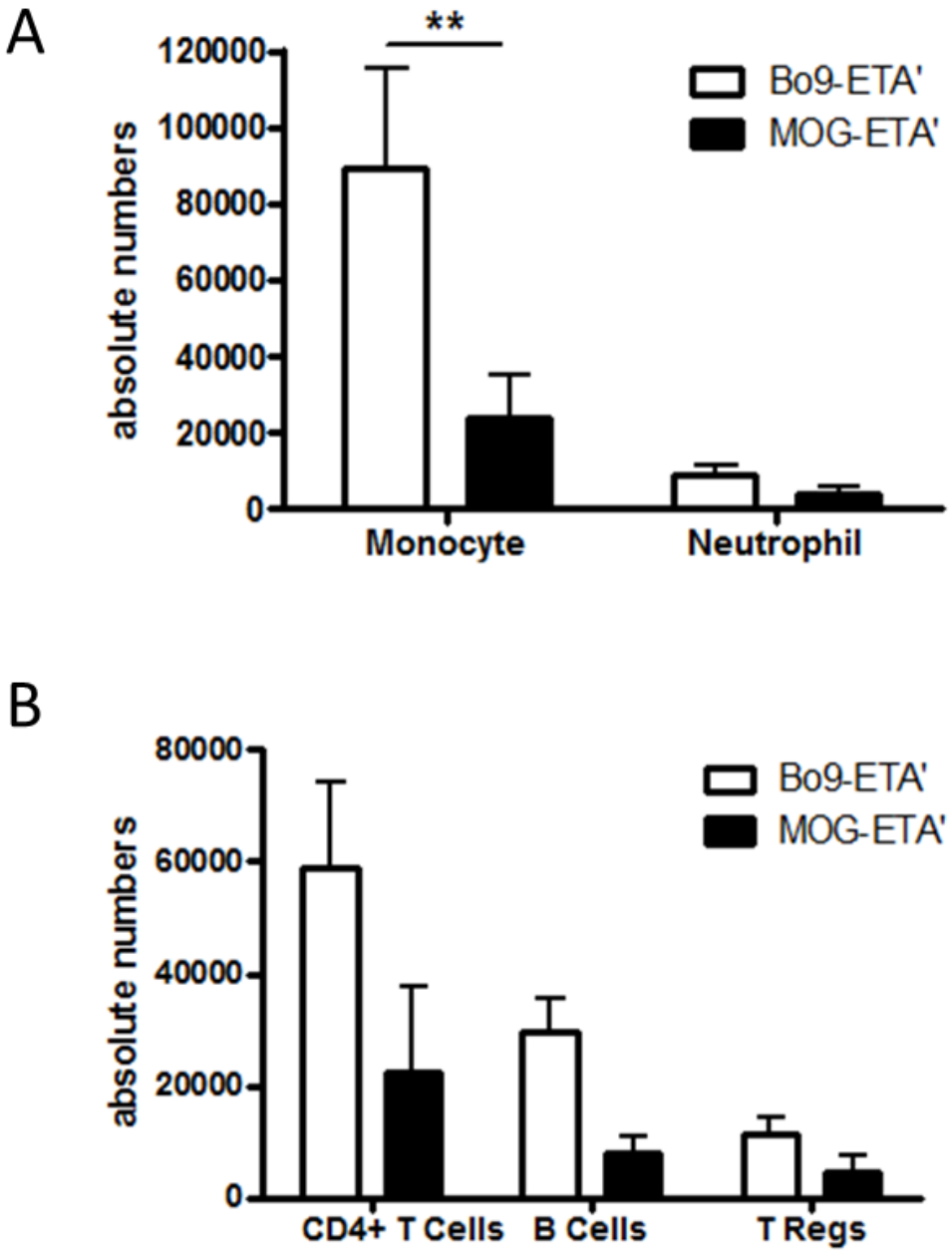


Figure 5.7. MOG-ETA' treatment reduced immune cellular infiltration into spinal cord.
Each single cell suspension was from 3 pooled spinal cords at termination of the experiment (11 d.p.i.). Cells were pre-gated on live $CD45^+$ single cells. (A) There was a significant reduction in infiltrating monocytes. Monocytes and neutrophils were stained with Ly6C and Ly6G respectively, which were pre-gated on $CD11b^+$. Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. **, $p < 0.01$. (B) There was an observable reduction in lymphocytes in the spinal cord. $CD4^+$ T cell populations were stained with CD4 and CD3, regulatory T cells stained with FoxP3 and CD25, which were pre-gated on $CD4^+$ T cells, B cells stained with B220, which were pre-gated on $CD5^-$ cells. Bar graphs represent mean ± SEM (n=3) of absolute numbers of cells in the spinal cord, representative of two individual experiments.

5.2.2 MOG-ETA' treatment is associated with a paradoxical MOG-specific pro-inflammatory response in periphery

5.2.2.1 MOG-ETA' induces peripheral MOG-specific autoimmunity

As MOG-ETA' treatment was shown to reduce disease severity and decrease cellular infiltration into the spinal cord, it suggested that the treatment was acting to ablate the expansion of an encephalitogenic immune response in the periphery. To assess this, MOG-specific proliferation and cytokine secretion by cells isolated from the subinguinal LNs of immunised mice were analysed. Interestingly, this revealed a dichotomy between a reduction in disease activity in the CNS and an increased peripheral MOG-specific immune response in MOG-ETA' treated mice. When LN cells isolated from the MOG-ETA' mouse group were incubated with $MOG_{1\text{-}125}$, they proliferated 3- to 4-fold times more than the Bo9-ETA'-treated controls, $p < 0.05$ (Figure 5.8). The supernatants from the cultured LN cells were subsequently screened using a semi-quantitative proteome array (Figure 5.9A). This revealed that the cells were secreting greater quantities of pro-inflammatory cytokines and chemokines, which could be observed by eye from the blots (Figure 5.9B). The arrays were quantified using TotalLab to attain arbitrary values (Table 5.1). The cytokine profile secreted by the MOG-ETA'-treated LN cells showed a selective increase in pro-inflammatory cytokines including IFNγ, TNFα and IL-17. The cytokine milieu appeared to be T_H1/T_H17 skewed with no evidence of a T_H2 or T_{Reg} response, for example there was no IL-4 or IL-10 secretion, respectively.

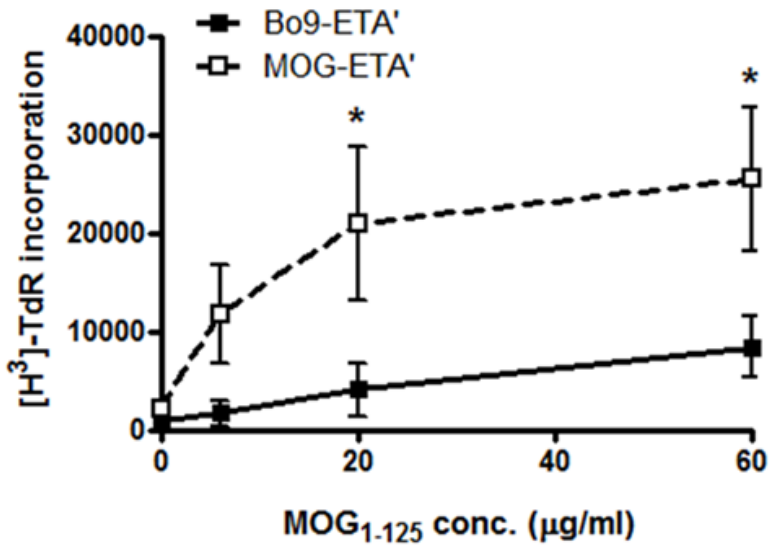


Figure 5.8. MOG-specific proliferation was increased in lymph node cells from MOG-ETA' treated mice.
Draining lymph nodes were collected from mice 11 d.p.i. (n=4), and mechanically dissociated into a single cell suspension and the recall response analysed. The cells were stimulated *ex vivo* with or without antigen (MOG_{1-125}). Antigen-induced proliferation was measured, as determined by incorporation of tritirated thymidine ($[H^3]$-TdR). Results are representative of 3 individual repeats. Data points represent mean (± SEM). Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. *, $p < 0.05$.

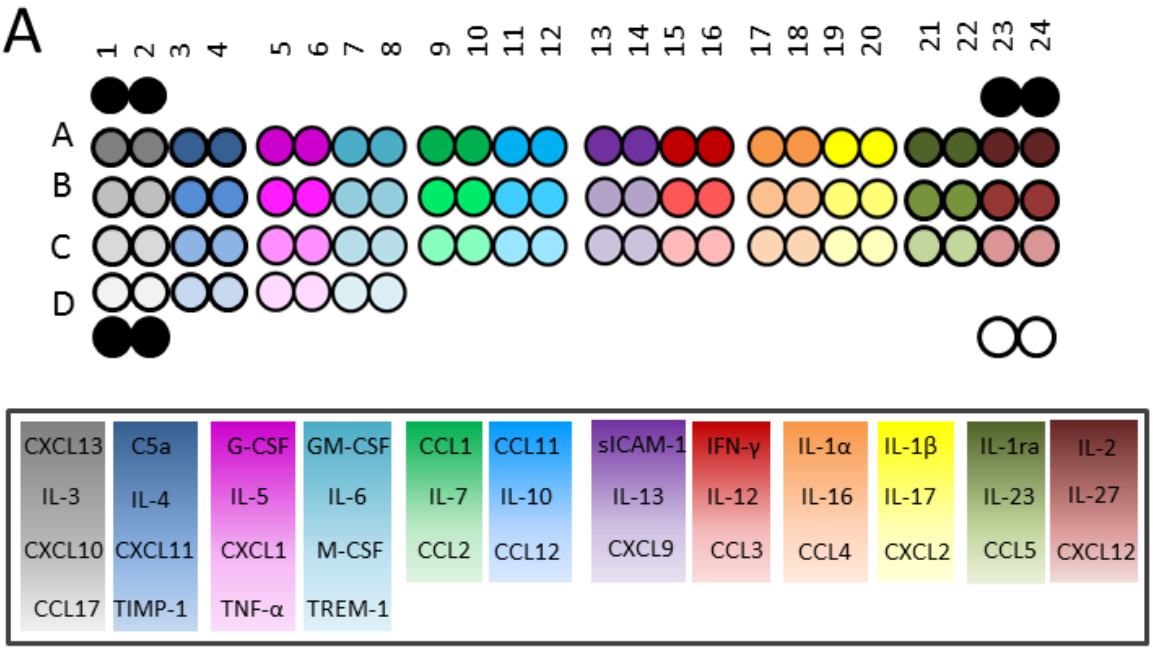


Figure 5.9. Cytokine and chemokine production from cells isolated from lymph nodes of Bo9-ETA' and MOG-ETA' treated mice.
Draining lymph nodes were collected and mechanically dissociated into a single cell suspension from mice 10 d.p.i. (n=4), and the recall response analysed. The cells were stimulated *ex vivo* with or without antigen (MOG_{1-125}). Antigen-induced cytokine and chemokine production (i.e. MOG_{1-125} minus medium alone) was measured from pooled supernatants of 4 mice and profile evaluated. Protein levels were measured using Mouse Cytokine Arrays as per manufacturer's instructions. (A) Mouse cytokine array coordinates are displayed above with corresponding cytokines and chemokines colour coded below. Black circles represent positive controls and clear circles represent negative controls. Not to scale. (B) Representative scans of proteome array.

Table 5.1. Peripheral MOG-specific cytokine response skewed towards pro-inflammatory in MOG-ETA' treated mice.
Draining lymph nodes were collected and mechanically dissociated into a single cell suspension from mice 11 d.p.i. (n=4), and the recall response analysed. The cells were stimulated *ex vivo* with or without antigen (MOG_{1-125}). Antigen-induced cytokine and chemokine production (i.e. MOG_{1-125} minus medium alone) was measured from pooled supernatants of 4 mice and profile evaluated. Protein levels were measured using Mouse Cytokine Arrays as per manufacturer's instructions. Arrays were analysed and quantified using TotalLab. Results are mean values representative of 3 biological repeats.

