The roles of anti-GM1 complex antibodies in autoimmune neuropathies

Gavin Robertson Meehan MSci (Hons).

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Institute of Infection, Immunity and Inflammation

College of Medical, Veterinary and Life Sciences University of Glasgow

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Declaration of authorship

All experiments are the work of the author unless specifically stated otherwise.

Gavin Robertson Meehan MSci (Hons).

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Abstract

Anti-ganglioside antibodies have been implicated in autoimmune neuropathies for several decades. They are thought to elicit injury through binding to sites in the peripheral nervous system, where they activate the complement pathway to induce cell death. Patient serum is therefore regularly screened for these antibodies to aid in the diagnosis of various conditions. Recent work has found that complexes composed of gangliosides and other glycolipids can improve the detection of these antibodies beyond the signals detected to the single ganglioside species.

In MMN research, complexes comprised of GM1 and GalC have been found to significantly enhance antibody detection in patient sera. In certain patients, however, antibody binding was only detected against these complexes and not the single antigens. This led some researchers to hypothesise that an unidentified class of antibody may have arisen that binds specifically to a neo-epitope formed by the combination of the two glycolipids. It has also been hypothesised that that this complex may be the true target of immune mediated attack in MMN.

This thesis sought to address this hypothesis by either cloning these antibodies directly from patient serum or through active immunisations with mice. Analysis of previously generated human monoclonal antibodies indicated that their behaviours were modified by complexes containing particular gangliosides or glycolipids. Furthermore, the antibodies behaviours were found to diverge, when they were screened against complexes comprised of gangliosides and different concentrations of accessory lipids. These findings suggested that the accessory lipids were interacting with the ganglioside headgroups to modify the presentation of different binding epitopes. This indicated that conformational modulation, rather than neo-epitope formation, may be responsible for complex enhancement

Cloning antibodies from patient sera was unsuccessful but examination of the screening techniques suggested that the appearance of complex-dependent antibodies may have been an artefact. Attempts to induce complex-specific responses in mice were similarly unsuccessful but several anti-ganglioside and anti-sulfatide antibodies were created. The subsequent chapters focused on the characterisation of these antibodies and indicated that most of them bound well to solid-phase assays, cells and tissue and may therefore be of use in future studies.

Taken together, the data from this thesis suggests that complex-dependent antibodies may not exist but are merely low concentrations of anti-ganglioside antibodies that are cis-enhanced by particular lipids. Future work should therefore focus on assessing how the ganglioside microenvironment modifies epitope presentation and how this affects the binding capabilities of anti-ganglioside antibodies.

Dedication

I dedicate this thesis to all autoimmune neuropathy patients past, present and future.

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Abbreviations

ACh	acetylcholine
AF	AlexaFluor
AIDP	acute inflammatory demyelinating polyneuropathy
ALS	amyotrophic lateral sclerosis
AMAN	acute motor axonal neuropathy
AN	autoimmune neuropathies
ANOVA	analysis of variance
ARAC	cytosine arabinoside
BBB	blood brain barrier
BGM	basic growth medium
BNB	blood-nerve barrier
BSA	bovine serum albumin
BTx	α-bungarotoxin
C. Jejuni	Campylocbacter Jejuni
CB	conduction block
CERT	ceramide transfer protein
CFP	cyan fluorescent protein
CGT	cerebroside galactose transferase
Chol	cholesterol
CIDP	chronic inflammatory demyelinating neuropathy
CMAP	compound muscle action potential
CMT	Charcot-Marie-Tooth syndrome
CSF	cerebrospinal fluid
CST	cerebroside sulfo transferase
CTx	cholera toxin b subunit
Cy5	cyanine-5
DCP	dicetyl phosphate
EFNS	European federation of neurological societies
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FAPP2	four-phosphate adaptor protein 2
FCS	foetal calf serum

FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GalC	galactocerebroside
GalNAcT	b1, 4-N- acetylgalactosamineyltransferase
GBS	Guillain-Barré syndrome
GD3 S	GD3 synthase
GFP	green fluorescent protein
GlcCer	Glucosylceramide
GSL	glycosphingolipids
HC	healthy controls
HRP	horse radish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
IP	intraperitoneally
IV	intravenously
IVIG	intravenous immunoglobulin
LacCer	lactosylceramide
LED	light emitting diode
LOS	lipooligosaccharides
mAbs	monoclonal antibodies
MAC	membrane attack complex
MADSAM	multifocal acquired demyelinating sensory and motor neuropathy
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MFS	Miller-Fisher syndrome
MHCII	Major Histocompatibility Complex 2
MMN	multifocal motor neuropathy
MND	motor neuron disease
MRC	medical research council
MRI	magnetic resonance imaging
MS	multiple sclerosis
N'ase	neuraminidase
NCS	nerve conduction studies
NF-Kβ	nuclear factor kappa-light-chain enhanced of activated B cells
NHS	normal human serum

NK	natural killer cells
NMJ	neuromuscular junction
NoR	node of Ranvier
OD	optical density
OND	other neurological diseases
OPC	oligodendrocyte progenitor cells
OPD	o-phenylenediamine
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PFA	paraformaldehyde PLP
PMT	photomultiplier tube
PNS	peripheral nervous system
PS	phosphatidyl serine
pSC	perisynaptic Schwann cell
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
SCIG	subcutaneous immunoglobulin
SEM	standard error or mean
SGM	Schwann cell growth medium
SM	sphingomyelin
SNAP	sensory nerve action potential
SNARE	Soluble NSF Attachment Protein REceptor
Sulf	sulfatide
TD	temporal dispersion
TLC	thin layer chromatography
TMSC	Terminal myelinating Schwann cell
TRITC	tetramethylrhodamine isothiocyanate
TS	triangularis sterni
WLE	whole lipid extract
WT	wild type
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 Autoimmune Neuropathies

Autoimmune neuropathies (AN) describe a diverse range of conditions in which an abnormal immune response results in inflammation of the peripheral nervous system (PNS). These conditions vary widely in their presentation, appearing in both acute and chronic forms, with symptoms affecting distal or proximal locations in a symmetrical or multifocal pattern. The inflammation produced can lead to demyelination or axonal degeneration resulting in permanent disability, respiratory paralysis or death.

The implications of such neuropathies are far reaching, having not only a substantial impact upon both the lives of patients and their families but also the healthcare system. A study showed that the pre-eminent acute AN, Guillain-Barré syndrome, was estimated to have cost the US economy $1.8 billion in 2004 alone, signifying the continued importance of the research and treatment of these conditions (Frenzen, 2008).

1.2 History

The first modern description of an autoimmune neuropathy was by Jean Landry in 1859 (Landry, 1859). He described a set of 10 patients suffering from ascending muscular paralysis accompanied by loss of reflexes and parasthesia. The onset of disease in one patient in particular was described in detail. This patient's condition deteriorated rapidly with the paralysis spreading to the trunk, resulting in asphyxia and death. No known cause was identified and autopsy results were inconclusive as nervous tissue was not examined. This condition was eventually termed Landry's paralysis.

In France in 1916, three physicians described a similar condition (Guillain et al., 1916). Georges Guillain, Jean Alexandre Barré and André Strohl were army physicians who examined two soldiers suffering from motor weakness and parasthesia. Upon examination of the cerebrospinal fluid they discovered raised

albumin levels but without pleocytosis indicating that no other infection was causing the symptoms. This was essential to distinguish the condition from Poliomyelitis which was the most common cause of acute flaccid paralysis at the time. Both patients recovered spontaneously and the condition was termed Guillain-Barré Syndrome (GBS) in 1927 (Dragnesco & Claudian, 1927).

For a period of time both diseases were considered separate entities. George Guillain, in particular, stressed the differences between Landry's paralysis and GBS due to the lack of pleocytosis in his patients (lumbar puncture had not been invented when Landry examined his patients) and the benign nature of their symptoms in comparison to those suffering from Landry's Paralysis. Despite his objections the diseases were grouped together in 1949 under the name of Landry-Guillain-Barré syndrome by Haymaker and Kernahan.

Research into GBS began to intensify following Haymaker and Kernahan's descripton of 50 patients with fatal GBS (Haymaker & Kernohan, 1949). At the same time a physician called Charles Miller-Fisher described several patients suffering from ophthalmoplegia, ataxia and areflexia (Fisher, 1956). Some of these patients suffered palsy and weakness but all recovered spontaneously. As some GBS patients suffer from ophthalmoplegia Miller-Fisher accurately concluded that his patients were suffering from a subtype of GBS. This was dubbed Miller-Fisher Syndrome (MFS), which is now known to be a specific subtype of GBS in which antibodies target gangliosides found enriched in the 3rd and 4th cranial nerves producing ophthalmoplegia.

Due to the increase in GBS cases associated with the 1976 swine flu outbreak in the United States, Asbury & Cornblath established strict diagnostic criteria for the condition (Asbury & Cornblath, 1990). These criteria cited motor weakness and areflexia as required symptoms for diagnosis which could be supported by different features often associated with the condition. These included electrophysiology which showed conduction slowing or block within the nerves. These data, alongside autopsy reports, were consistent with demyelination of the nerve roots and trunk which led to the assumption that GBS was a homogenous condition synonymous with Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP).

It was not until the description of a seasonal outbreak of a GBS-like illness in rural China that further subtypes of GBS were described. This condition, originally called Chinese paralytic syndrome, produced acute ascending flaccid paralysis leading to quadraparesis and respiratory failure (McKhann et al., 1990). It was commonly mistaken for GBS but electrophysiological examination suggested that the patients were suffering from denervation rather than demyelination of the motor nerves, whilst the sensory nerves remained unaffected. Research into this condition intensified and it was found to be an axonal variant of GBS which was more prevalent in Asian countries(Yuki, Yoshino, & Miyatake, 1993; McKhann et al., 1993). It was eventually termed acute motor axonal neuropathy (AMAN) (Ho et al., 1995).