Pro-inflammatory cytokine and chemokine secretion			
	MOG_{1-125} stimulated cells		
Chemokines	Bo9-ETA'	MOG-ETA'	Fold change in MOG-ETA' treated group
CCL1	22301	38710	1.74
CCL3	22852	53484	2.34
CCL4	19897	45080	2.27
CCL5	6930	20533	2.96
CXCL2	35521	42594	1.20
CXCL9	429	4847	11.30
CXCL10	4513	17548	3.89
Cytokines			
G-CSF	7874	19898	2.53
IL-1α	1848	12085	6.54
IL-1ra	1082	15183	14.03
IL-3	13824	36644	2.65
IL-13	3316	7279	2.20
1L-17	28676	53840	1.88
IFNγ	7715	21034	2.73
TNFα	7628	16133	2.11

5.2.2.2 MOG-ETA' treatment led to increased titres of demyelinating MOG-specific antibodies

Proliferation and cytokine data showed that MOG-ETA' treated mice had an amplified anti-MOG cellular activity compared to the Bo9-ETA' treated mice. The next question posed was to ask if there was also an enlarged anti-MOG antibody response in the serum. This was investigated by analysis of mouse sera obtained 11 d.p.i. using an ELISA. The results revealed that MOG-ETA' treatment induced a 3-fold increase in the MOG-specific antibody titre (Figure 5.10A). It is now well recognised that anti-MOG reactivity determined by ELISA does not necessarily translate into evidence of a pathogenic antibody response. These antibodies could be binding to linear MOG peptides or epitopes that are usually not exposed *in vivo*. Therefore, next it was examined if there was also an increased anti-MOG response to correctly folded, native MOG in MOG-ETA' treated mice using MOG_{1-125} transfected LTK cells. These results showed that MOG reactivity still remained significantly higher in the MOG-ETA' mouse group (Figure 5.10B).

To determine if the MOG-ETA' treatment was inducing a demyelinating autoantibody response, serum was assayed using myelinating cultures to see if the antibodies were capable of demyelination. The sera were harvested from MOG-ETA' and Bo9-ETA' treated mice (11 d.p.i.) and incubated for 16 hours with 2% rat serum on 28 DIV myelinating cultures. There was significant myelin loss in the cultures incubated with sera from the MOG-ETA' treatment group (1:100, Bo9-ETA': 36.3 ± 16.7%; MOG-ETA': 75.3 ± 7.7%, $p < 0.001$) (Figure 5.11). There was no significant difference in myelination at the lower dilutions of sera, suggesting that the pathogenic antibodies exist at low titres, as a high concentration of sera was required to get an observable effect. Cultures were also treated with 2% rat serum alone as a control and results showed negligible differences in % myelin loss compared to untreated control (2% rat sera = 4.42% ± 7.42; untreated control = 5.00% ± 8.67). Therefore, this indicates that the immunotoxin treatment triggers a

pathogenic autoantibody response in the periphery, which is paradoxical to the reduction of cellular infiltrate observed in the CNS and decreased EAE severity.

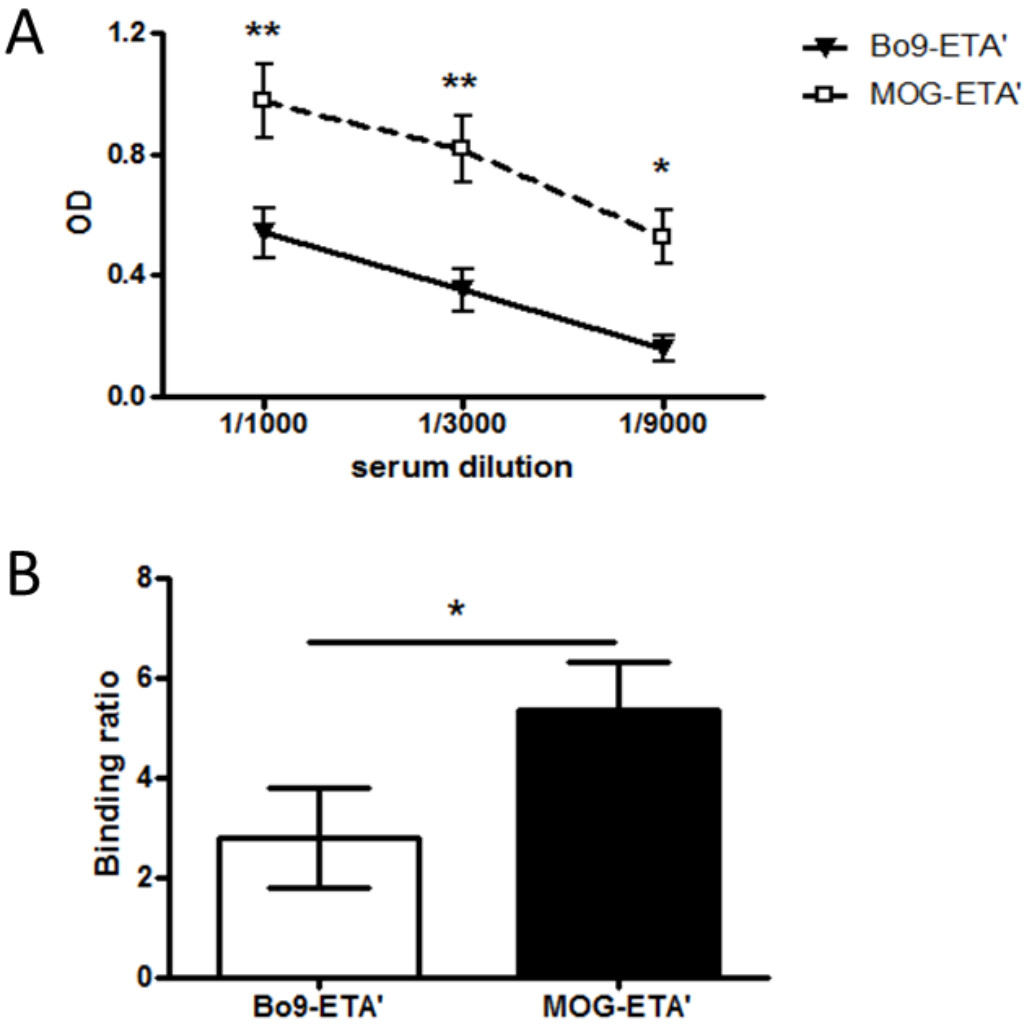


Figure 5.10. Increase in serum MOG reactivity and demyelinating activity.
Sera from mice 11 days post immunisation were analysed. (A) Serum levels of anti-MOG mouse IgG was measured by ELISA (n=8). Results representative of 3 individual experiments (mean ± SD). Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. *, $p < 0.05$, **, $p < 0.01$. (B) To investigate if these antibodies could bind to native MOG they were incubated with MOG transfected LTK cells. Serum samples were used at a 1:30 dilution (n=5). Antibody binding was detected using FITC-labelled anti-mouse IgG antibodies. FACS data represents the binding ratio (MOG-LTK transfected cells mean fluorescent intensity (MFI) divided by the control LTK cells MFI). Cells were pre-gated on live single cells. Bar graph represents mean ± SD. Significance was determined by a two-tailed unpaired Students' t test. *, $p < 0.05$.

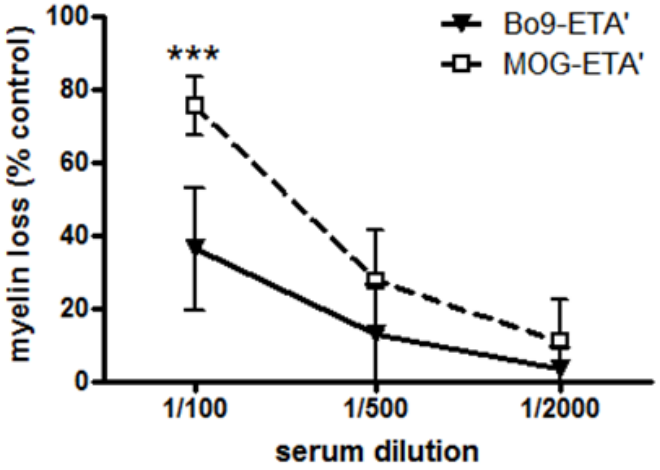


Figure 5.11. Increased demyelinating activity in serum from MOG-ETA' treated mice.
Serum from mice 11 d.p.i. was analysed to investigate demyelinating activity. Myelinating cultures (28 DIV) were incubated for 16 hours with pooled mice sera (n=6) in a series dilution plus 2% rat sera. Untreated cultures were used as controls. Cultures were stained with anti-MBP and anti-SMI31 to detect the myelin and axons, respectively. These were then identified using secondary fluorescently-labelled antibodies. Myelin loss was quantified using CellProfiler. Full methodology is described in Materials and Methods (Section 2.4.2 and 2.4.3). Experiment was performed in triplicate, results shown are from 2 biological repeats. Data points represent mean (± SD). Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. ***, $p < 0.001$.

5.3 DISCUSSION

Antigen-specific immunotherapies are the Holy Grail for the treatment of MS. Currently MS treatments encompasses broad immunosuppression, using drugs like IFNβ, Mitoxantrone, Fingolimod and Natalizumab. Their efficacy is compromised by an increased risk in opportunistic infections, like JC virus which causes PML (Bloomgren et al., 2012), treatment-related fatalities (Lindsey et al., 2012, Pelletier and Hafler, 2012), and other adverse effects. Global B cell depletion is an emerging therapeutic approach with great potential for people with MS. The first B cell-depleting drug was anti-CD20 (Rituximab), which was initially licensed to treat lymphoma and rheumatoid arthritis, but was later discovered to reduce disease activity in the CNS of patients with RRMS (Cross et al., 2006, Hauser et al., 2008). The next generation of humanised anti-CD20 treatments are currently in development and have been effective at slowing disease progression and suppressing CNS inflammation in recent clinical trials with RRMS patients (Kappos et al., 2011, Sorensen et al., 2014). Unfortunately, these therapies were also limited by an increased risk of PML due to JC virus infection (Carson et al., 2009), and other adverse effects associated with continual depletion of the B cell repertoire. However, the clinical successes of these drugs have reignited research into the B cells role in MS and B cell targeted treatments.

Apart from B cells role in autoantibody production, they can also potentiate disease via cytokine secretion and are highly efficient at antigen presentation to cognate T cells (Antel and Bar-Or, 2006), as discussed in the general introduction (Section 1.4.2). This information taken together led to the proposal that selective depletion of autoantigen-specific B cells could provide a novel strategy in which the successes seen with global B cell depletion could be recapitulated but without the elimination of other important components of the B cell repertoire; namely recently identified immunoregulatory protective B cell populations (Matsushita et al., 2008, Matsushita et al., 2010, Mauri and Bosma, 2012, Shen et al., 2014). This

study utilised the recent advancements in recombinant immunotoxin technologies and knowledge to investigate the efficacy of an antigen-specific therapy in EAE.

The autoantigen-specific immunotoxin MOG-ETA' was previously shown to effectively target and deplete MOG-specific B cells *in vitro* and also eliminate splenic cells from IgH_{MOG} transgenic mice *ex vivo* (Nachreiner et al., 2008). In this proof of principle study presented in this thesis it was shown that MOG-ETA' treatment significantly reduced clinical severity in a chronic animal model of MS despite the complex effector mechanisms involved. The experiment was terminated at day 11 due to the severity of the model. If there were no time constraints and more MOG-ETA' available this experiment would have been repeated in a less severe mouse model, which would have allowed a longer disease course and the possibility of therapeutic experiments.

The clinical effect was associated with reduced inflammation in the spinal cord, which was evident by a decrease in the global cellular infiltration into the CNS. Most pronounced was the significant reduction in recruitment of monocytes. Decrease of these peripheral monocyte/macrophage populations may be partly responsible for the disease suppression as macrophages constitute a large proportion of the immune cells found in active lesions (Lucchinetti et al., 2000). In addition to this, the depletion of macrophages in EAE has been shown to decrease disease severity (Huitinga et al., 1995, Bauer et al., 1995). Treatment-associated decrease in effector cell recruitment to the CNS could be a result of reduced antigen-specific B cell-T cell interactions due to MOG reactive B cell depletion. B cells are highly efficient antigen presenting cells for their cognate antigen (van der Veen et al., 1992). Studies in B cell deficient mice have shown that T cell priming and expansion was dependent on B cell antigen presentation and as a result T cell responses were greatly decreased (Rivera et al., 2001). This T cell activation could act to establish a positive feedback loop, where products secreted by the T cells then increase the B cells ability to process and present antigen (Harp et al., 2008,

Lund, 2008). Therefore, MOG-ETA' could be diminishing MOG reactive T cell activation and expansion via cognate B cell depletion. The decrease in effector T cells could also be responsible for the decline in monocyte numbers as activated T cells secrete IFNγ and TNFα which are required for macrophage activation and their subsequent production of pro-inflammatory cytokines (Nacy and Meltzer, 1991, Cantor and Haskins, 2006).

MOG-ETA' induced depletion of B cells was predicted to mediate numerous effects that could have contributed to the disease suppression observed. As well as the direct elimination of pathogenic B cells it was hypothesised that this would induce effects on the T cell repertoire that would skew the MOG-specific response from an encephalitogenic "pro-inflammatory" T_H1/T_H17 response to a regulatory phenotype, including the expansion of the regulatory $FoxP3^+$ T cell population (Weber et al., 2010, Hamel et al., 2011). Paradoxically, although MOG-ETA' significantly reduced disease severity there was no evidence of a potentially compensatory expansion of the regulatory T cell repertoire. Furthermore the *ex vivo* experiments using cells isolated from the peripheral lymph nodes showed an increase in MOG-specific proliferation. Analysing the supernatants from these experiments showed that the production of cytokines and chemokines were indicative of a T_H1/T_H17 response, including IFNγ, TNFα and IL-17.

In association with the increase in MOG-specific cellular activity there was also a significant increase in MOG-specific autoantibodies found in the blood. Not only was there a greater anti-MOG titre it was also discovered there was a higher frequency of demyelinating autoantibodies present in the MOG-ETA' treated mice. It was not enumerated whether it was the MOG-specific antibodies responsible for the demyelination but future experiments would first remove the anti-MOG antibodies before treating the cultures to elucidate this. These results clearly demonstrated that the MOG-ETA' was not eliminating all MOG-specific B cells, suggesting the treatment regime used in this study was not sufficient. In addition

the increased MOG-specific antibodies could be binding to the immunotoxin and preventing it from binding and killing target B cells. However, it was previously shown that the immunotoxins effects were not compromised by circulating anti-MOG immunoglobulin in an experiment where MOG-specific B cells were still eliminated, even when the cells were incubated with MOG-ETA' plus MOG-specific antibodies (Nachreiner et al., 2008). Another reason could be, because plasma B cells no longer present immunoglobulin on their cell surface they would be unlikely to uptake the immunotoxin. This is a similar problem to that faced by Rituximab as long-lived plasma cells, which are $CD20^-$, remain after treatment (Warde, 2010, Bluml et al., 2013, Pieper et al., 2013).

What is still unclear is that since MOG-ETA' treatment induces a MOG-specific T_H1/T_H17 response in peripheral immune organs and an increased anti-MOG titre with demyelinating activity, then why does this not induce more severe EAE than Bo9-ETA'? It is possible that the decrease of immune cellular infiltration into the CNS could mean that there has been little inflammation or disruption of the BBB, therefore even though there is a higher titre of MOG-specific antibodies, they do not access the CNS to potentiate disease. In addition to this it is known that even in disease only a small percentage of antibodies pass into the CNS, therefore calling into question the usefulness of blood borne antibodies as disease markers and indicating that future experiments should investigate anti-MOG antibody levels in the CNS.

As disease reduction cannot be entirely due to B cell depletion, another explanation could be the introduction of excessive soluble MOG antigen, as a result of the degradation of MOG-ETA', which may be inducing tolerance. Excessive, inappropriate antigen-specific stimulation of MOG-reactive T cells could induce T cell anergy or activation-induced cell death (AICD) (Critchfield et al., 1994, Racke et al., 1996). This would reduce circulating numbers of effector cells, thereby decreasing inflammation in the CNS and limiting access of autoantibodies.

Soluble peptide and protein-induced tolerance as a prospective treatment of MS is already an active area of research with many successful EAE studies and patient clinical trials (Lutterotti et al., 2008).

MOG-ETA' treatment obviously had a protective effect but its efficacy needs to be improved. Rapid clearance of the immunotoxin could be limiting its ability *in vivo* due to a small active time frame or quick degradation of the immunotoxin as ETA-based immunotoxins typically have a 0.5-3 hour half-life. Future experiments would assess using higher doses and shorter time intervals to counteract MOG-ETA's short half-life, as more regular treatments may increase its efficacy. However, this would need to be balanced with increased risks of non-specific toxicities like hepatotoxicity and vascular leak syndrome (VLS) and the development of neutralising antibodies (Choudhary et al., 2011). Unfortunately, regardless of altering the MOG-ETA' treatment regime, more antigen would still be being introduced into the system.

There have been previous studies of immunotoxins in MBP-induced EAE in C57BL/6 mice. This group administered immunotoxin using cationic liposome-embedded encoded plasmids, therefore leading to *in vivo* expression. Both studies targeted and eliminated activated T cells through their chemokine receptors CCR5 and CXCR3 using diphtheria toxin conjugated to CCL5 or IP-10, respectively (Jia et al., 2006, Chen et al., 2007). These immunotoxins both delayed onset of disease and reduced EAE burden. They further showed a decrease in $CD3^+$ T cells infiltrating into the CNS, which are similar to results observed with MOG-ETA' treatment. In contrast, their immunotoxin also reduced peripheral inflammation as IFNγ serum levels were decreased but interestingly also appeared to stimulate a more protective T_H2 response indicated by increased levels of IL-4. These studies suggest that a future consideration would be to use a MOG_{79-96}-ETA immunotoxin (the encephalitogenic T cell epitope for DBA/1j mice), since perhaps targeting MOG reactive T cells would provide increased disease suppression without the

adverse peripheral anti-MOG response. In addition to this, implicating their method of immunotoxin delivery may also offer better disease protection as this strategy showed immunotoxin still present up to 72 hours after administration. Therefore, there would be more MOG-ETA' available and less i.p. injections necessary, which are very stressful for the mice; this would also be an advantage when treating MS patients. Other groups have had success suppressing EAE with MBP fused to GM-CSF, therefore targeting and presenting MBP in a tolerogenic manner to antigen-presenting cells (APCs) (Abbott et al., 2011, Blanchfield and Mannie, 2010). Therefore, considering effective targeting of the MOG-ETA' would be worthwhile as the immunotoxin has a short half-life so little time to encounter and deplete target cells.

In summary this novel MOG-specific therapeutic strategy warrants further research as data shows clinical benefits of the treatment, although these are compromised by some drawbacks. However, this antigen-specific approach is the future of autoimmune treatments as broad immune suppression is not sustainable for long term use in many patients. The challenge now, in complex heterogeneous diseases like MS, will be finding the right antigens to target.

CHAPTER SIX

SOLUBLE MOG_{1-125} ANTIGEN-SPECIFIC THERAPY: A STRATEGY TO TREAT MULTIPLE SCLEROSIS

6 SOLUBLE MOG_{1-125} ANTIGEN-SPECIFIC THERAPY: A STRATEGY TO TREAT MULTIPLE SCLEROSIS

6.1 INTRODUCTION

The current therapeutic approaches in MS are based on broad immunosuppression, which can be effective but have the disadvantage of adverse effects. The previous chapter presented new findings using MOG-ETA' as an antigen-specific immunotherapy. The treatment reduced disease burden but was associated with a paradoxical increase in MOG-specific autoreactivity. In addition it was clear that immunotoxin mediated B cell depletion could not fully account for the disease suppression as there was evidence of increased anti-MOG autoantibody titres. This chapter investigated whether the MOG component of the MOG-ETA' treatment would be sufficient alone to reduce EAE severity via antigen-specific tolerising mechanisms, without incurring other adverse peripheral MOG reactivity.

The use of antigen-specific tolerance-based therapies have been shown to suppress disease activity in multiple disease models, including non-obese diabetic (NOD) mice, collagen-induced arthritis (CIA) and EAE (Miller et al., 2007). In EAE a range of different antigen-specific strategies have been shown to inhibit or suppress the development of EAE: -

i. *Soluble studies.* The first pioneering studies were performed by Levine et al. (1968). EAE was prevented by the i.v. infusion of MBP when given either before or just after the transfer of MBP-specific T cells. More recent studies have also confirmed EAE suppression after i.v. administration of MBP (Ishigami et al., 1998, Odoardi et al., 2007). Soluble peptide treatment has also been investigated. These studies revealed EAE progression could be prevented when an i.p. injection of immunodominant MBP peptides was given 10 days after EAE induction (Gaur et al., 1992). As there is no

dominant antigen in MS and antibody specificity is heterogeneous a research group designed a "multipleantigen/multiepitope" protein containing MBP, PLP and MOG for treatment of EAE (Zhong et al., 2002). This successfully suppressed PLP-induced EAE but also complex EAE induced by T cells reactive against MBP, PLP and MOG. In more recent studies using a similar multiple-epitope method, it was shown to induce effective, long lasting immune suppression through tolerogenic mechanisms including $FoxP3^{+}CTLA4^{+}$ T_{Reg} cells and immune deviation from T_H1/T_H17 to a T_H2 cell response, evident by secretion of IL-4 and IL-10 (Kaushansky et al., 2011).

ii. *Oral studies.* Oral administration of MBP in EAE studies using Lewis rats showed that the treatment could inhibit or stop the development of EAE and suppress *in vitro* lymphocyte proliferation (Bitar and Whitacre, 1988). Subsequent studies investigating the immunosuppression mechanisms showed evidence of clonal anergy having an importance in oral tolerance (Jewell et al., 1998, Whitacre et al., 1991).

iii. *DNA-vaccination.* Tolerance-based antigen-specific therapies have also used naked DNA as a method of local gene delivery. A study injecting DNA encoding $PLP_{139-151}$ into SJL/J mice suppressed EAE and reduced $PLP_{139-151}$-specific proliferation and cytokine secretion, most likely due to T cell anergy (Ruiz et al., 1999). Further attempts have investigated co-vaccination of $PLP_{139-151}$ DNA in conjunction with *IL-4* DNA which suppressed disease by inducing a T_H2 response (Garren et al., 2001).

iv. *Antigen coupled to APCs.* Other attempts at an antigen specific therapy coupled MBP with monocyte rich peripheral blood myeloid cells using a chemical fixative, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI), which when incubated with T cells specifically tolerised T_H1 cells inducing anergy and apoptosis but did not have this effect on T_H2 cells (Vandenbark et al., 2000).

The mechanisms involved in disease suppression due to antigen-specific therapies have been studied in depth but are still not clearly defined. Critchfield et al. (1994) were the first to discover that high doses of protein antigen drove antigen-specific T cells into activation-induced cell death (AICD). They showed autoreactive T cell depletion by soluble protein *in vitro* and *in vivo*. AICD was shown to be due to IL-2-induced cell cycling in parallel with T cell receptor restimulation with high doses of antigen. Another study using a similar approach determined that AICD of autoreactive T cells was in the target organ, i.e. the CNS specifically, and did not occur in the peripheral organs (Ishigami et al., 1998). MBP treatment increased the number of Fas^+ and $FasL^+$ apoptotic T cells in the CNS, suggesting apoptosis is Fas-mediated.

Subsequent investigations into the mechanisms behind soluble protein-induced disease suppression used live-video and two-photon *in situ* imaging in Lewis rats which were intravenously (i.v.) injected with MBP (Odoardi et al., 2007). Using $T_{MBP\text{-}GFP}$ cells they showed that the response to MBP treatment occurred incredibly quickly. Within 10 minutes there was deceleration in T cell migration, which then formed clusters around splenic APCs. This response was dependent on antigen recognition as the behavioural changes could be blocked with anti-MHC class II molecules and needed the relevant antigen. It was also not just specific to MBP as the same results were seen with MOG- and OVA-specific GFP-tagged T cells. This data showed that soluble protein treatment could inhibit the re-circulation of antigen-specific T cells and induce AICD.

Investigations into the cytokine response after soluble protein treatment have been contradictory. An earlier study elucidated that there was an increase in gene expression of pro-inflammatory cytokines IFNγ and TNFα and inducible nitric oxide synthase (iNOS) one hour after treatment (Weishaupt et al., 2000), although there were no changes detected in IL-10 levels. In contrast, a recent study showed the opposite, with immune suppression being associated with an increase in T_H2

cytokines IL-4 and IL-10 and an enlarged $FoxP3^{+}CTLA4^{+}$ T_{Reg} population (Kaushansky et al., 2011). Taking this data together, it appears that immune suppression is mediated through rapid changes in T cell behaviour, T cell AICD and immune deviation to a regulatory response.

These antigen specific therapies have been tested in patients with MS but with less success than in EAE. Early attempts at using whole soluble human MBP as an immunotherapy in MS patient trials had opposing results. On one hand a study with 64 patients in a double-blind trial treated candidates with 5 mg protein once a week for 30 months generating positive results; where some patients had a feeling of well-being and others better bladder control or decreased fatigue (Campbell et al., 1973). On the-other-hand a very similar study failed to observe any clinical effects of soluble MBP treatment (Gonsette et al., 1977).

Later clinical studies were carried out in which patients were treated i.v. with 500mg of synthetic MBP peptide, equivalent to amino residues 82-98, which is the immunodominant binding epitopes for B and T cells in patients with the HLA haplotype DR2 (Warren et al., 2006). This was trialled in a 2-year double-blind placebo-controlled study in 32 patients with PPMS, which included a 5-year follow-up treatment period. This treatment significantly reduced disease progression in patients with HLA haplotypes DR2 and/or DR4 and also decreased CSF anti-MBP autoantibodies independent of their HLA-DR haplotype.