As research developed into acute subtypes of GBS, clinicians began to describe chronic forms of autoimmune neuropathies. These chronic conditions are all closely related and can be considered to be part of a spectrum of disease. Chronic inflammatory demyelinating polyneuropathy (CIDP) was the first to be described by Austin in 1958. He examined patients from the literature and two of his own who had suffered from recurring bouts of paralysis. These patients tended to present with muscle weakness in the extremities, which eventually spread to the trunk with increased severity. Following treatment with cortisone and prednisone he was able to control the symptoms in his own patient but was not able to prevent relapse (Austin, 1958).

Clinicians began to report patients with similar chronic neuropathies that had differing symptoms. Lewis and Sumner reported five patients who were suffering from a variant of CIDP (Lewis et al., 1982). The patients presented with asymmetric sensorimotor weakness focussed in the upper extremities with multifocal nerve involvement. This condition, known as Lewis-Summer syndrome or multifocal acquired demyelinating sensory and motor neuropathy (MADSAM), can be distinguished from CIDP by the electrophysiology data, namely the prolonged conduction block seen in the MADSAM patients. However, the two conditions are closely related and share many of the same diagnostic criteria.

The most recent chronic neuropathy to be described was multifocal motor neuropathy (MMN). MMN was first observed in five patients in 1982 who presented with an asymmetric motor neuropathy predominantly affecting the upper limbs. Diagnostic tests revealed the presence of conduction block in the

motor nerves but none in the sensory nerves. Two of the patients were treated with steroids and it was assumed that the condition was a variation of CIDP (Lewis et al., 1982).

Between 1985 and 1986 three separate papers also reported patients with similar symptoms (Chad et al., 1986; Parry & Clarke, 1988; Roth et al., 1986) but it was not until 1988 that Pestronk et al discovered the presence of antibodies targeting structures predominantly found in the peripheral nervous system called gangliosides, in particular GM1 (Pestronk et al., 1988). Treatment of these patients with immunomodulating therapy was shown to be successful, indicating the role of these antibodies in producing the aforementioned muscle weakness.

1.3 Multifocal Motor Neuropathy

Although not widely researched, the incidence rate of MMN is between 1 and 2 people per 100,000 population, similar to that of GBS. It is found more commonly in males than females in a 2.6:1 ratio with an average age of onset of approximately 40 years (Slee et al., 2007).

1.3.1 Clinical Criteria

1.3.1.1 Core Criteria

There is no definitive test for MMN; rather patient symptoms must fulfil certain diagnostic criteria (Table 1 and 2). Most patients initially present with slowly progressing asymmetric muscle weakness, most commonly in the fore arms. Although no paralysis occurs, this weakness results in the patients experiencing foot drop, wrist drop and grip weakness. These are commonly accompanied by fasciculations and loss of tendon reflexes within the affected limb. Sensory symptoms, other than minor vibration sense abnormalities, should not be present although parasthesia and minor numbness may be experienced in a small number of patients, particularly as the disease progresses (Léger & Gavriliuc, 2012). As symptoms vary significantly between patients, these criteria can be substantiated by various diagnostic tests including electrophysiology and serology.

1.3.1.2 Electrophysiology

Nerve conduction studies (NCS) are one of the main diagnostic tests performed to confirm the presence of MMN. They allow for the detection of conduction block (CB), demyelinative slowing or loss of motor axons. This is achieved through stimulation of the nerves by an electrode which generates an action potential recorded at a distant point on the same nerve. This same electrical impulse activates the target muscle supplied by the nerve and produces a compound muscle action potential (CMAP). As shown in Table 1, a proximal CMAP with an area reduction of at least 30% compared to the distal CMAP suggests the presence of CB.

Table 1-1 - Electrophysiological Requirements for Motor Conduction Block

1	**Definite Motor CB***
Negative peak CMAP area reduction on proximal versus distal stimulation of at least 50 % whatever the nerve segment length (median, ulnar, peroneal). Negative peak CMAP amplitude on stimulation of the distal part of the segment with motor CB must be >20 % of the lower limit of normal and >1mV. Increase of proximal to distal negative peak CMAP duration must be ≤30 %	
2	**Probable Motor CB***
Negative peak CMAP area reduction of at least 30 % over a long segment (e.g. wrist to elbow or elbow to axilla) of an upper limb nerve with an increase of proximal to distal negative peak CMAP duration ≤30 % Or: negative CMAP area reduction of at least 50 % (same as definite) with an increase of proximal negative CMAP duration of >30 %	
3	**Normal Sensory Nerve Conduction in Upper Limb Segments with CB**

Electrophysiological Requirements for Motor CB as defined by Joint Task Force of the European Federation of Neurological Societies and the Peripheral Nerve Society (EFNS/PNS), 2010 *Evidence for CB must be found at sites distinct from common entrapment and compression sites. CB = conduction block; CMAP = compound muscle action potential. (EFNS/PNS MMN Guideline, 2010)

Conduction block is defined as the failure of action potential propagation at a given site in a structurally intact axon (Kaji, 2003). The CMAP area is used to determine CB over CMAP amplitude as the latter can be greatly influenced by a process known as temporal dispersion (TD). Temporal dispersion is a result of loss of synchronisation between nerve fibres which leads to phase cancellation. It therefore strongly mimics the effects of CB with some animal studies

Table 1-2 - Clinical criteria for MMN diagnosis

Definite MMN
Clinical criteria 1, 2 and 8–11 and electrophysiological criteria 1 and 3 in one nerve (see Table 1)
Probably MMN
Clinical criteria 1, 2 and 8–11 and electrophysiological criteria 2 and 3 in two nerves (see Table 5); clinical criteria 1, 2 and 8–11 and electrophysiological criteria 2 and 3 in one nerve and at least two supportive criteria 1–4 (see Table 1)
Possible MMN
Clinical criteria 1, 2 and 8–11 and normal sensory nerve conduction studies and supportive criteria 4; clinical criteria 1 with clinical signs present in only one nerve, clinical criteria 2 and 8–11 and electrophysiological criteria 1 or 2 and 3 in one nerve (see Table 1)
Clinical Criteria
Core Criteria (Both must be present)
1. Slowly progressive or stepwise progressive, focal, asymmetric* limb weakness, that is, motor involvement in the motor nerve distribution of at least two nerves for more than 1 month.** If symptoms and signs are present only in the distribution of one nerve, only a possible diagnosis can be made.
2. No objective sensory abnormalities except for minor vibration sense abnormalities in the lower limbs***
Supportive Clinical Criteria
3. Predominant upper limb involvement †
4. Decreased or absent tendon reflexes in the affected limb ‡
5. Absence of cranial nerve involvement §
6. Cramps and fasciculations in the affected limb
7. Response in terms of disability or muscle strength to immunomodulatory treatment
Exclusion Criteria
8. Upper motor signs
9. Marked bulbar involvement
10. Sensory impairment more marked than minor vibration loss in the lower limbs
11. Diffuse symmetrical weakness during the initial weeks
Supportive Criteria
1. Elevated IgM anti-ganglioside GM1 antibodies
2. Laboratory: increased CSF protein (<1 g/l)
3. MRI showing increased signal intensity on T2-weighted imaging associated with a diffuse nerve swelling of the brachial plexus
4. Objective clinical improvement following IVIg treatment

Clinical criteria for MMN diagnosis as defined by by Joint Task Force of the European Federation of Neurological Societies and the Peripheral Nerve Society (EFNS/PNS), 2010 *Asymmetric: a difference of 1 Medical Research Council (MRC) grade if strength is MRC >3 and 2 MRC grades if strength is MRC ≤3. **Usually more than six months. ***Sensory signs and symptoms may develop over the course of MMN. † At onset, predominantly lower limb involvement accounts for nearly 10 % of the cases. ‡Slightly increased tendon reflexes, in particular in the affected arm, have been reported and do not exclude the diagnosis of MMN provided criterion 8 is met. § Twelfth nerve palsy has been reported. (EFNS/PNS MMN Guideline, 2010)

suggesting that a reduction in CMAP amplitude of up to 80% can be attributed to TD alone. (Rhee et al., 1990)

The use of CMAP area is therefore required for an accurate diagnosis although a decreased CMAP area in proximal versus distal locations of up to 50% can still be caused by TD. This has led to strict guidelines determining that a definitive diagnosis of MMN must have a CMAP area reduction of 50% or above.

Smaller reductions can still indicate MMN but must be supported by other diagnosis criteria. This includes the measurement of sensory nerve action potentials (SNAPs) in nerves with conduction block. The presence of motor CB alongside normal SNAPs is a strong indication of MMN whereas decreased SNAPs would be more indicative of MADSAM.

1.3.1.3 Treatment

Part of the supportive clinical criteria of a MMN diagnosis is an improvement in disability or muscle strength in response to immunomodulating agents. The most effective of these treatments is intravenous immunoglobulin (IVIG), which consists of pooled IgG antibodies from more than a thousand different blood donors. Patients undergoing treatment with IVIG show a marked clinical improvement with up to 94% of patients showing an improvement in disability score and muscle strength (Cats, van der Pol, et al., 2010). This significant improvement reinforces the hypothesis that antibodies are responsible for disease pathogenesis in MMN.

The mechanism of action of IVIG has yet to be fully elucidated but it is considered to exert its effects upon several different pathways. Several studies have indicated that it prevents B cell proliferation and antibody production and inhibits the ability of these antibodies to cross the blood-nerve barrier (BNB) (Hartung, 2008; Kondo et al., 1994; Stohl & Elliot, 1996). It is also thought that IVIG can interfere with the complement cascade and thus prevent complement induced injury to nerves (Jacob & Rajabally, 2009). It is unclear whether it is just one of these mechanisms or a combination of them all that helps prevent autoantibody induced damage but as serum antibody levels tend to remain stable during treatment it has been hypothesised that IVIG acts to prevent antibody binding and complement activation (Malik et al., 1996).