DNA vaccinations have also been tested in MS patient studies. This randomised, double-blind, placebo-controlled trial with a plasmid encoding full length human MBP was the first DNA vaccine used to treat MS in humans (Bar-Or et al., 2007). The treatment was successfully tolerated with no adverse effects in the patients. Results showed that lesion activity was reduced, although this was not significant. In addition analysis of the blood and CSF showed a reduction in MBP-specific immune responses. Most recent clinical trials induced immune tolerance using MS patients' blood cells coupled with seven myelin peptides derived from MOG, MBP

and PLP (Lutterotti et al., 2013). This was a small phase I trial with only 9 patients to establish feasibility and safety. The treatment was well tolerated and patients on the higher dose of treatment showed a reduction in antigen-specific T cell responses, establishing this as a potential future therapeutic.

Another recent clinical trial had success also using a mixture of 1 mg MOG_{35-55}, MBP_{85-99} and $PLP_{139-151}$. However, this treatment was administered transdermally via a skin patch (Walczak et al., 2013). The outcome of this small trial of 16 treated patients and 10 placebos showed that treatment ameliorated MS. This was concluded from a 66.5% reduction in gadolinium-enhanced lesions and a significantly lower annual relapse rate. This treatment was tolerated well, therefore presenting a new antigen-targeted, non-invasive therapy.

There are drawbacks with the antigen-specific approach, primarily in identifying the immunodominant antigen targeted in MS autoimmune pathogenesis. Some clinical studies have been problematic due to toxicity. A phase II clinical trial tested an MBP altered peptide ligand (APL), CGP77116, which was administered s.c. (Bielekova et al., 2000). Of the 24 patients enrolled only one completed the study due to a range of reasons, namely, exacerbations of MS and allergic shock responses. Hypersensivity reactions were also seen in a similar phase II trial (Kappos et al., 2000). Another difficulty, evident from EAE studies, is epitope spreading that occurs with T and B cells (Vanderlugt and Miller, 2002, Robinson et al., 2003). However, research taken together present antigen-specific therapies as an effective mode of immune suppression in EAE models and having a range of success in treating patients thus far, with increasing efficacy as technology and knowledge have advanced.

Previous soluble protein therapeutic studies have focused on the T cell arm of the immune response. However, MOG is a special case as it stimulates a pathogenic autoreactive T cell and autoantibody response. In the previous chapter MOG-ETA' was able to reduce disease severity. A question raised was whether this reduction

in disease was in fact due to induction of MOG-specific tolerance instead of depletion of cells via the immunotoxin moiety. Thus, the aim of this chapter was to investigate the effects of low doses of soluble MOG protein alone using the same protocol used in the MOG-ETA' studies. This was in the expectation of reducing disease by inducing MOG-specific tolerance response.

6.2 RESULTS

6.2.1 Low dose soluble MOG_{1-125} treatment significantly reduced disease severity in MOG-induced EAE

To investigate the hypothesis that soluble MOG treatment can induce similar clinical results as with MOG-ETA' treatment, the same EAE model was adopted as used in Chapter 5; where mice were immunised with 100 µL complete Freund's adjuvant containing 50 µg/mL MOG_{1-125} and 150 µg heat killed *M. tuberculosis*. The mice were then treated 2 d.p.i. and every 48 hours after with 25 µg MOG_{1-125} i.p. or PBS as a control. Again, due to the severity of this MOG-induced EAE model the experiment was terminated at 10 days post immunisation to ensure a large enough group of mice for analysis at the end of the experiment. Despite this the MOG_{1-125} treatment significantly decreased the mean clinical score on day 9 and 10 (Figure 6.1A). Soluble MOG_{1-125} treatment also significantly reduced the mean cumulative disease score, and the total cumulative score was approximately halved (PBS = 48 ± 1.65; MOG_{1-125} = 27 ± 0.75) (Figure 6.1B). Therefore, indicating treatment with soluble MOG clearly reduced disease severity despite these mice having a severe model of EAE, which incorporates both the T and B cell arms of the immune system. MOG-ETA' also decreased disease severity illustrating the fact that both treatments had a similar effect, although soluble MOG was slightly more effective than MOG-ETA' as significant suppression was seen from 9 d.p.i. whereas in experiments using MOG-ETA' the effects was seen from 10 d.p.i.

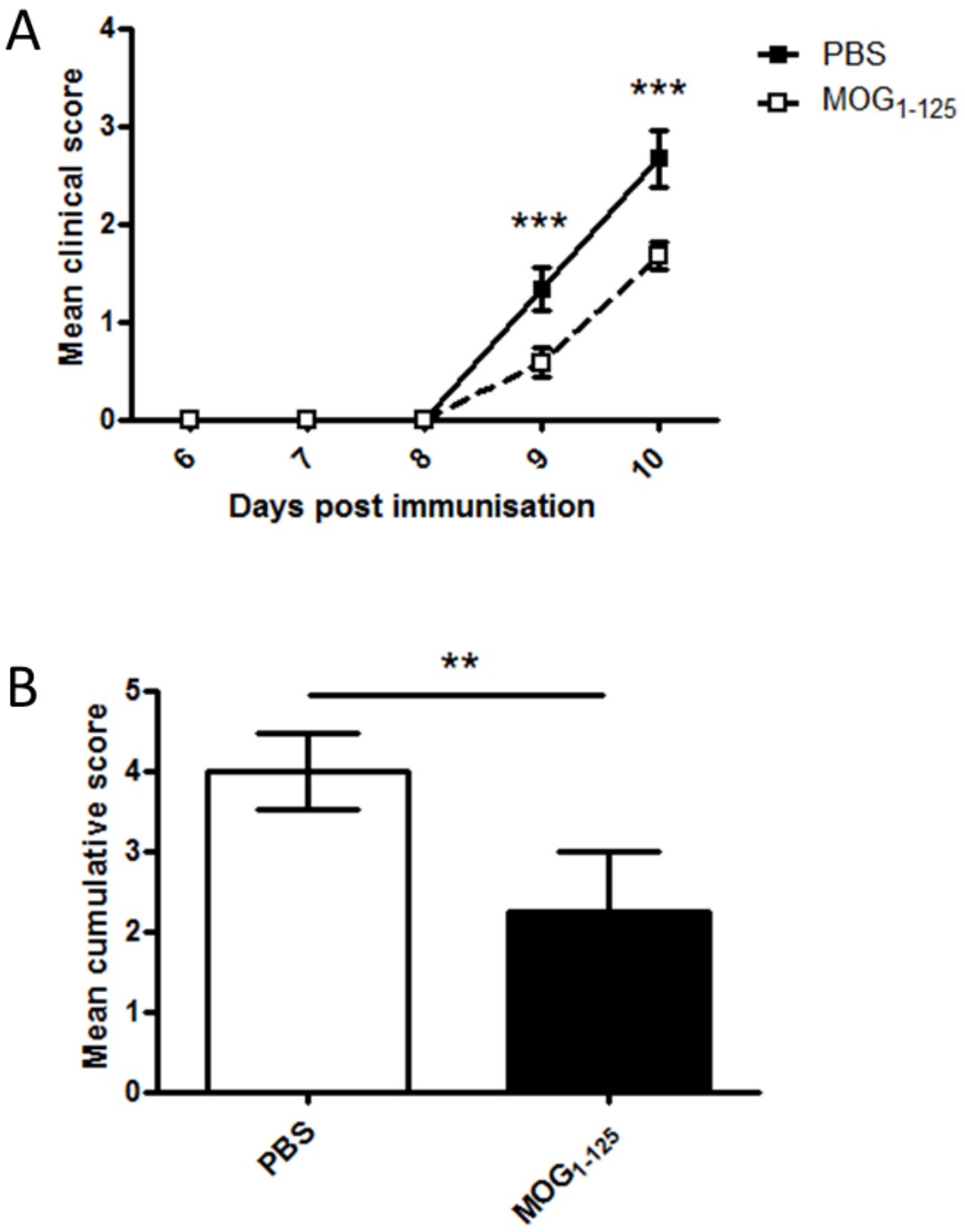


Figure 6.1. Soluble MOG_{1-125} treatment decreases EAE disease activity
EAE was induced in 7-8 week old DBA/1j mice by s.c. immunisation with 100 µL complete Freund's adjuvant containing 50 µg MOG_{1-125} and 150 µg heat killed *M. tuberculosis*. Mice were treated i.p. with 25 µg MOG_{1-125} or PBS on day two and every 48 hours after. (A) Clinical data shows reduction of EAE severity with MOG_{1-125} treatment. Data points represent mean clinical scores ± SEM (n=12). Data is representative of three individual repeats. Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. ***, $p < 0.001$. (B) Mean cumulative score was calculated from the addition of daily clinical scores of all mice (n=12). Bar graph represents mean cumulative score from three biological repeats. Significance was determined by a two-tailed students t test. **, $p < 0.01$.

6.2.2 Treatment reduced immune cellular composition in the CNS

To elucidate the immune mechanisms that might be contributing to disease suppression the cellular infiltrate present in the spinal cord was analysed. Flow cytometry was used to investigate if this was affected by the immune suppression presented in Figure 6.1. Results showed that treatment was associated with a global reduction of inflammatory cells in the spinal cord. Specifically, $CD4^+$ T cells, B cells, and $FoxP3^+$ T regulatory cells were all significantly reduced (Figure 6.2A). In addition to lymphocytes, innate immune cells monocyte/macrophages were also significantly reduced (Figure 6.2B). Neutrophils were decreased but this did not reach significance. These data suggest that treatment with soluble MOG_{1-125} is associated with an overall decrease in cellular infiltrate into the CNS, suggesting that the treatment is somehow dampening the immune response, potentially via tolerogenic mechanisms. Again, MOG-ETA' treatment had similar effects on immune cell recruitment but it was not as pronounced as results observed with soluble MOG treatment.

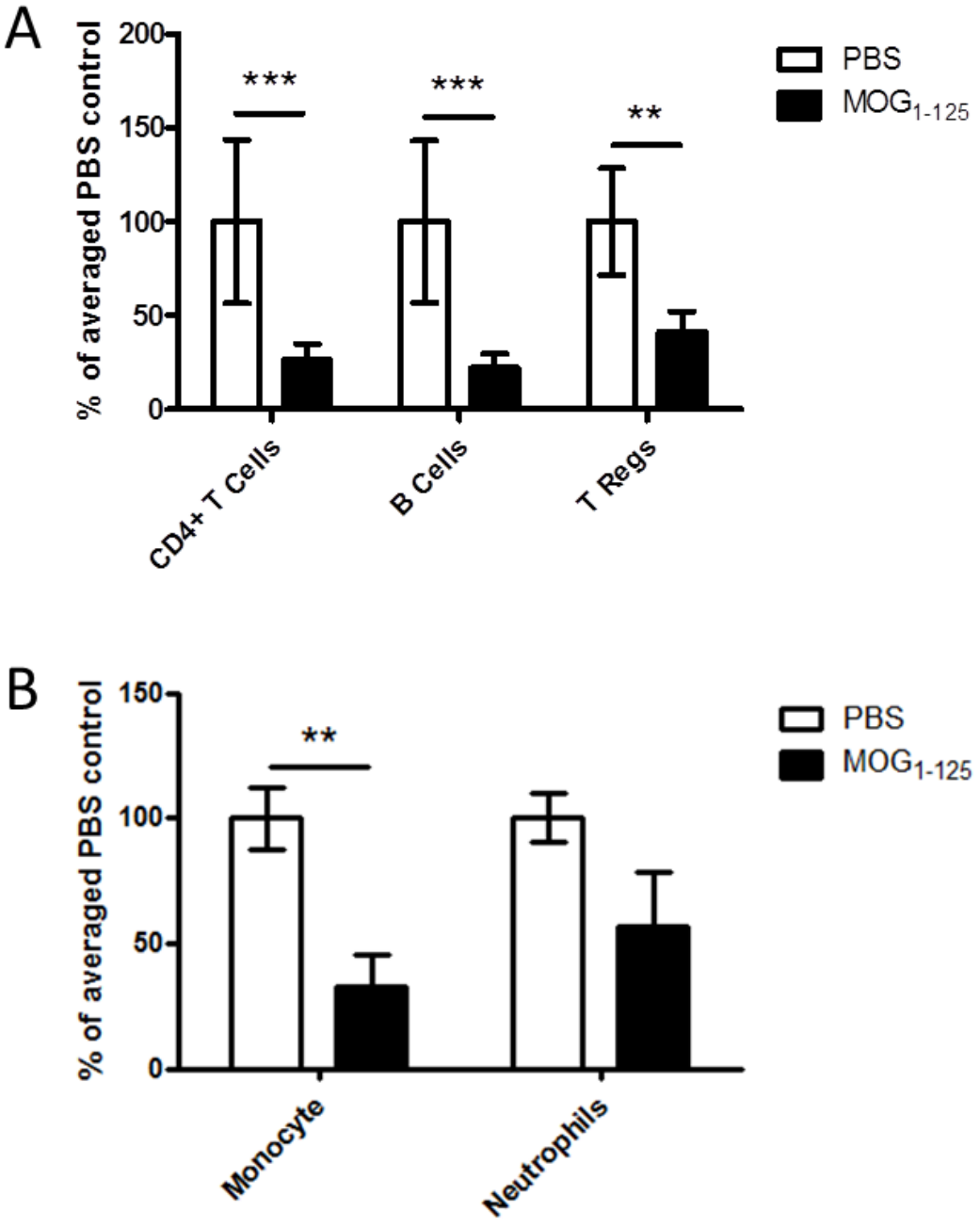


Figure 6.2. Spinal cord immune cell infiltration is decreased in MOG_{1-125} treated mice.
Spinal cord single cell suspensions were pooled from 4 mice 10 days after MOG_{1-125} immunisation. Cell suspensions were pre-gated on live, $CD45^+$, single cells. (A) Results show a significant decrease in the number of cells to have infiltrated into the spinal cord after MOG_{1-125} treatment. Flow cytometry was used to identify and analyse $CD4^+$ T cell populations, which were stained with CD4 and CD3; regulatory T cells were stained with FoxP3 and CD25, which were pre-gated on $CD4^+$ T cells; B cells were stained with B220, which were pre-gated on $CD5^-$ cells. Bar graph indicates mean (±SD) % of averaged PBS control (n=9). (B) Spinal cord was also stained with CD45, CD11b, Ly6C and Ly6G to gate on $Ly6C^+$ monocytes and $Ly6G^+$ neutrophils. Bar graph indicates mean (±SEM) % of averaged PBS control (n=9). Both graphs show results from three biological repeats performed in triplicate. Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. **, $p < 0.01$, ***, $p < 0.001$.

6.2.3 Soluble MOG treatment associated with an increase in $CD1d^{high}CD5^{+}$ regulatory B cells

Despite this overall reduction of immune cells in the CNS an enrichment of $CD1d^{high}CD5^{+}$ cells was detected, which are markers for B regulatory cells (Figure 6.3) (Yanaba et al., 2008). To definitively confirm these cells as B regulatory cells future experiments would need to show that they were able to produce IL-10 (Bouaziz et al., 2008). The frequency of these $CD1d^{high}CD5^{+}$ B cells was also investigated in the peripheral draining lymph nodes and spleen. This showed that this population was specifically enlarged in the spinal cord and not in the periphery, suggesting there was selective enrichment in the CNS (Figure 6.4). Since the global B cell population was depleted, this regulatory population represented approximately 10% of the remaining B cells. This indicated that MOG_{1-125} treatment may be inducing an enlargement of this regulatory B cell population, which may play a role in the suppression of EAE through the secretion of IL-10 (Yanaba et al., 2009). Effects on this regulatory population were not observed with MOG-ETA' treatment, so it appears to be specific to the soluble MOG infusion.

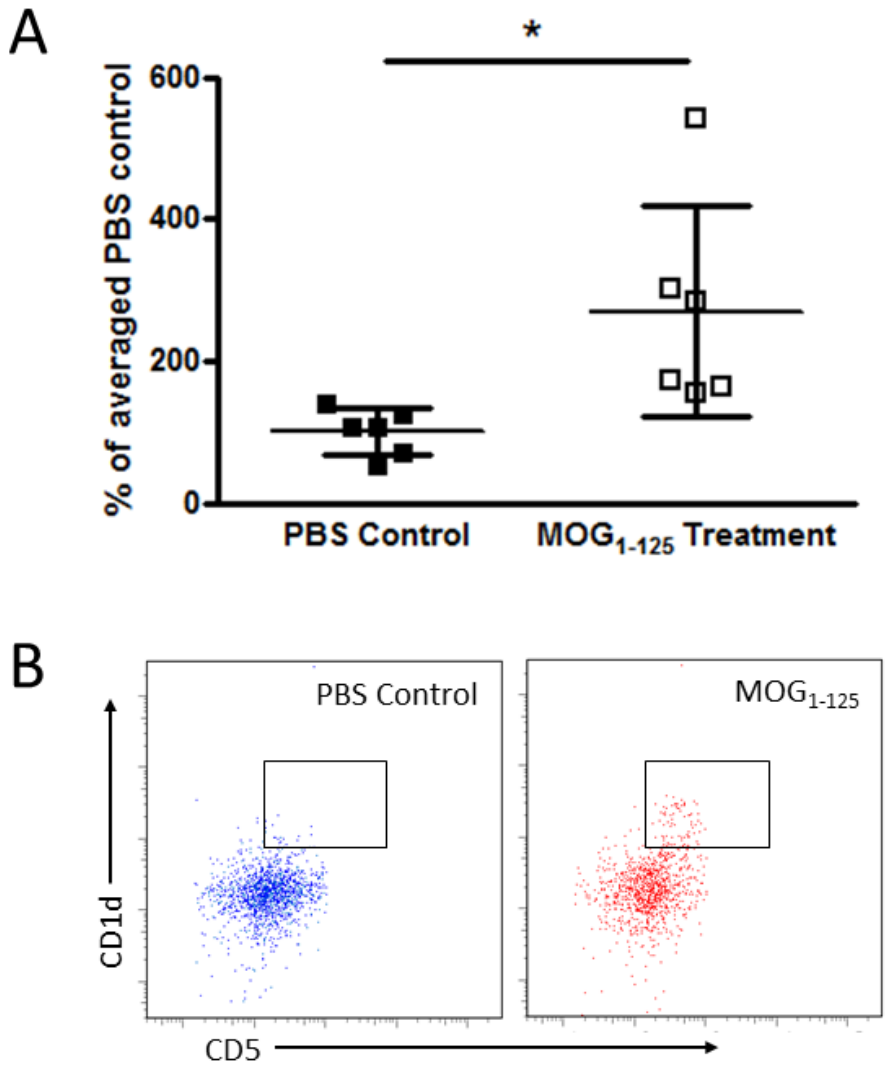


Figure 6.3. Soluble MOG_{1-125} treatment increases the frequency of $CD1d^{high}CD5^{+}$ B Cells.
Spinal cord single cell suspensions were pooled from 4 mice 10 days after MOG_{1-125} immunisation. Cell suspensions were pre-gated on live $CD45^{+}$ singlets. Cells were stained CD1d, CD5, and B220 expression. (A) Scatter plot indicates mean (±SD) % of averaged PBS control of $CD1d^{high}CD5^{+}$ B cells (n=6). Results of two biological repeats performed in triplicate. (B) FACS plots show representative result of total numbers of $CD1d^{high}CD5^{+}$ regulatory B cells, highlighted within the indicated gates, which were pre-gated on $B220^{+}$ B cells.

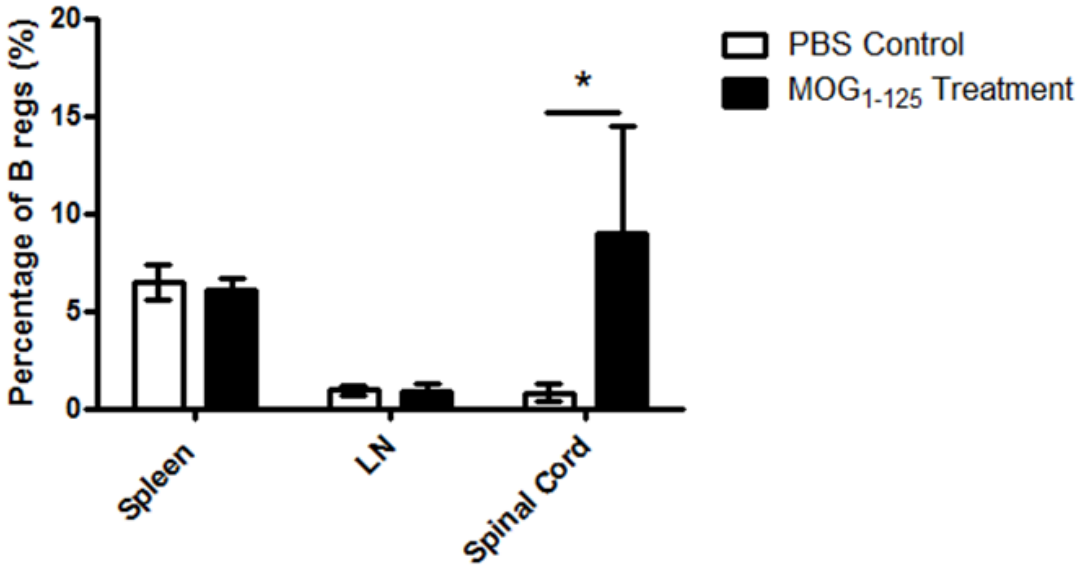


Figure 6.4. Regulatory $CD1d^{high}CD5^{+}$ B Cells are selectively enriched in the spinal cord. Experiment was performed as described in Figure 6.3. LN single cell suspensions were pooled from 4 LNs per mouse. Cell suspensions were pre-gated on live $CD45^{+}$ single cells. Cells were stained CD1d, CD5, and B220. Results show that the frequency of regulatory B cells was significantly increased with MOG_{1-125} treatment but only in the spinal cord. Bar graph shows frequency of $CD1d^{high}CD5^{+}$ cells within the $B220^{+}$ B cell population. Values representative of two biological repeats, plotted as mean ± SD (n=3).

6.2.4 MOG_{1-125} treatment associated with a reduced MOG-specific cytokine response

To assess if the immune suppression was associated with a tolerised peripheral immune anti-MOG response, the MOG-specific chemokine and cytokine response was analysed using cells isolated from the subinguinal and para-aortic LNs. The supernatants from this recall experiment were evaluated using a semi-quantitative mouse proteome array, which could rapidly detect 40 different cytokines and chemokines, detailed in Figure 6.5A. Observing the blots by eye showed that there were more cytokines and chemokines being secreted from cells isolated from the PBS treated mice after re-stimulation with MOG_{1-125} (Figure 6.5B). The values calculated from the proteome arrays using TotalLab reflected the initial observations made by eye (Table 6.1). There was an increase in proinflammatory chemokines and cytokines being produced by the re-stimulated cells from the PBS treated mice, including CXCL9, CXCL10 and IL-17, IFNγ, TNFα. These are indicative of a T_H1/T_H17 immune response.

A small number of products in the supernatant were increased in the MOG_{1-125} treated group after MOG-specific stimulation; IL-1ra, CCL5 and CXCL13. IL-1ra was approximately doubled in the MOG_{1-125} treated mice (PBS = 15,946.66; MOG_{1-125} = 30,542.96). IL-1ra is a natural antagonist to the pro-inflammatory cytokines IL-1α and β, therefore complementing the rest of the cytokine array data. CCL5 and CXCL13 function as a promiscuous pro-inflammatory chemokine and for B cell recruitment to follicles, respectively. Despite these two pro-inflammatory chemokines the predominant MOG-specific response from the soluble MOG treated mouse group was anti-inflammatory/suppressed.

Taken together, these results suggest that treatment with soluble MOG_{1-125} tolerises the peripheral immune cells against MOG. Unfortunately there was no proliferation data available for the soluble MOG treatment due to technical issues. Overall, this peripheral cellular response correlates with the literature published

on soluble protein treatment. This result opposes the data retrieved from the MOG-ETA' experiments, which induced an increased MOG-reactive cellular response. This dichotomy suggests that there are different mechanisms responsible for the suppression of EAE with these separate treatments.