There are several drawbacks of IVIG treatment despite its effectiveness. The increased off-label use of the treatment for a wide number of conditions has led to a worldwide shortage of the drug, which has also led to a substantial increase in its cost (Deparment of Health, 2011; O'Riordan et al., 2010). This coupled with the requirement of patients to attend hospital regularly to receive treatment has resulted in a drive to investigate other immunotherapies (Nobile-Orazio & Gallia, 2013).

Subcutaneous immunoglobulin (SCIG) is a relatively new treatment option that addresses the issues with intravenous administration. SCIG contains the same dose of immunoglobulin as standard IVIG but can be self-administered by patients at home. This results in reduced costs for healthcare systems who no longer need to provide staff to administer the immunotherapy and also results in an improvement in the quality of life for the patient (Ozerovitch, 2013).

Studies have shown that SCIG is as effective at maintaining muscle strength and disability score as IVIG but was scored higher by patients in regards to their health related quality of life (Eftimov et al., 2009; Harbo et al., 2009; Misbah et al., 2011). Further studies are needed to establish the long term safety profile of SCIG but it is becoming popular with physicians and patients as an alternative form of treatment.

Aside from immunoglobulin therapy, researchers have also examined the effectiveness of B cell depletion in treating MMN. This has primarily been carried out using the humanised monoclonal antibody Rituximab, which specifically targets the B cell surface antigen CD20 (Anderson et al., 1997). Once bound, this antibody is able to initiate cell death via complement activation, antibody-dependent cell-mediated toxicity and apoptosis (Kosmidis & Dalakas, 2010).

Although it was initially approved for treating B cell lymphoma, Rituximab has been shown to effectively treat a variety of autoimmune conditions including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. It has also been demonstrated to be effective in treating MMN, with several studies showing an improvement in muscle strength and a reduction in circulating anti-GM1 antibodies following administration (Gorson et al., 2007; Pestronk et al., 2003; Rüegg et al., 2004). Despite this work, the antibody has only been shown

to be effective alongside IVIG and has not yet been proven as a useful standalone therapy.

Very limited research has also been performed with complement inhibitors, with one study examining their effects on MMN patients (Fitzpatrick et al., 2011). The study used a humanised monoclonal antibody, ecluzimab, which targets and neutralises terminal complement component 5 (C5). This prevents the formation of the membrane attack complex (MAC) on cell membranes and thus reduces complement induced injury. When used in conjunction with IVIG treatment it showed an improvement in conduction block and select motor performance tests in a number of patients. This effect appeared independent of the IVIG benefits but a fully blinded study would need to be performed on a larger number of patients to establish the full effectiveness of complement inhibitors.

Certain therapies used to treat other ANs are not suitable for MMN patients and can actually exacerbate symptoms. A prime example is plasma exchange in which the plasma is removed from the body and filtered to extract auto-antibodies. This treatment is commonly used to reduce nerve injury and demyelination in GBS and CIDP patients but in MMN patients it causes a worsening of their symptoms (Carpo et al., 1998). It is unclear why plasma exchange is unsuccessful in treating MMN, although it has been hypothesised that filtering the plasma also removes beneficial antibodies and cytokines which regulate the pathogenic antibodies. The pathogenic antibodies would therefore be able to produce higher levels of inflammation and cell death, which would result in a deterioration in the patient's condition (Claus & Specht, 2000). This reiterates the importance of an accurate diagnosis as plasma exchange is an undesirable treatment option for a MMN patient.

1.3.2 Exclusion Criteria

As MMN shares many of the symptoms of other neurological diseases, it is necessary to exclude particular symptoms, which may be indicative of other conditions. This is important to prevent the misdiagnosis of patients who may be suffering from conditions with much worse prognoses such as motor neuron disease (MND).

Motor neuron disease is a rapidly progressive condition in which the motor neurons become damaged leading to muscle weakness and eventual paralysis and death. Although the symptoms of both are very similar in the earlier stages, there are certain symptoms unique to MND which exclude patients from a MMN diagnosis. These include weakness in the absence of fasciculations, involvement of large groups of muscles, brisk reflexes, and a positive Babinski sign.

These symptoms would suggest the involvement of upper motor neurons which are not affected in MMN. Other exclusion criteria include marked bulbar involvement, where patients have difficulty swallowing, chewing and breathing, which would also be suggestive of a MND subtype known as bulbar palsy.

Sensory symptoms, beyond those already discussed, would be more suggestive of a different chronic autoimmune neuropathy such as CIDP or MADSAM. The presence of any of these exclusion criteria would prevent a diagnosis of MMN and would be more suggestive of different conditions affecting the lower motor neurons.

1.3.3 Supportive criteria

There are certain other diagnostic and laboratory tests which can support a MMN diagnosis but are not specific enough to act as essential criteria. These include measurements of CSF protein, MRI scans and serology screening to identify anti-ganglioside antibodies.

1.3.3.1 Raised Cerebrospinal Fluid Protein

A lumbar puncture is performed to test for the presence of raised CSF protein, which is indicative of an autoimmune neuropathy. The main function of the test is to preclude any infective agents such as poliomyelitis as possible causes of the symptoms (Hadden & Hughes, 2003). If these were the cause then the CSF protein would expect to be within its normal range and the physician could search for an alternative diagnosis.

1.3.3.2 Magnetic Image Resonance

Limited research has been performed using magnetic resonance imaging (MRI) scanners to detect nerve swelling in MMN patients. This takes the form of an increased signal in T2-weighted images of the brachial plexus, which is similar to that seen in CIDP patients. The main benefit of this test is to distinguish MMN from lower motor neuron disease (Van Es et al., 1997).

1.3.3.3 Serology

The presence of anti GM1 antibodies in patient sera is a distinguishing feature in MMN but, due to the high variability between studies, it is not part of the required diagnostic criteria. Publications suggest that antibodies targeting GM1 can range from between 30 to 80% in different laboratories which could be an indication of differences in population dynamics or simply differences in laboratory technique (Harschnitz et al., 2014; Nobile-Orazio et al., 2013). This number tends to increase when the serum is screened against complexes composed of GM1 and other gangliosides or glycolipids with some studies detecting antibodies in 100% of patients (Galban-Horcajo et al., 2013).

Currently there is no specific biomarker for MMN but it is hoped that the use of ganglioside complexes may reveal an epitope that has not been previously discovered. This would be greatly beneficial in MMN diagnosis as it would alleviate the requirement for the more specialist tests previously mentioned and reduce diagnosis time.

1.3.4 Pathophysiology

Although the presence of anti-GM1 antibodies and the response to immune modulating treatments are good indications that MMN is an autoimmune condition, no studies have directly demonstrated the pathophysiological mechanisms responsible for the onset of disease.

1.3.4.1 Molecular Mimicry

Part of the difficulty in assessing the pathogenicity of MMN is that the cause of the disease is unclear. One possibility is that the condition is a post infectious disease, in which the development of an immune response to a previous infection leads to autoimmunity. The best example of this is GBS, which tends to arise 2-3 weeks following exposure to a triggering antigen. These can range from vaccinations to upper respiratory and gastrointestinal infections, but GBS is most commonly associated with Campylobacter jejuni (C. jejuni) (Nyati & Nyati, 2013; Willison & Yuki, 2002).

This bacterium is commonly found in animal faeces and as a result tends to affect rural communities more than those found in urban areas. During the original descriptions of AMAN, it was noted that outbreaks tended to occur at the same time as the seasonal C. jejuni infections, which peaked in the summer months (McKhann et al., 1990). This gave rise to the theory that the bacterium was responsible for triggering AMAN. Subsequent research proved this theory by demonstrating that a degree of molecular mimicry existed between the lipo-oligosaccharide coats of the bacterium and human gangliosides, particularly GM1 and GD1a (Figure 1.3) (Aspinall et al., 1994; Yuki et al., 2004).

From an evolutionary stand point, it appears that the bacteria has evolved to resemble gangliosides in an effort to mask itself from the immune system (Moran et al., 1996). Unfortunately, in certain patients, this results in the antibodies cross reacting with host gangliosides bringing about neurological injury.

This has also been demonstrated in animal models, where immunisations with C. jejuni led to the development of specific anti-ganglioside antibodies (Goodyear et al., 1999), which, in the case of rabbits, led to acute flaccid paralysis (Yuki et al., 2004). Molecular mimicry is therefore a major factor in the development of anti-ganglioside antibodies and may be of relevance in MMN.

1.3.4.2 Adaptive Immune Response

The mechanisms responsible for the development of autoimmunity in GBS are well established. Upon exposure to bacteria, such as C. jejuni, epithelial cells in the gut secrete chemokine ligand 20 and interleukin 8 in order to recruit antigen presenting cells to the site of infection. Dendritic cells in the gut then rapidly internalise the bacterium, which triggers the release of cytokines, NF-Kβ and tumour necrosis factor-α (Jones et al., 2003). These cells then either undergo apoptosis or migrate to the mesenteric lymph nodes, where they trigger a predominant Th1 response to aid in bacterial clearance (Willison & Goodyear, 2013).

In addition, the immune response also triggers IgA secretion by B cells, which aids in resolving the infection. In individuals that are susceptible to GBS, it is thought that this response is altered due to differences in the gut microbiota (Willison & Goodyear, 2013). This results in a more substantial antibody response and the involvement of different classes including IgM, IgG1 and IgG3 (Willison & Veitch, 1994).

This is surprising, especially considering that carbohydrate antigens are typically of the IgG2 subclass (Willison & Veitch, 1994). In addition, class switching strongly implies that T cell help is involved but it is unclear how the carbohydrate antigens are presented to the immune system.