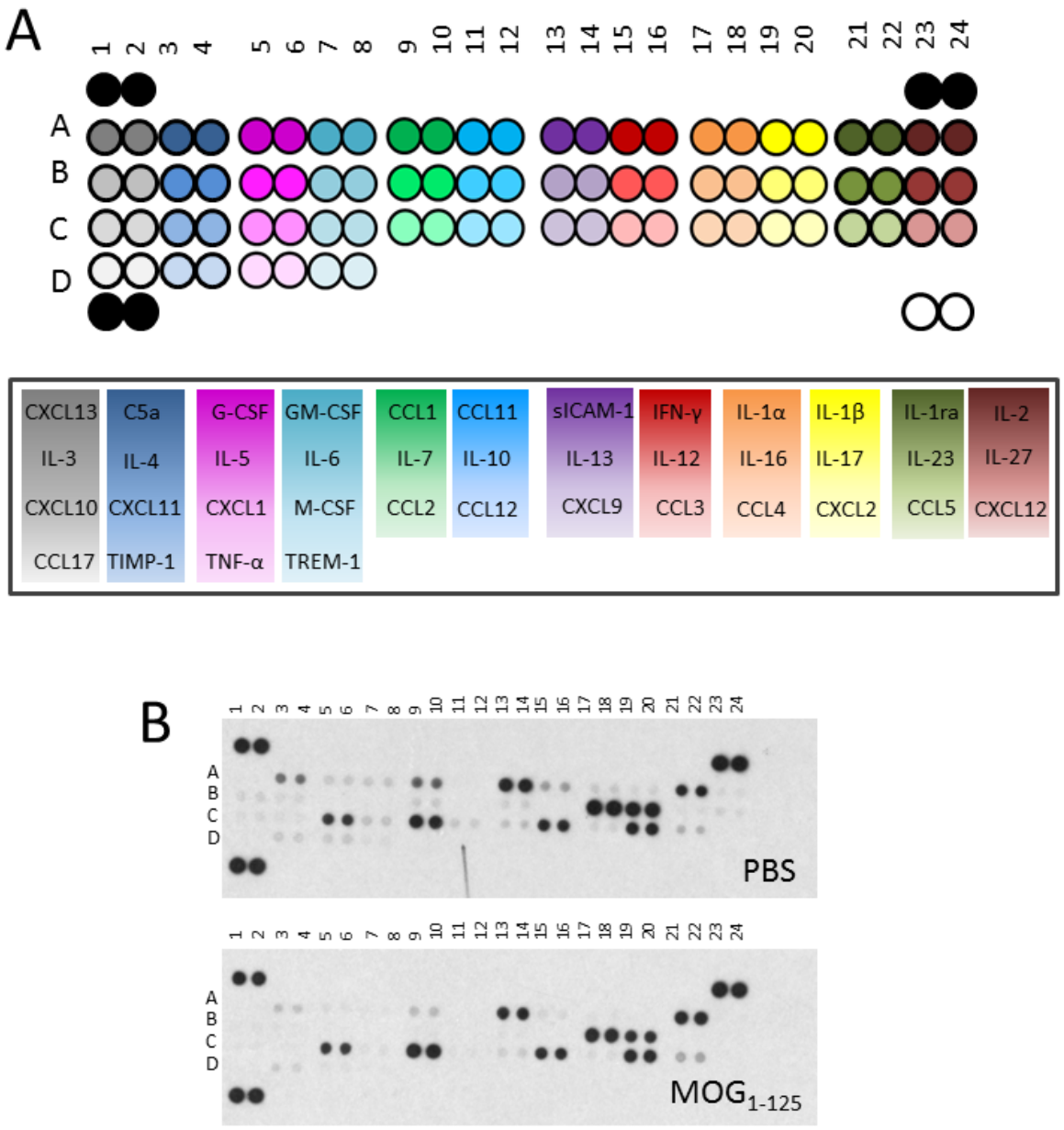


Figure 6.5. Cytokine and chemokine production from cells isolated from lymph nodes of PBS and MOG_{1-125} treated mice.
Draining lymph nodes were harvested and made into a single cell suspension 10 days after MOG_{1-125} immunisation and the recall response analysed. The LN cells pooled from each mouse were stimulated *ex vivo* with or without antigen (MOG_{1-125}) (n=4). Antigen-induced cytokine and chemokine production (i.e. MOG_{1-125} minus medium alone) was measured from pooled supernatants of 4 mice from each treatment group and profile evaluated. Protein levels were measured using Mouse Cytokine Arrays as per manufacture instruction. (A) Mouse cytokine array coordinates are displayed above with corresponding cytokines and chemokines colour coded below. Black circles represent positive controls and clear circle represents the negative control. Not to scale. (B) Representative scans of proteome array.

Table 6.1. MOG_{1-125} treatment reduces the MOG-specific cytokine and chemokine response in cell extracted from the lymph nodes.
Supernatants were acquired and cytokine and chemokine levels measured as explained in Figure 6.5. Protein levels were measured using Mouse Cytokine Arrays as per manufacture instruction and results quantified using TotalLab. Ag-induced cytokine and chemokine production values (i.e. MOG_{1-125} minus medium alone) were expressed as mean values representative of 2 biological repeats.

Pro-inflammatory cytokine and chemokine secretion			
	MOG_{1-125} stimulated cells		
Chemokines	PBS	MOG_{1-125}	Fold change in PBS treated goup
CCL1	9243.94	3908.78	2.36
CCL3	29056.86	23955.80	1.21
CCL12	2872.16	386.75	7.43
CXCL9	3160.04	2607.53	1.21
CXCL10	1218.10	386.40	3.15
Cytokines			
G-CSF	3131.86	1343.47	2.33
GM-CSF	2299.06	1232.65	1.87
IL-3	1834.84	898.98	2.04
IL-7	2125.05	351.39	6.05
IL-13	1477.11	314.77	4.69
IL-17	36985.27	9045.90	4.09
IFNγ	7793.59	1411.21	5.52
TNFα	2890.96	12.36	233.94

6.2.5 MOG_{1-125} treatment led to an increased MOG-specific pathogenic antibody response

The MOG_{1-125} treatment appeared to be reducing EAE severity by reducing infiltration of immune cells into the CNS and decreasing anti-MOG cellular activity in the periphery. The next question was to observe if the treatment affected the MOG-specific antibody response. The results showed that disease reduction was accompanied by a significant increase in anti-MOG antibody serum titres, which was detected in mice sera harvested 10 days post immunisation by ELISA (Figure 6.6). Unfortunately MOG-specific binding to native protein expressed on transfected LTK cells was not tested due to technical difficulties.

To clarify if these antibodies were pathogenic their functional role was investigated using the *in vitro* myelinating culture system described in previous chapters. Mice sera were harvested from mice 10 d.p.i. and incubated with the myelinating cultures (28 DIV) in a series dilution for 16 hours with 2% rat serum as a source of exogenous complement. Sera from the soluble MOG_{1-125} treated mice induced greater myelin loss compared to the PBS treated control mice group. This difference was significant at the lower dilutions of 1/100 and 1/500 (1/100, $p < 0.001$; 1/500, $p < 0.05$). These results revealed that despite soluble MOG treatment significantly reducing clinical disease and suppressing the antigen-specific response in peripheral immune cells, there was an increase in MOG-specific antibody titres and pathogenic autoantibodies found in the blood.

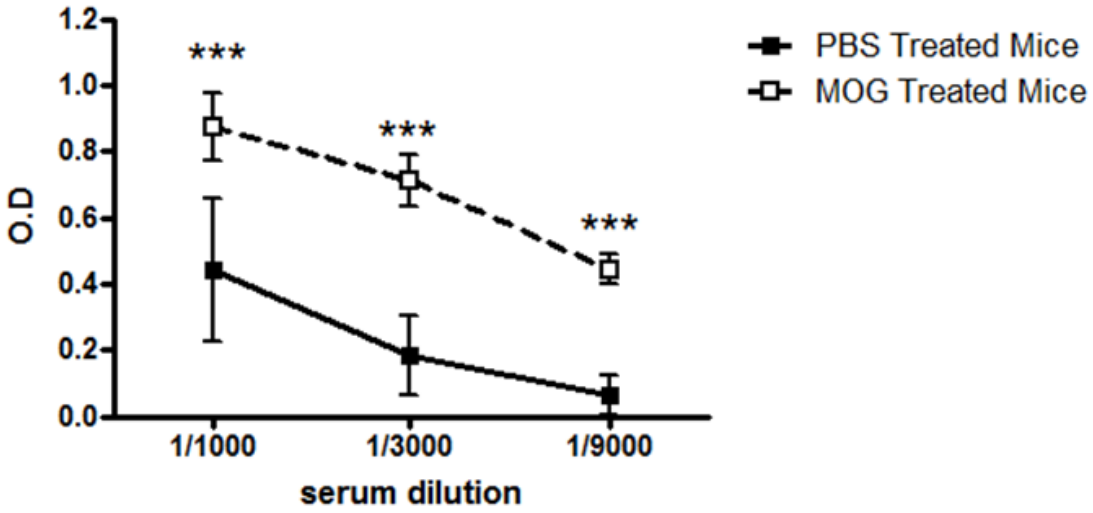


Figure 6.6. Anti-MOG reactivity was increased in serum after MOG_{1-125} treatment.
Sera from mice 10 days post immunisation were analysed (n=12). Serum levels of anti-MOG mouse IgG was measured by ELISA. Results are from 2 individual repeats with each mouse tested in triplicate. Data points represent mean (± SEM). Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. ***, $p < 0.001$.

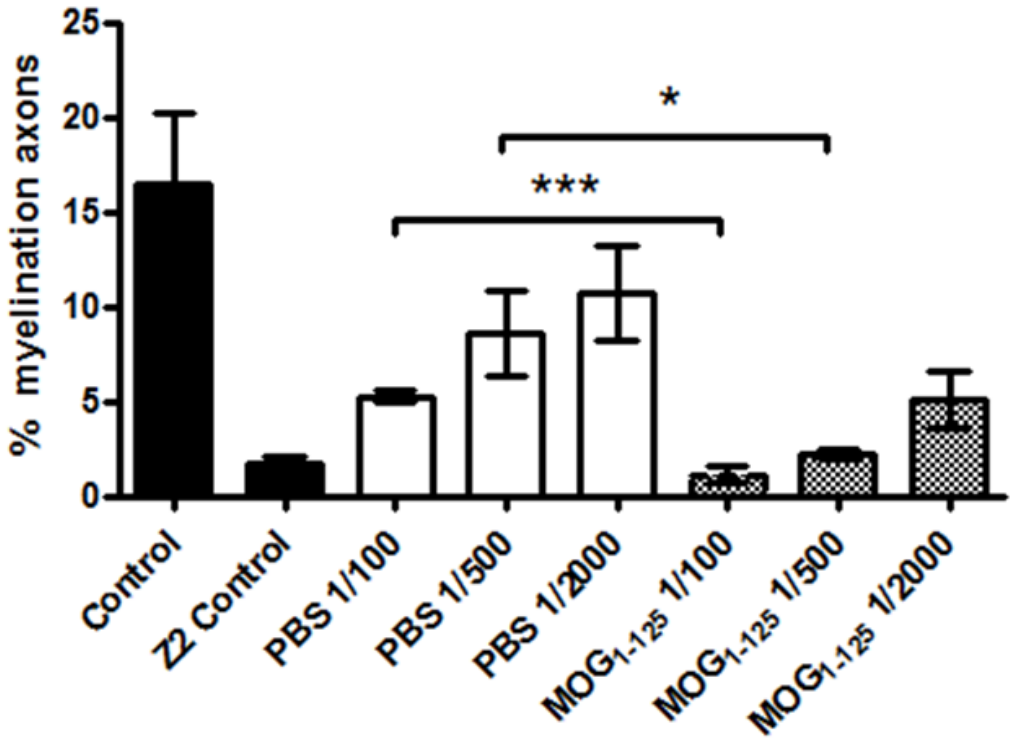


Figure 6.7. MOG_{1-125} treatment led to increased serum demyelinating activity.
Sera from mice 10 days post immunisation were analysed. Myelinating cultures (28 DIV) were incubated for 16 hours with pooled mice sera (n=6) from the PBS and MOG treated groups. Sera were used in a series dilution plus 2% rat serum. Experiment was done in triplicate, results representative of 2 biological repeats. Data points represent mean (± SD). Significance was determined by a student's two-tailed t test. *, $p < 0.05$, ***, $p < 0.001$.

6.3 DISCUSSION

Antigen-specific therapy is an important area of research in MS, as there are many adverse effects associated with long-term immune suppression, including currently unknown risks for young children treated with these DMTs. A promising therapeutic approach is the application of soluble antigen, which induces a tolergenic response. Early studies had limited effects in patients despite promising EAE studies (Campbell et al., 1973, Gonsette et al., 1977), but there has been great advances in knowledge and technology available and current studies using multi-epitopes-coupled to patients' red blood cells are now showing promising results in early MS patient clinical trials (Lutterotti et al., 2013).

In this study the effects of low doses of soluble MOG_{1-125} treatment in MOG-induced EAE in DBA1/j mice was investigated. The results showed MOG was able to significantly suppress pathology in this complex, severe EAE murine model. Disease reduction was associated with decreased immune cell populations located in the spinal cord after 10 days, which was associated with global reduction in antigen-specific responses in the periphery. However, despite this there was an increased titre of MOG-specific antibodies, which when tested on the myelinating cultures were pathogenic.

The majority of previous animal studies have treated EAE with MBP or PLP, predominantly using peptides and not the whole protein. Whole MOG protein has not been investigated in DBA/1j mice before but MOG peptide has previously been tested to investigate if it had any suppressive effects on EAE. It has been shown that disease can be reduced when MOG_{41-60} peptide is injected i.v. into MOG-induced EAE in (PL/J X SJL)F_1 mice (Leadbetter et al., 1998). Significant disease suppression using the same murine model was also observed with intravenous injections of another MOG peptide, MOG_{91-110} (Devaux et al., 1997). In this study treatment was stopped at day 15 but the therapeutic effect continued for 25 days, at which point the experiment was terminated, suggesting that the treatment

offered prolonged disease suppression. Both these MOG peptide studies were targeting T cells, as MS was perceived as predominantly a T cell mediated disease with little consideration of the B cells role in pathogenesis. The EAE study presented in this thesis was novel, as we treated DBA/1j mice with the whole extracellular domain of MOG. This EAE model was chosen as it incorporates both arms of the adaptive immune response unlike other models commonly used (Abdul-Majid et al., 2000, Svensson et al., 2002, Abdul-Majid et al., 2003). The use of whole MOG was with the intention of targeting B and T cells, although the purified MOG was unfolded so is therefore likely to have a greater effect of T cells.

Our clinical data (Figure 6.1) showed significant reduction of mean cumulative score and mean clinical scores from day 9 between the MOG_{1-125} and PBS treated mice. This suggests that the soluble MOG effect could be responsible for the disease suppression recorded with MOG-ETA' treatment. Although preliminary data in which mice were treated with 8.3 µg MOG (the exact amount of MOG_{1-125} contained in the MOG-ETA immunotoxin), it did not have any effects on disease (data not shown). To test if MOG alone was more effective than MOG-ETA' at suppressing disease a control experiment could be to synthesise a MOG-ETA' construct with a mutated, non-functional ETA' domain. This current study did not investigate the therapeutic effects of soluble MOG protein treatment as the disease severity was too great in the control group to extend the time course but this will be addressed in the future using a less severe animal model. This would have been interesting as another group, investigating the lasting therapeutic effects of MOG treatment in marmosets, showed that disease was actually exacerbated after treatment was ceased (Genain et al., 1996).

Our results investigating cellular composition of immune cells in the spinal cord demonstrated that there was a significant reduction in T cells, B cells and $FoxP3^+$ regulatory T cells compared to the PBS treated controls. Depletion of effector T cell data is in keeping with current literature on soluble protein treatment *in vitro* and

in vivo as it has been shown that soluble MBP treatment leads to apoptosis of autoreactive T cells (Critchfield et al., 1994, Ishigami et al., 1998). Another explanation that could explain the reduction of T cells detected in the spinal cord after MOG_{1-125} treatment was elucidated using live-video and two-photon *in situ* studies (Odoardi et al., 2007). This experiment showed rapid effects on T cell motility in response to soluble MBP treatment and that these cells were being sequestered in the spleen. Therefore presenting a range of mechanisms that could be involved in the decrease in the CNS of T cells. Interestingly, there was no expansion of $FoxP3^+$ regulatory T cells associated with disease reduction as these cells were found in reduced numbers in the active treatment mouse group, suggesting EAE amelioration was not due to regulatory T cell immune modulation. In addition to the lymphocytes, innate immune cells monocytes and neutrophils were also decreased in MOG_{1-125} treated mice. Monocyte/macrophages are found in great numbers in active MS lesions (Lucchinetti et al., 2000), and when depleted in EAE the mice have reduced disease severity (Huitinga et al., 1990, Huitinga et al., 1995), suggesting they have a major role in EAE.

This thesis study showed that one cell group was found to be selectively enriched in the treated group, namely $CD1d^{high}CD5^+$ "B regulatory cells", which indicated they may have played a role in reducing the disease burden. This enhancement specifically occurred in the spinal cord, therefore selective to where disease pathogenesis was occurring. Although B cell depleting drugs like Rituximab have been effective in treating autoimmune diseases like RA and MS (Caporali et al., 2009, Hauser et al., 2008), there is evidence to suggest that B cell populations can also have a regulatory phenotype. In mice genetically deficient in B cells it was shown that EAE disease severity was greater, suggesting a role in immune modulation (Wolf et al., 1996). It was observed in another study that anti-CD20 B cell depletion before induction of EAE actually increased disease severity (Matsushita et al., 2008). Taken together these studies and others have brought

about great interest and stimulated research into describing the "regulatory B cell".

Trying to fully characterise these cells is an on-going challenge and is still under debate (Gray and Gray, 2010). One important feature is that they suppress the immune response by producing IL-10, an anti-inflammatory cytokine that has a key role in EAE suppression (Ray et al., 2011). IL-$10^{-/-}$ mice have a higher susceptibility to EAE (Bettelli et al., 1998), whereas mice that over express it are resistant to disease (Cua et al., 1999). It will be possible to confirm if these cells are regulatory B cells by investigating their production of IL-10, since this is a feature that defines the cell type. This enlarged cell population in our experiments could be partly responsible for the EAE suppression observed as studies have shown that increased disease burden from B cell depletion was due to the reduced number of $CD1d^{high}CD5^{+}$ cells, and adoptively transferring these regulatory cells before disease induction normalised EAE (Matsushita et al., 2008). They also showed that these cells only appeared to have a suppressive effect at the beginning of the disease. As this study only analysed cellular infiltration at one time point (day 10) it would be interesting to investigate if they were still enriched in the CNS at later time points. A recent publication identified the importance of IL-35 secreting B cells in the suppression of the immune response, as well as IL-10 (Shen et al., 2014). In addition, they showed that plasma cells were the predominant B cell subset secreting these regulatory cytokines.

To investigate the peripheral immune response to MOG, after soluble antigen treatment, peripheral immune cells were re-stimulated with MOG_{1-125}. This showed that cells from the MOG_{1-125} treated mouse group secreted reduced amounts of pro-inflammatory cytokines and chemokines, therefore suggesting these cells may be tolerised against MOG. Results presented in this thesis showed that one of the cytokines found in larger quantities in PBS treated mice included IL-17, which has been identified in having a pro-inflammatory role in many

autoimmune diseases (Hu et al., 2011), including MS (Lock et al., 2002, Tzartos et al., 2008). Other hallmark T_H1 cytokines IFNγ and TNFα were also found increased in this group. Interestingly, IL-1ra was increased in MOG_{1-125} treated mice after antigen-specific stimulation. IL-1ra is a naturally occurring IL-1α and β antagonist; therefore, the increase in IL-1ra secretion may have played a role suppressing these pro-inflammatory cytokines. Animal studies showed treatment with recombinant IL-1ra lead to a milder EAE in DA rats (Badovinac et al., 1998). Also IL-1ra levels rose in response to IFNβ treatment *in vitro*, suggesting it could have a function in the disease suppression seen with this MS treatment (Sciacca et al., 2000, Nicoletti et al., 1996). In addition, treatment of RA patients with recombinant human IL-1ra moderately reduced disease burden (Furst, 2004).

This was different to the results seen with MOG-ETA', where treatment was associated with an increase in MOG-specific cellular activity, suggesting that immunosuppressive mechanisms may be different between the two antigen-specific therapies (Table 6.2). Table 6.2 highlights that the soluble MOG_{1-125} treatment was associated with a decrease in the release of pro-inflammatory cytokines after MOG stimulation (highlighted in orange), with the exception of IL-1ra, whereas MOG-ETA' was mostly associated with an increase (highlighted in green). Future experiments would investigate antigen-induced proliferation as these results would help elucidate soluble MOG's mode of EAE reduction. Taken together these data suggest this antigen-specific therapy was modulating peripheral immune cells by shifting them from a T_H1 response to a more regulatory phenotype. This suppression of MOG-reactivity could be an important mechanism in the disease suppression observed with this treatment.

Table 6.2. Differences in chemokine and cytokine secretion from lymph node cells after MOG_{1-125} stimulation from the different animal treatment groups.
This table shows the different patterns of chemokine and cytokine secretion from the lymph nodes after stimulation with MOG_{1-125} (protocol described in Figure 6.5). Orange boxes represent a decrease in secretion of protein between control and active treatment and green boxes represent an increase. In both experiments protein levels were measured using Mouse Cytokine Arrays as per manufacture instruction and results quantified using TotalLab. Table demonstrates mean values of ag-induced cytokine and chemokine production (i.e. MOG_{1-125} minus medium alone). Soluble MOG treatment values are representative of two individual experiments and MOG-ETA' treatment values from three.

Comparison of cytokine and chemokine secretion between both antigen-specific treatments				
	MOG_{1-125} stimulated cells			
	Soluble MOG treatment		MOG-ETA' treament	
Chemokines	PBS	MOG_{1-125}	Bo9-ETA'	MOG-ETA'
CCL1	9244	3909	22301	38710
CCL3	29057	23956	22852	53484
CXCL9	3160	2608	429	4847
CXCL10	1218	386	4513	17548
Cytokines				
G-CSF	3132	1343	7874	19898
IL-1α	-	-	1848	12085
IL-1ra	15947	30543	1082	15183
IL-3	1835	899	13824	36644
IL-13	1477	315	3316	7279
IL-17	36985	9046	28676	53840
IFNγ	7794	1411	7715	21034
TNFα	2891	12	7628	16133

This treatment, like MOG-ETA', was associated with an increased anti-MOG. ELISA data showed a significantly greater anti-MOG response in the MOG_{1-125} treated mouse group. These antibodies were shown to have pathogenic properties in an *in vitro* bioassay. Whole protein was used in this study in the hope of suppressing MOG-reactive B cells, but the antibody titre results clearly show that this has not been effective. One possibility to explain these results is that the protein used was

unfolded, and in this from would not have affected B cells specific for epitopes that require the correct protein folding. Previous studies in EAE have shown that conformational epitopes are a major target for pathogenic MOG-specific antibodies (Breithaupt et al., 2008). What is interesting is that despite this increase in demyelinating antibodies; the soluble protein treated group still had a less severe clinical disease. This could be because there was less inflammation occurring in the CNS, so the BBB was not comprised, therefore the pathogenic antibodies could not enter. This increase in autoantibody titres has been observed by another group investigating MOG-specific immune tolerance in MOG-induced EAE in marmosets (Genain et al., 1996). In this study treatment was associated with EAE suppression, T cell suppression and immune deviation from a T_H1 to T_H2 cytokine response. However, in parallel with this there was also an increase in MOG-specific antibodies, which once treatment ended, induced a lethal demyelinating disorder. This disease exacerbation could also occur in this study but due to time constraints the disease course was not observed post treatment. Future experiments would cease MOG_{1-125} treatment and then observe animals to examine if these increased antibody titres would lead to a severe demyelinating disorder.

When comparing the two antigen-specific therapies, results showed that both significantly ameliorated disease and were associated with a reduction in immune cellular infiltration into the CNS. The mechanisms involved in disease suppression were not elucidated in this study but appear to be different as although the global reduction in CNS immune cells were superficially similar it was likely that different mechanisms were occurring. This was evident as soluble MOG treatment was coupled with an increase in $CD1d^{high}CD5^{+}$ regulatory B cells. In addition to this there was a dichotomy in the MOG-induced cytokine response between the two treatments. However, the major drawback of both these MOG-specific therapies is the induction of increased titres of potentially pathogenic autoantibodies.

To circumvent this issue of enlarged anti-MOG titres a treatment strategy targeting and eliminating T cell mediated inflammation could be more effective, for example infusing the mice with soluble peptides. The hypothesis being that treatment with peptide would tolerise the T cells without stimulating a problematic anti-MOG response. Paradoxically preliminary data assessing the effects of low dose soluble MOG peptide, MOG_{79-96}, using the exact same methodology significantly increased the severity of disease (see appendix). This is in contrast to other published studies using MOG peptides, which have been shown to suppress EAE (Yuan et al., 2014, Devaux et al., 1997, Leadbetter et al., 1998). In these studies different amino acid motifs were used and in different murine models, which may explain the contrasting results. Future experiments would also assess the treatment regime and dosage. Studies in rats using i.p. administration of 50 µg and 100 µg MBP showed that disease suppression was dose dependant, where increasing doses of MBP had an increasing effect on immunosuppression (Ishigami et al., 1998), suggesting better immunosuppression might be observed with higher concentrations.

Overall, this result provides further support for the use of antigen-specific therapies in MS. There is still a great need for more specific therapeutic approaches to be researched as although current treatments involving broad immunosuppression have increased life expectancies in patients with MS and reduced patient mortality they are associated with many other adverse effects; The full extent of which has not yet been assessed in paediatric patients, now the most common form of neurological disorder in children. Therefore antigen-specific treatment approaches could effectively modulate pathogenic immune responses meanwhile sparing other important immune functions.