One possible mechanism is that antigen presenting cells process and present the carbohydrates on their MHCII receptors to trigger CD4 T cell activation (Cobb et al., 2004). Alternatively, it is possible that the cells migrate to the spleen where they trigger plasmablast differentiation in marginal zone B cells in a T cell independent manner.

A newly discovered population may also be able to aid B cells generate anti-ganglioside antibodies without the aid of T cells. This neutrophil population, known as inducible B cell helper neutrophils ($iN_{BH}s$), are able to prime marginal zone B cells to produce IgM antibodies (Puga et al., 2011). Furthermore, the presence of lipo-oligosaccharides allows these cells to interact with the marginal zone B cells to induce non classical class switching (Willison & Goodyear, 2013).

This may lead to the break down in tolerance that is found in GBS; however, the specific mechanisms responsible are still not clear.

Although the adaptive immune response appears to be responsible for the onset of GBS, the slow progression and chronic nature of MMN make this a difficult aspect to study within this disease. Furthermore, unlike GBS, MMN is not associated with any specific infection (Terenghi et al., 2002), which suggests that the adaptive immune system may not be responsible for the development of anti-ganglioside antibodies. In fact, recent evidence appears to suggest that the source of these antibodies may be an abnormality in the natural B cells of the innate immune system.

1.3.4.3 Innate Immune Response

In mice B1 cells are responsible for the secretion of a subset of antibodies that do not require prior immune activation. These antibodies are termed “natural” antibodies as they are present from birth without external antigenic exposure (Grönwall et al., 2012). As they recognise self-antigens it has been proposed that they evolved for immunoregulatory roles, particularly the suppression of the innate immune system and clearance of apoptotic cells (Grönwall et al., 2012).

The B1 cells themselves are found predominantly in the peripheral and pleural cavities (Hayakawa et al., 1986) but it is splenic B1 cells that are thought to secrete the majority of immunoglobulin. There is some debate as to whether a human equivalent of B1 cells has been discovered (D. O. Griffin et al., 2011) but this topic is highly controversial (Covens et al., 2013; Tangye, 2013). Regardless of this, natural antibodies are still found in humans and evidence suggests that they may have roles in the development of certain autoimmune neuropathies.

In particular, it has been proposed that abnormal proliferation of these B cells may be responsible for the development of anti-GM1 antibodies in MMN (Harschnitz et al., 2014). This same process is also thought to occur in the development of anti-MAG antibodies in various neuropathies.

It is possible in these conditions that the antibody secreting B cell is either mutated or transformed leading to immortalisation (Steck et al., 2013). These immortal cells will secrete large titres of IgM antibodies, which will eventually

build up in the circulation and begin targeting their respective antigens. This will lead to cell injury and thus neurological dysfunction.

Analysis of the anti-GM1 antibody populations in MMN support this hypothesis as they have shown restricted immunoglobulin light chain use, which suggests that they arise from a limited number of B cell clones (Cats et al., 2015).A few studies have also shown that lymphoma patients can develop MMN (Lefaucheur et al., 2003; Noguchi et al., 2003; Stern et al., 2006), which further supports the theory that the condition may arise due to an abnormal B cell. Unfortunately, bone marrow populations have not yet been examined in MMN patients so the roles of B cell proliferation in the disease have yet to be fully elucidated.

1.3.4.4 Motor nerve susceptibility

It has been demonstrated that the titres of anti-GM1 antibodies in MMN correlate with the severity of muscle weakness (Cats et al, 2010). From this observation it could be assumed that GM1 is expressed solely by the motor nerves, which would explain why the sensory nerves remain unaffected in the condition.

Examination of GM1 distribution in different nerves, however, produced conflicting results. Whilst one group found higher GM1 expression in the ventral roots (Ogawa-Goto et al., 1992), other researchers found little difference between either tissue (Gong et al., 2002; Svennerholm et al., 1994).

Even if the motor nerves did have a higher expression of GM1, the lack of injury in the sensory nerves cannot be explained. They will still express the ganglioside to a certain degree, suggesting that another feature of the nerve must protect it from injury.

One possibility is that the molecular composition of GM1 may differ between tissues. This has been demonstrated previously, with a study showing that the ceramide core of gangliosides differs in the sensory nerves compared to the motor nerves, characterised by an increase in the number of long chain fatty acids (Ogawa-Goto et al., 1990). This may impact how the gangliosides are distributed in the plasma membrane, which may in turn alter the presentation of different binding epitopes.

Similarly, the content of the gangliosides microenvironment has also been shown to influence the orientation of GM1, which may also affect the availability of binding epitopes (Fantini et al., 2013; Greenshields et al., 2009).

Alternatively, it is possible that the antibodies are capable of binding both tissues but, due to an unknown property, are less able to injure sensory nerves. There is some support for this theory, as patients with chronic MMN have an increased chance of developing minor sensory abnormalities (Cats, van der Pol, et al., 2010; Léger & Gavriliuc, 2012); however, further research is needed.

1.3.4.5 Anti-GM1 antibody immune mediated injury

As with GBS, it is thought that the axons are injured in MMN through activation of the complement cascade. This is based upon research, which has found a significant correlation between the high complement activating capacity of anti-GM1 antibodies and more severe muscle weakness and axonal loss (Vlam et al., 2015).

Unfortunately, there is no animal model of the condition so it has not been possible to replicate these findings in live tissue. However, there are animal models of AMAN, the motor form of GBS, which is thought to have a similar mechanism of disease (Harschnitz et al., 2014).

In rabbits it was found that immunisations with purified GM1 or *C Jejuni* with GM1 like structures led to the development of axonal polyneuropathy in the motor nerves, resulting in acute flaccid paralysis (van Sorge et al., 2007). The conclusion from this study was that AMAN only occurs in animals that develop anti-GM1 IgG antibodies with pro-inflammatory responses.

Assuming that a similar process occurs in MMN, it could be postulated that anti-GM1 antibodies will bind GM1 enriched target sites, such as the nodes of Ranvier (NoR) and activate the complement cascade to bring about cell death. This will result in disruption of the sodium channels leading to CB (Franssen & Straver, 2014; Susuki et al., 2007).

1.3.4.6 Complement

Complement is the name given to a series of distinct plasma proteins that form one of the main arms of the innate immune system (Janeway et al., 2001). It has three main pathways dubbed the classical, alternative and mannose-binding lectin pathway but in MMN it appears that only the classical pathway is activated by anti-GM1 antibodies (Piepers et al., 2010; Yuki et al., 2011).

In the classical pathway, once a complement fixing antibody binds its target, it is recognised by complement protein C1q. This produces a conformational

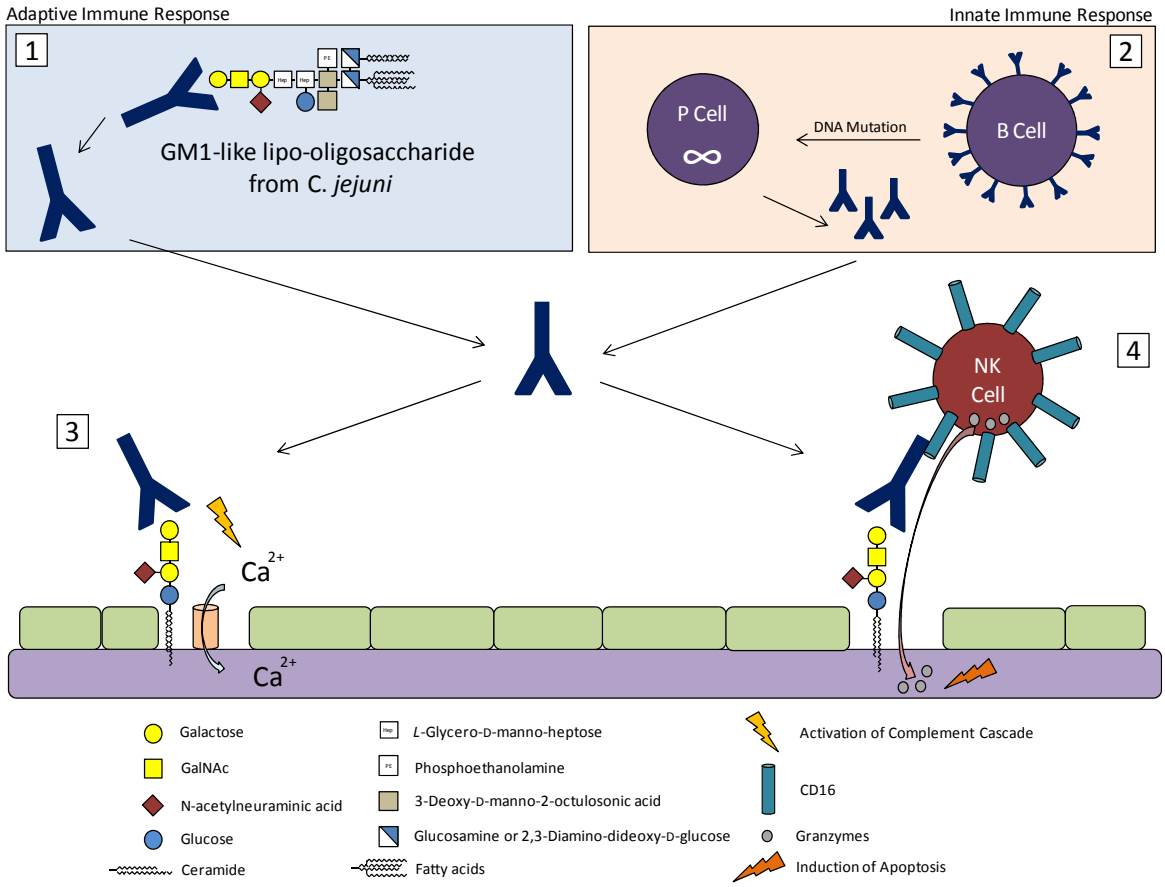


Figure 1.1 – Auto-Antibody Mediated Injury

Auto-antibodies can arise through two different mechanisms. 1: Infections, such as C. *jejuni*, can give rise to antibodies that target LOS, which closely resembles GM1. This molecular mimicry causes the antibodies to target the ganglioside. 2: The B cells that produce natural anti-ganglioside antibodies can mutate to form immortal plasma cells that produce antibodies indefinitely. 3: Once bound, antibodies from both pathways can kill cells by activating the complement system. This leads to MAC pore deposition, which results in a large calcium influx and by extension cell death. 4: Alternatively, NK cells can bind the antibody's Fc portion via CD16. Once bound the NK cell releases granzymes, which invade the antigen presenting cells to induce apoptosis.

change that triggers a cascade reaction culminating in the formation of a membrane attack complex (MAC) (Muller-Eberhard, 1986). This MAC pore inserts into the plasma membrane of a cell causing an uncontrolled influx of extracellular fluid, which disrupts the osmotic balance. This causes cell lysis and thus death (Figure 1.1).

The role of complement in GBS is well established particularly in mouse models, where the use of complement inhibitors has been shown to abrogate antibody mediated injury (Goodfellow et al., 2005; Halstead, Humphreys, et al., 2005; McGonigal et al., 2010). Its roles in MMN, however, are less well established due to the lack of animal models but it is thought to initiate cell injury in the same manner as GBS (Harschnitz et al., 2014).

1.3.4.7 Antibody-dependent cell-mediated cytotoxicity

Instead of activating the complement cascade, antibodies can induce cell death via NK cells in a process known as antibody-dependent cell-mediated cytotoxicity (Figure 1.3). NK cells express the cell surface antigen CD16, which specifically binds the Fc portion of IgG antibodies. Upon binding the antibody molecule, NK cells release granzymes and perforins, which are able to enter the target cell to induce apoptosis (Seidel et al., 2013).

Whilst it may have been assumed that this mechanism would be irrelevant in MMN due to the presence of IgM antibodies, it appears that NK cells are raised in MMN patient blood (Mizutani et al., 2005). This may be related to the breakdown of the BNB as NK cells have been shown to bind vascular endothelial cells and induce lysis (Damle et al., 1987). Their presence would therefore exacerbate symptoms through secondary injury. This may also explain the effectiveness of IVIG, as NK cells are able to bind and inactivate monomeric IgG (Sulica et al., 1993). The NK cells would therefore be removed from circulation, which would lead to a possible improvement in the patient's condition.

1.4 The Peripheral Nervous System

The various components of the peripheral nervous system (PNS) can be targets of antibody mediated attack in autoimmune neuropathies. It is therefore essential

to understand the normal structure and function of the PNS to truly appreciate how these antibodies elicit their pathogenic effects.

1.4.1 Axons

All voluntary movements are transmitted to the periphery via axons, which are lengthy extensions of the cell bodies of neurons that originate in the CNS. Due to the distance between the terminals and the cell bodies, axons are insulated from one another and bundled together by 3 protective tissue layers. Axons, Schwann cells and endoneurial components are grouped together and surrounded by the perineurium to form a nerve fascicle. These fascicles in turn are bundled together by epineurial tissue to form a nerve (Topp & Boyd, 2006).

1.4.2 Nodes of Ranvier (NoR)

Within the endoneurium, a single Schwann cell is intimately associated with a single myelinated axon to form an internode. The gaps between these internodes are called Nodes of Raniver (NoR). These are specialised domains of the myelinated axon, which facilitate propagation of action potentials along the nerve.

The presence of CB in MMN is a good indication that anti-GM1 antibodies are targeting the NoR; however, they may also produce this effect through segmental demyelination of the axon. It has been shown that the NoR in distal sites are more susceptible to anti-ganglioside antibody mediated attack than proximal locations, which correlates with the distal dominant pattern observed in MMN (McGonigal et al., 2010). However, there is still no definitive evidence showing the site of injury in this condition.

1.4.3 Blood Nerve Barrier

The PNS like the CNS is an immune privileged site. It is protected from leukocyte infiltration by an interface, known as the blood nerve barrier (BNB),which forms between the endoneurial microenvironment and the surrounding extracellular space (Kanda, 2013). Despite the assumption that the BNB is weaker than the

blood brain barrier (BBB), evidence suggests that both are equally matched in preventing molecules entering the neural parenchyma (Poduslo et al., 1994).

There is some evidence to suggest that BNB disruption may be a factor in MMN pathogenesis, particularly due to the large size of IgM molecules. Limited experimental work *in vitro* has shown that addition of MMN patient sera to BNB cultures resulted in disruption of the tight junction molecules between epithelial cells in the BNB (Shimizu et al., 2014). This was thought to be related to the autocrine secretion of the cytokine VEGF but research into this area is limited.

1.4.4 Neuromuscular Junction

Although the neuromuscular junction (NMJ) is not thought to be targeted in MMN, it is a site of particular interest in autoimmune neuropathies due to the absence of any BNB (Yu, 2011). The NMJ is a specialised synapse, which facilitates the transmission of electrical impulses from the nerve terminal to the skeletal muscle. It comprises three main parts: the presynaptic region, the synaptic cleft and the postsynaptic surface (Hughes et al., 2006).

When the nerve terminal receive an action potential, vesicles containing pools of acertylcholine (ACh) fuse with the plasma membrane to release the neurotransmitter into the synaptic cleft. A large number of proteins including SNARE then recycle this transmitter and traffic it back to reserve pools to allow for continuous muscle stimulation (Südhof, 2004). This site is also enriched with a variety of gangliosides, which are easily accessed by anti-ganglioside antibodies. Immune mediated damage to the NMJ is therefore thought to be partially responsible for the acute flaccid paralysis observed in GBS (Goodfellow et al., 2005; Halstead, Humphreys, et al., 2005; Halstead, Morrison, et al., 2005).

Once Ach is released, it diffuses across the synaptic cleft and binds to the nicotinic acetylcholine receptor. This results in contraction of the innervated muscle.

1.5 Glycosphingolipids

Most autoimmune neuropathies are associated with antibodies which target a family of cell surface receptors known as glycosphingolipids (GSL) (Chiba et al., 1992a; Ilyas et al., 1991; Kusunoki et al., 1995; Nobile-Orazio et al., 1992; van den Berg et al., 1992). These are specialised amphipathic molecules that contain a lipid that is anchored to the cellular membrane and a carbohydrate group that projects into the extracellular space. The three main types of GSLs are globosides, cerebrosides and gangliosides, which are all ubiquitously expressed throughout the body but are particularly enriched within the nervous system. They have various roles in cell-cell interactions, signal transduction and are strongly associated with the maintenance and repair of the nervous system. (Furukawa et al., 2004; Hakomori et al., 1998; Kasahara et al., 1997; Kittaka et al., 2008)

1.5.1 Biosynthesis

All sphingolipids contain a hydrophobic core called ceramide, which anchors the molecules to the cell membrane. Ceramide is synthesised on the cytosolic side of the endoplasmic reticulum (ER) but is capable of alternating between the cytosolic and luminal sides via a carrier protein called FAPP2 (D'Angelo et al., 2007) (Figure 1.2). This transference of ceramide allows it to be used by luminal enzymes such as cerebroside galactose transferase (CGT) which forms galactocerebroside (GalC) through the addition of a galactose molecule. After transport to the Golgi apparatus, GalC can be further modified by the enzyme cerebroside sulfotransferase (CST) which adds a sulfate group to the 3-O-position of GalC to form sulfatide (Eckhardt, 2008).

Ceramide can also be translocated by the transport protein CERT to the golgi apparatus, where it can be glycosylated to form gangliosides (Hanada et al., 2007). The enzyme glucosylceramide (GlcCer) synthase adds a glucose group to the ceramide which is then flipped inside the golgi body by flippase. The addition of a galactose group by Gal T-1 then forms lactosyl ceramide (LacCer), which can then be sialyated by various enzymes to form the simple gangliosides.