CHAPTER SEVEN

GENERAL DISCUSSION

7 GENERAL DISCUSSION

The possibility antibodies play a role in the pathogenesis of MS was first discussed over seventy years ago, but there is still no consensus as to their specificity, mode of action or even their clinical significance. Nonetheless, the identification of MOG-specific autoantibodies in children with MS or ADEM led to immediate speculation they would cause more severe disease (McLaughlin et al., 2009, Brilot et al., 2009). This hypothesis was based on experimental studies demonstrating MOG-specific autoantibodies mediate widespread demyelination and exacerbate disease severity in animal models of MS (Linington et al., 1988). However, recent studies indicate no correlation exists between disease severity and the presence of absence of MOG-specific autoantibodies in children with MS or ADEM (Mayer et al., 2013). Moreover experiments discussed in this thesis, Chapter 3, were unable to demonstrate the presence of demyelinating antibodies in sera from anti-MOG seropositive patients. This was attributed to the antibody-titre being simply too low to induce complement-mediated demyelination *in vitro*, and by extrapolation probably unable to contribute significantly to the mechanisms involved in causing clinical deficits in patients. However, this is not to say this MOG-specific response plays no role in disease pathogenesis.

MOG-specific antibodies recognise MOG exposed at the membrane surface and their dominant isotype is IgG1, characteristics predicted to cause some degree of myelin/oligodendrocyte damage even if this does not lead to demyelination *per se*. Moreover this MOG-specific antibody response persists, albeit at low levels, in children with MS, but is lost rapidly in cases of ADEM (Mayer et al., 2013). These observations suggested the clinical significance of low titres of MOG-specific autoantibodies might be to maintain a chronic inflammatory response in the CNS, a function that might not necessarily be dependent on their ability to mediate complement-mediated demyelination.

Experiments performed in the course of this thesis indicate that this may well be the case, as they suggest formation of antibody/antigen complexes within the CNS may induce a pro-inflammatory chemokine response. Importantly, this response is not restricted to antibodies recognising antigens exposed at the myelin surface, but might be triggered by any antigen/antibody complex generated in the CNS compartment. This concept is based on the demonstration that pre-formed OVA/OVA-specific antibody complexes, as well as antibodies recognising accessible epitopes of sulphatide, PLP or MOG all induced expression of CCL5 in myelinating cultures.

If confirmed this observation has important implications with respect to our understanding of how a chronic inflammatory environment is maintained in the CNS of patients with MS. One of the classical features of MS is its association with an intrathecal antibody response maintained by clonally expanded B cells sequestered in the CNS, which result in OCBs of immunoglobulins seen when patient CSF samples are analysed by IEF. The specificity profile of this intrathecal antibody response is complex, and as yet no dominant single reactivity has been discovered that is disease specific. In contrast, it appears the response is heterogeneous with individual patients harbouring a variety of different specificities in the CNS. These may include antibodies specific for: myelin-associated lipids (Villar et al., 2005, Podbielska and Hogan, 2009), in particular sulphatides (Ilyas et al., 2003, Kanter et al., 2006, Brennan et al., 2011, Haghighi et al., 2013), combinations of myelin proteins, neuronal antigens and cytoskeletal components (Lambracht-Washington et al., 2007, Quintana et al., 2012), viruses (Owens et al., 2011), and many more.

This range of specificities together with the presence of soluble and membrane bound antigens in CSF suggest there is a high probability that antibody/antigen complexes can be generated that may trigger a chemokine response similar to that observed *in vitro*. This provides a mechanism that might explain reports showing that the presence of OCBs or high levels of intrathecal Ig synthesis correlate with

more rapid disease progression and higher MS conversion rates (Stendahl-Brodin and Link, 1983, Sastre-Garriga et al., 2003, Bourre et al., 2012, Ferraro et al., 2013).

In principal such "triggering" complexes would not require antibody-recognition of cell surface exposed epitopes, as antigen enters the CSF as a consequence of tissue damage e.g. myelin-derived vesicular debris (Scolding et al., 1989), axonal proteins (Burman et al., 2014). However, autoantigens are also found in the CSF of healthy individuals in the form of exosomes and other vesicular material (Street et al., 2012, Chiasserini et al., 2014). This raises the intriguing possibility that myelin/oligodendrocyte-derived exosomes may not only provide a source of antigen required to maintain an intrathecal sulphatide-specific B cell response in MS (Kramer-Albers et al., 2007), but if in complex with antibody may trigger a pro-inflammatory chemokine response predicted to recruit effector cells into the CNS. At present there is no formal evidence to support this might occur in MS, but the circumstantial evidence is strong. Not only do many patients exhibit a strong intrathecal response to myelin-derived lipids (Kanter et al., 2006, Brennan et al., 2011), but these can form antigen/antibody complexes *in vivo* (Kasai et al., 1986).

Future studies must address this issue, as this mechanism could play a pivotal role in maintaining a low grade chronic inflammatory response in the parenchyma of patients with progressive forms of MS who do not benefit from currently available disease modifying treatments such as the β-interferon's, Tysalbri or Gilenya. The obvious starting point is to determine whether or not patient CSF contains antibody and/or antigen/antibody complexes that trigger a similar pattern of chemokine expression in myelinating cultures. The experiments described in this thesis took CCL5 as an exemplar, but as already apparent from Proteome and qPCR assays several other chemokines are up regulated along with CCL5 (Figures 4.5 and 4.7, respectively). This raises the possibility non-biased micro array analysis of antibody treated cultures might identify a fingerprint of transcriptional changes defining the presence of functionally relevant antibody/antigen complexes in clinical samples. Such experiments should however be performed together with

studies designed to decipher which cells and molecular pathways are responsible for this response *in vitro*. The obvious cellular targets are microglia and astrocytes as both can be induced to express a range of chemokines, and logically one may speculate the effect is Fc mediated. However, we observe a similar response to myelin-specific IgG and IgM antibodies and although several studies are available discussing the cellular expression of Fcɣ receptors on astrocytes and microglia, little is known about the Fcμ receptors and their cellular expression in the CNS.

It should also be appreciated that this may provide a more general mechanism that might promote disease activity in other neurological diseases, as suggested by studies indicating autoantibodies from patients with NMO and neuropsychiatric lupus can induce chemokine expression in astrocytes (Howe et al., 2014), and microglia (Santer et al., 2009), respectively.

If proved correct the hypothesis outlined above will have major implications with respect to the development of antigen-specific therapies for MS. This was the other major theme addressed in this thesis, which was based on the assumption antigen-specific deletion of B cells would inhibit disease activity in EAE without disrupting the entire B cell repertoire. Although preliminary clinical data suggests this approach may be effective there was a significant complication in that the introduction of additional autoantigen stimulated an increased pathogenic MOG-specific antibody response. This is a significant drawback as these antibodies would exacerbate disease activity if they gain access to the CNS, a problem encountered in another study in which soluble MOG was used to “tolerised” the MOG specific repertoire in a primate model of MS (Genain et al., 1996).

However although this approach might eventually be effective in models of MS driven by an autoimmune response to a single antigen, it is unlikely this will be useful in patients with MS. The major issue being it appears increasingly likely MS is a poly-specific disease in which multiple specificities contribute to disease pathogenesis. These may not only differ between patients but increase in

complexity during the course of disease due to “epitope spreading”. Therefore, “antigen-specific” therapies may be much more effective if designed to target a combination of disease relevant antigens. This approach is already being investigated using combinations of CNS antigens and a variety of different delivery strategies, including multi-epitope targeted therapy (Kaushansky et al., 2011), and transdermal application of myelin peptides (Walczak et al., 2013). These studies also demonstrate the importance of developing better immune monitoring assays to detect and follow treatment responses as the disease develops (Lutterotti and Martin, 2014). Despite the lack of success in elucidating disease-specific antigens it is still imperative to continue to define pathogenic components of the disease associated autoantibody repertoire, as demonstrated by the recent, exciting identification of KIR1.4 as a novel and apparently common target for pathogenic autoantibodies in MS (Kraus et al., 2014).

The past decades saw real progress in characterising and understanding the pathogenesis of MS which resulted in the introduction of effective treatments for MS. Reliable historical data is difficult to extract from the literature but it is clear that the life expectancy of MS patients has been increasing. Nowadays, studies have shown that people with MS die 5-10 years earlier than that of the general population (Runia et al., 2012), which is a vast improvement and this gap appears to be decreasing (Webpage 1). Nonetheless we still have no cure for MS and are unable to halt accumulation of disability in patients with progressive forms of the disease; with approximately 100 people being diagnosed with MS every week the need for better therapeutics has never been more pertinent (Webpage 2). Aggressive targeting of B cell dependent disease mechanisms in combination with modern stem cell treatments (Connick et al., 2012), to replenish or repair the B cell compartment may provide a new generation of even more successful therapies.

CHAPTER EIGHT

APPENDICES

8 APPENDICES

8.1 LOW DOSE SOLUBLE MOG_{79-96} TREATMENT SIGNIFICANTLY INCREASED DISEASE SEVERITY IN MOG-INDUCED EAE

To circumvent the issue of the enlarged anti-MOG titres which developed with soluble MOG_{1-125} treatment the mice were instead injected with low doses of soluble MOG_{79-96}. The same course of treatment was carried out as before with induction of MOG-induced EAE then 25 μg i.p. injections on day two post immunisation and every 48 hours after. The hypothesis being that treatment with peptide would tolerise the T cells, therefore leaving the B cells with no stimulation, so no induction of antibody production. In contrast peptide treatment significantly enhanced EAE severity in comparison to PBS treatment (Figure 8.1). This was also significant in comparison to MOG_{1-125} treatment, which was also run in parallel (data not shown). Due to time constraints further analysis was not performed at termination of experiment to examine the cellular infiltration and antibody production but this will be addressed in the future.

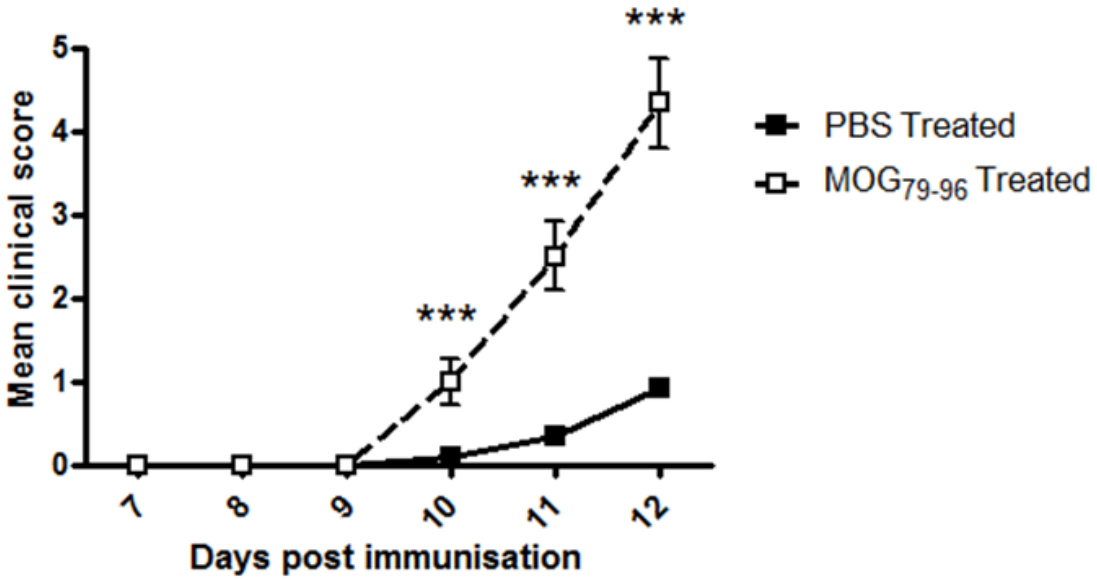


Figure 8.1. MOG_{79-96} treatment increased EAE severity.
7-8 weeks old DBA/1j mice were immunised with 100 µl complete Freund's adjuvant containing 50 µg MOG_{1-125} and 150 µg heat killed *M. Tuberculosis* to establish EAE s.c. at the base of the tail. Mice were treated i.p. with 25 µg MOG_{79-96} or PBS on day two and every 48 hours after. Clinical data, with each point representing the pooled mean scores ± SEM (n=12). It showed significant increase of EAE severity with MOG_{79-96} treatment. Data points from one biological repeats. Significance was determined by two-way repeated measures ANOVA with Bonferroni post-tests. ***, $p < 0.001$.

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