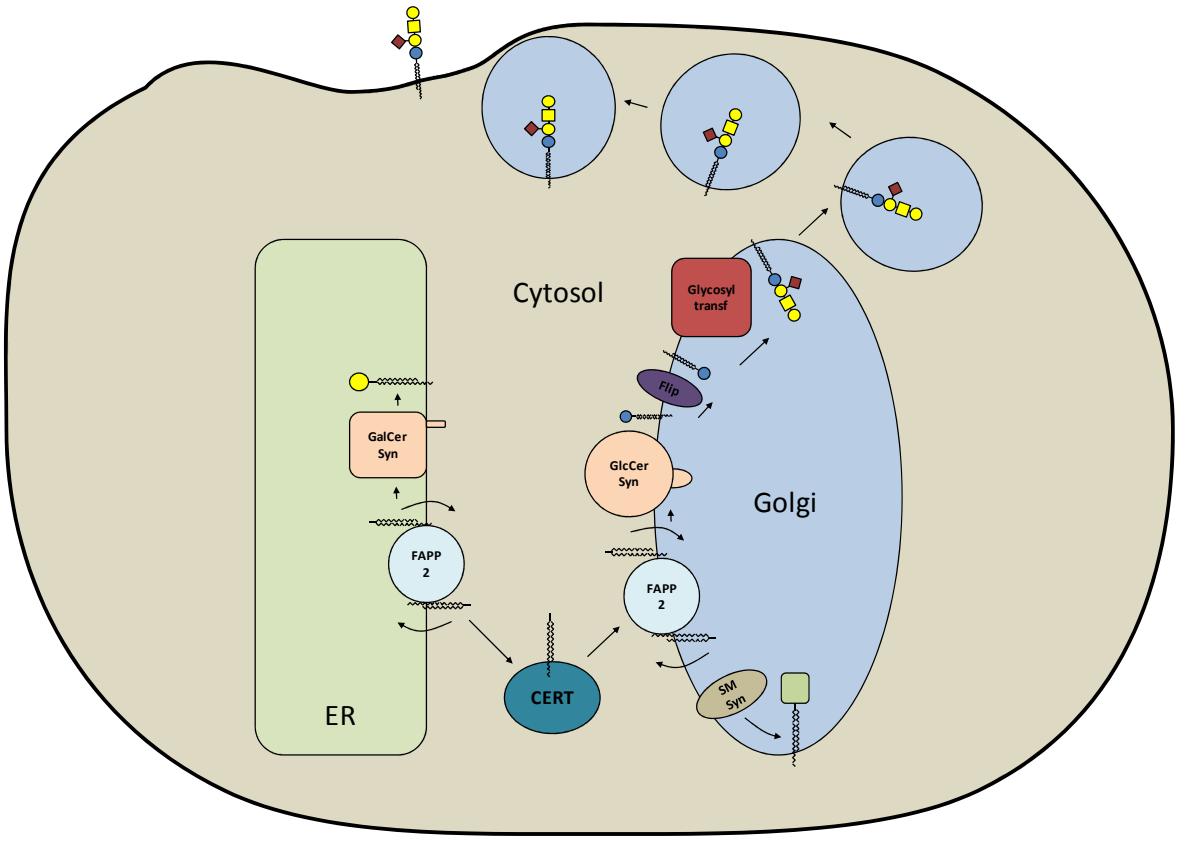


Figure 1.2 - Synthesis of Glycosphingolipids

Ceramide is synthesised in the cytosolic ER and can be internalised into the lumen by FAPP2 where GalCer Synthase can synthesise GalC. CERT can also transport ceramide to the golgi apparatus where it can be converted into SM or GlcCer. GlcCer is internalised by flippase and can be glycosylated by sialyltransferases to form gangliosides. These lipids migrate via transport vesicles to the plasma membrane where they are displayed to the extracellular environment. ER: endoplasmic reticulum; FAPP2: four-phosphate adaptor protein 2; GalCer: galactose cerebroside; CERT: ceramide transfer protein; SM: Sphingomyelin; GlcCer: Glucose Ceramide

The complex gangliosides of the O, a, b and c series are then formed by the step by step addition of sugar and sialic acid moieties by several different glycosyltransferases (Figure 1.3). Alternatively instead of forming gangliosides, the ceramide on the golgi apparatus can flip into the luminal side and be converted to sphingomyelin (SM) by the enzyme sphingomyelin synthase.

Once synthesised, these newly formed lipids concentrate in the luminal side of transport vesicles, which then migrate to the plasma membrane (Crespo et al., 2004). The vesicles fuse with the membrane and the embedded lipids are displayed into the extracellular environment where they are able interact with other cells.

1.5.2 Gangliosides

The most diverse species of GSL are gangliosides. These lipids have the same basic characteristics as both globosides and cerebrosides but have a large degree of variation in their headgroup and are unique in their expression of sialic (N-

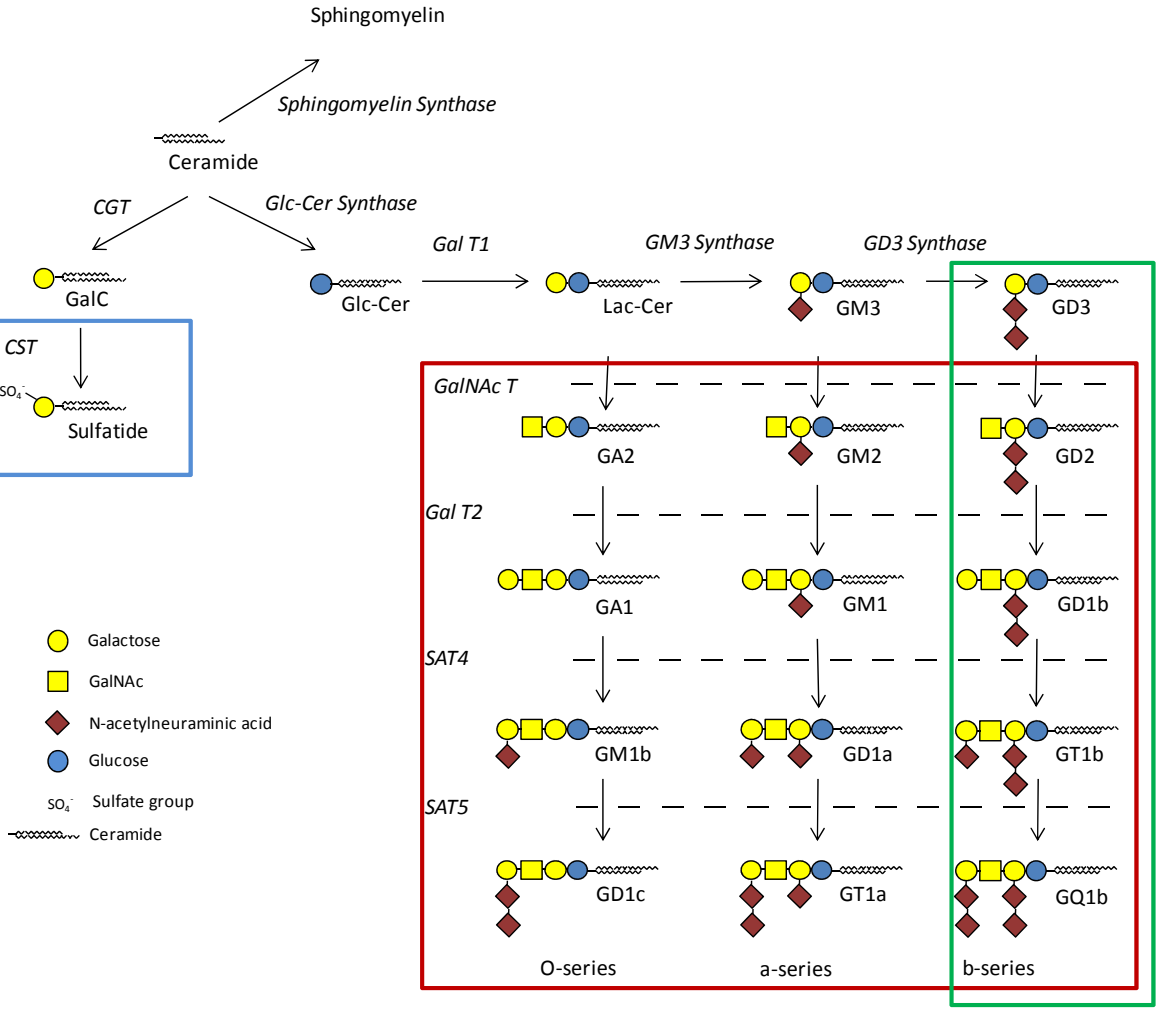


Figure 1.3 – Biosynthesis of Cerebrosides and Gangliosides

All cerebrosides and gangliosides are formed from a ceramide backbone. The addition of a galactose molecule by CGT forms GalC, which can by further modified by CST to form sulfatide. Alternatively sphingomyelin synthase can add a phosphocholine group to ceramide to form sphingomyelin. Gangliosides are formed by the addition of different oligosaccharide moieties to ceramide through the actions of several different glycosyl transferases. Several knockout mice have been created that are unable to synthesise particular glycolipids (outlined in boxes). These include CST-/- mice, which are unable to synthesise sulfatide (blue), GalNAcT-/- mice which are unable to synthesise complex gangliosides (red) and GD3 S-/- mice which are unable to synthesise b-series gangliosides (green).

acetylneuraminic) acid residues (Figure 1.3). They were first isolated from the brain of a patient suffering from Tay Sachs disease (Klenk, 1935) but it was not until subsequent characterisation studies that they were found to be enriched in the ganglion cells of the brain, leading to the adoption of the name gangliosides (Klenk, 1942).

1.5.2.1 Nomenclature

There are now over 200 described gangliosides which have been named according to the nomenclature laid out by Svennerholm (Svennerholm, 1964; Yu et al., 2007). This simplified system employs the use of a code where "G" denotes a ganglioside, whilst the use of a numeral prefix represents the number of sialic acids e.g. mono – M, di – D, tri – T, quad – Q. This is followed by a number which is determined by the migration of the lipids on TLC and is related to the size of the oligosaccharide chain. Finally, the use of a lowercase letter denotes the isometric arrangement of the sialic acids on the headgroup.

1.5.2.2 Distribution

Gangliosides are widely distributed in most vertebrae tissues (Table 1.3). They are particular enriched in the grey matter of the brain but are also found to be abundantly expressed in most tissues of the central and peripheral nervous systems (Rueda & Gil, 1998). The diversity of the ganglioside species are higher in these tissues compared to those elsewhere in the body, which is presumably related to their different functions.

GM1, for example, has been shown to act a co-receptor for fibroblast growth factor by binding and exposing the ligand for receptor binding. A similar process has been proposed to occur with neurotransmitters, such as glutamate and GABA, where GM1 binds the ligand and pulls it into a closer association with the membrane to optimise binding and neurotransmission (Reviewed by Fantini & Barrantes, 2009). This may explain the higher expression of GM1 in neural tissues; however, this has yet to be definitively proven.

Aside from differences in the ganglioside distribution, it has also been noted that the ganglioside profiles in tissues, particularly within the brain, change during

Table 1-3 – Ganglioside Distribution in Various Rat Tissues

Tissue	GM3	GM2	GM1	GD3	GD2	GD1a	GD1b	GT1b	GQ1b	Others
Cerebrum	Tr	Tr	9.4	1.5	Tr	40.2	16.4	24.3	8.2	
Cerebellum	Tr	Tr	4.5	3.5	7.9	31.2	8.8	30.0	13.6	
Spinal Cord	Tr	Tr	9.3	4.3	Tr	14.3	22.1	31.1	18.8	
Thymus	15.2			7.1						77.7
Lung	72.9			2.2		16.8				8.4
Heart	93.1	Tr		2.4		2.5				2.0
Liver	52.3	Tr	2.5	0.7		32.0	5.2			7.3
Stomach	62.9		Tr	14.8		10.5				11.8
Spleen	55.7	Tr	Tr	4.0		30.7	2.4			7.2
Intestine	74.9	Tr		20.7		1.2				3.2
Kidney	64.9	Tr		28.8		0.8				4.3
Testis	16.4					66.8				16.8
Bone Marrow	27.6	7.1	1.6			59.7				4.0
Buffy Coat						100.0				
Erythrocytes			36.5	22.3		41.2				

Gangliosides are expressed throughout the body but their distribution varies between tissues. Distribution is expressed as a percentage of total ganglioside content. Others refers to unidentified gangliosides. Tr: Trace Amounts

development and aging. The predominant species during embryogenesis is the simple gangliosides GM3 and GD3; however, at later stages the ganglioside profiles switch to contain significantly more complex gangliosides (Yu & Saito, 1989). The distribution of these gangliosides decreases again during aging (Posse de Chaves & Sipione, 2010), with their loss being linked to a number of neurodegenerative disorders, such as Alzheimer's and Parkinson's disease (Yamamoto et al., 2008).

1.5.2.3 Function

Gangliosides have a wide array of functions ranging from the modulation of protein activity and signalling to the maintenance of myelin-axon interactions and the formation of glycosynapses (Posse de Chaves & Sipione, 2010). The essential roles of gangliosides in the normal function of the nervous system have been best demonstrated through the generation of GalNAc T-/- mice, which lack the ability to synthesise complex gangliosides (Chiavegatto et al., 2000; Takamiya et al., 1996).

These knockout mice have been shown to develop tremors and deficits in balance, strength and coordination. As would be expected they also experience Wallerian degeneration and myelination defects, which indicates the essential roles complex gangliosides have in maintaining normal neural physiology.

1.5.3 Sulfatide

Sulfatide is another GSL that is often implicated in autoimmune neuropathies. It is composed of a GalC molecule that is sulfated in the 3-O position and is highly enriched in a variety of tissues including the kidneys, gastrointestinal tract, islets of Langerhans, trachea and myelin (Takahashi & Suzuki, 2012). It is a major component of the myelin sheath, where it forms approximately 6% of the total lipid content (Norton & Cammer, 1984) and is therefore of significant interest to autoimmune neuropathy researchers.

Prior to the creation of CST-/- mice, which lack the ability to synthesise sulfatide, very little was known about the glycolipids biological function. This was due to the limitations of *in vitro* experiments, which were only able to establish its most basic characteristics, such as its biosynthesis and its interactions with other proteins and lipids (Ishizuka, 1997).

Once the CST-/- mice were created, the physiological roles of sulfatide became clearer, particularly within the nervous system. Initial studies indicated that the glycolipid had a minimal role in development, as the mice were healthy up until the age of 6 weeks. However, after this point they began to develop hindlimb paralysis, pronounced tremor and progressive ataxia, which suggested that sulfatide was responsible for myelin sheath maintenance (Honke et al., 2002).

Surprisingly, the compact myelin was preserved in these mice but abnormalities were observed in the paranodal junctions, which were found to turn away from the axon. Subsequent studies also shown that certain nodal proteins such as neurofascin 155 and Caspr were incorrectly localised, suggesting that sulfatide has a role in protein trafficking (Ishibashi et al., 2002; Schafer et al., 2004).

These findings correlated well with observations made in patients suffering from demyelinating conditions, which are often associated with anti-sulfatide antibodies (Alpa et al., 2007; Andersson et al., 2002; Ilyas, 2003; Souayah et al.,

2007). This suggests that they may be responsible for disease pathogenesis; however, this has yet to be proven due to a lack of experimental evidence.

1.5.4 Plasma Membrane

The fundamental structure of the plasma membrane is the lipid bilayer. It consists of a number of major phospholipids and GSLs, which due to their amphipathic nature are able to form a barrier between the cytoplasm and the extracellular environment. This prevents the free diffusion of ions and proteins, which allows the cell to maintain its osmotic balance (Cooper & Sunderland, 2000).

The four major phospholipids of the plasma membrane include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM), (Cooper & Sunderland, 2000). These lipids are asymmetrically distributed between the two layers, with the inner leaflet consisting mainly of PS and PE; whilst PC and SM form the majority of the outer leaflet.

1.5.4.1 Lipid Rafts

The classical hypothesis for the organisation of the plasma membrane was the fluid mosaic model (Singer & Nicolson, 1972). In this model, proteins were likened to icebergs freely floating in a sea of lipids but subsequent research has established that membranes are much more highly organised than originally proposed (Pike, 2003).

They are now known to contain lipid rich microdomains called lipid rafts. Unlike the general membrane of the outer leaflet, which is enriched with PC, rafts are found to express high quantities of Chol, SM, GSLs and lipid associated proteins. The identification of these rafts, however, has been a controversial topic due to the methods by which they were first isolated (K. Simons & Ikonen, 1997).

Initially research focussed on identifying raft components based upon their resistance to non-ionic detergent solubilisation at 4°C. If these components were solubilised following treatment with methyl-β-cyclodextrin, then it strongly supported their classification as raft proteins (K. Simons & Gerl, 2010). The

artificial nature of these techniques, however, coupled with the inability to directly observe these rafts in cells, led some researchers to dispute their existence (Shaw, 2006).

Recent advances in new technology appear to have addressed these doubts. Biophysical analysis, in particular, has demonstrated the presence of two membrane phases: liquid ordered and liquid disordered. It appears that raft lipids, such as SM, Chol and GSLs, are associated with the liquid ordered phase, whilst other lipids such as PC are found in the liquid disordered phase (Feigenson, 2006). Studies using giant unilamellar vesicles have demonstrated how altering the composition of the lipid mixture can change the size of these domains (Elson & Genin, 2010). This acts as proof that they can spontaneously form in membranes; however, this has yet to be directly observed in biological tissue.

Additional improvements in microscopy techniques and the employment of fluorescently labelled lipids and proteins have also provided good evidence for the existence of rafts but further work is required to localise them in the plasma membrane.

1.6 Anti-Glycolipid Antibodies

As discussed previously, anti-glycolipid antibodies are associated with a wide range of autoimmune neuropathies. These antibodies can target various antigens depending upon their initial causative factor and, as such, can bind and injure a host of different tissues throughout the body.

1.6.1 Anti-Sulfatide Antibodies

Anti-sulfatide antibodies have been associated with a variety of different diseases including systemic lupus erythematosus, GBS, MS and type 1 diabetes (Alpa et al., 2007; Andersson et al., 2002; Ilyas, 2003; Souayah et al., 2007). The extensive range of these antibodies is related to the diverse expression of sulfatide, which is found in many tissues but is particularly enriched in the myelin sheath.

It is the roles of antibodies which bind to this tissue that are of significant interest to neuropathy researchers. This is due to evidence that patient's with demyelinating neuropathies produce anti-sulfatide antibodies that are capable of binding and injuring myelin (Petratos et al., 2000). Similarly, experimental evidence has shown that the pre-eminent mouse anti-sulfatide antibody, O4, can produce demyelination and dysmyelination in cultures (Elliott et al., 2012; Rosenbluth & Moon, 2003) and is capable of inducing spinal cord lesions when implanted *in vivo* in a model of MS (Rosenbluth et al., 2004).

Despite this evidence, certain studies have suggested that human anti-sulfatide antibodies may be incapable of binding live tissue (Brennan et al., 2011). This may be related to antibody diversity; however, this has yet to be definitively proven as most experimental studies have limited themselves to the use of O4. Further studies of anti-sulfatide antibodies are therefore required to more fully establish their roles in disease.

1.6.2 Anti-Ganglioside Antibodies

The first study to associate anti-ganglioside antibodies with disease was performed in 1992, in which researchers discovered high titres of anti-GQ1b antibodies in the sera of MFS patients (Chiba et al., 1992b). Subsequent studies established that the cranial nerves responsible for ocular control were enriched in GQ1b, which suggested that the antibodies themselves were responsible for the ophthalmoplegia that is strongly associated with the disease (Chiba et al., 1993, 1997).

Leading on from this discovery, other autoimmune neuropathies were associated with specific anti-ganglioside antibodies. AMAN was associated with IgG antibodes targeting GM1 and GD1a (Yuki & Hartung, 2012), whilst MMN was associated with IgM antibodies targeting GM1 (Pestronk et al., 1997). As with MFS, researchers began associating the presence of these antibodies with specific symptoms as it was assumed that they were the cause of pathogenesis.

This led to some anomalies, however, as certain gangliosides are located throughout the nervous system but not all tissues are targeted in disease. Several theories were put forth to explain these behaviours including differences in the gangliosides molecular composition, local microenvironment and density

(Corbo et al., 1992; Ganser et al., 1983; Ogawa-Goto et al., 1990) but it was not until the use of ganglioside complexes that researchers were able to adequately explain differences in antibody binding patterns.

1.6.2.1 Ganglioside Complexes

Ganglioside complexes are a relatively new feature of clinical research (Figure 1.4). They were first employed in a study in 2004 in an attempt to increase antibody detection in GBS patient serum (Kaida et al., 2004). In this study antibodies were detected against complexes composed of GD1a and GT1b but minimal signals were detected against the single species. It was therefore concluded that the antibodies were binding to clustered epitopes formed by the combination of the two gangliosides.

Subsequent research by the same group found that 17% of GBS patients had antibodies against a series of ganglioside complexes (Kaida et al., 2007). Furthermore, they identified an association between the presence of antibodies against GD1a:GT1b and GD1a:GD1b complexes and the requirement for mechanical ventilation. This suggested that ganglioside complexes may be useful indicators for the severity of the disease and could be useful for establishing effective treatment plans.

Following on from these findings other researchers found that the interactions of different gangliosides may shield binding epitopes from antibody access. This was first demonstrated in a study using an anti-GM1 antibody that was incapable of binding live tissue (Greenshields et al., 2009). Examination of the antibody's binding patterns in solid phase assays indicated that it was inhibited from binding GM1 when it was in complex with most other gangliosides. GD1a was found to cause the most inhibition, which led the researchers to postulate that this ganglioside may be preventing the antibody from accessing its binding epitope. This was confirmed by treating the tissue with neuraminidase, which cleaves the terminal sialic acid of GD1a to form GM1. Antibody binding was detected following this treatment, proving that GD1a was having an inhibitory effect.

This same phenomenon was discovered in a clinical study of MMN patient sera. GM1:GD1a complexes were found to inhibit or severely reduce the binding ability

of anti-GM1 antibodies in all MMN positive patients (Nobile-Orazio et al., 2010). This raised the possibility that the restricted binding patterns observed in this condition were related to antibody access.

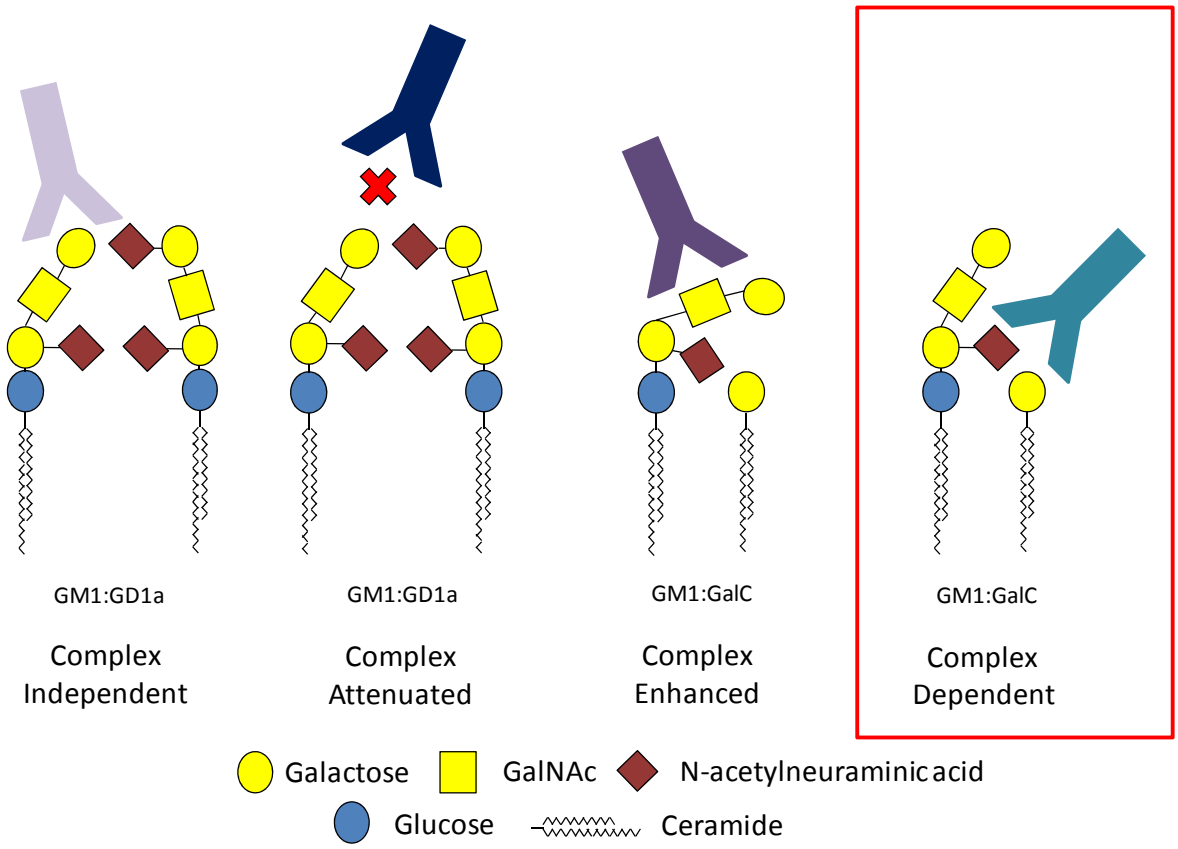


Figure 1.4 – Impact of Ganglioside Complexes on Antibody Binding

The presence of other gangliosides or glycolipids influences the presentation of different binding epitopes on the GM1 headgroup. This has differential effects on the binding abilities of antibodies as follows: complex-independent antibodies can bind to GM1 regardless of its configuration; complex-attenuated antibodies are unable to bind GM1 in the presence of particular glycolipids; complex-enhanced antibodies are better able to access their epitope when certain lipids are present. Another antibody subtype, termed complex-dependent antibodies, has been proposed to exist (orange box). These antibodies bind to a neo-epitope formed by the combination of two different lipids.

Further analysis of ganglioside complexes in MMN led to the discovery of the enhancing effects of GM1:GalC. Although this had been described almost 15 years previously (Pestronk et al., 1997), the findings from this study had been largely overlooked by researchers. It was not until 2013 that there was a resurgence of interest in GM1:GalC complexes following a study that found

antibody enhancement in 100% of patients screened (Galban-Horcajo et al., 2013).

These results were confirmed by other research groups (Nobile-Orazio et al., 2013), but there was some debate as to the cause of enhancement. Whilst some researchers believed that GalC was altering the orientation of GM1 to better expose certain binding epitopes, others thought that the two glycolipids were interacting to form a neo-epitope.

There was some evidence to support the latter theory as a subset of patients in these studies were found to have antibodies that bound solely to the GM1:GalC complex. This suggested that a complex-dependent antibody was forming in these patients but this is an antibody class that has yet to be isolated from either patients or animal models.

1.7 Aims

The discovery of antibodies that appear to bind specifically to GM1:GalC complexes has opened up a new avenue of research in MMN. As these antibodies appear to be more prominent in patient sera than those against single gangliosides, we have hypothesised that they may be responsible for the pathogenesis of the disease. Furthermore, it may be that GM1:GalC complexes are the true targets of immune mediated attack in MMN, which may explain the selective injury of the motor nerves found in this condition.

To investigate the existence of these antibodies this thesis therefore proposed the following:

- Detailed analysis of the binding patterns of current human monoclonal antibodies to establish their cis-inhibitions and cis-enhancements
- Determine the effect that secondary lipid concentration has upon antibody binding
- Clone a complex-dependent antibody directly from MMN patient serum
- Clone a complex-dependent antibody through active immunisations with mice
- Establish a high throughput screening method for hybridoma supernatant that is optimised for detecting complex-dependent antibodies

- Characterise the binding behaviours of any new antibodies through the use of solid phase assays, tissue section staining and *ex vivo* preparations

In addition to these aims, this thesis also sought to address several important questions relating to CST-/- mice and sulfatide. As such it also aimed to:

- Determine the susceptibility of CST-/- mice to immunisation with sulfatide liposomes
- Produce a series of new anti-sulfatide antibodies
- Determine the binding behaviours of these antibodies to solid phase assays and live tissue

2 METHODS

2.1 Materials

2.1.1 Buffer Solutions

Phosphate buffered saline (PBS) was prepared in deionised water at a final concentration of 140 mM NaCl, 1.5mM KH_2PO_4 , 2.7 mM KCl and 77 mM Na_2HPO_4

Ringer's solution was prepared to a final concentration of 116 mM NaCl, 4.5 mM KCl, 23 mM $NaHCO_3$, 1 mM NaH_2PO_4 , 11 mM glucose, 1 mM MgCl2 and 2 mM $CaCl_2$ in distilled water. Oxygen was bubbled through the solution for 10 minutes prior to its use. Its pH was adjusted to approximately 7.2 using 1mM HCl.

2.1.2 Glycosphingolipids

All gangliosides and some glycolipids including GalC, sulfatide, and PS were derived from bovine brain and were purchased from Sigma Aldrich (St Louis, MO, USA). Cholesterol and DCP were also purchased from Sigma Aldrich. Sphingomyelin and PC were derived from egg and purchased from Carbosynth (Compton, UK) and Avanti Polar Lipids (Alabaster, USA) respectively. LM1 and whole lipid extract (WLE) were kindly gifted by Robert Yu (Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, USA).

2.1.3 Secondary Antibodies

A variety of secondary antibodies were used for imaging solid phase assays, cells, fixed tissue sections and live tissue preparations. These antibodies are listed in Table 2.1 alongside their specificities, manufacturers and working concentrations.

2.1.4 Fluorescently Labelled Markers

Cholera toxin B subunit (CTx) conjugated to Alexafluor 647 acted as a ganglioside marker, specifically GM1, and α-bungarotoxin (BTx) conjugated to either Alexafluor 555 or Alexafluor 488 was used to delineate the NMJs by

Table 2-1 – List of Secondary Antibodies

Target Antigen	Target Species	Host Species	Conjugate	Manufacturer	Function	Concentration or Dilution
IgG (Fcγ)	Mouse	Goat	FITC	Southern Biotech (Birmingham, AL, USA)	Tissue Staining	3.33µg/ml
IgG (Fcγ)	Mouse	Goat	TRITC	Southern Biotech (Birmingham, AL, USA)	Tissue Staining	3.33µg/ml
IgG (Fcγ)	Mouse	Goat	AF 555	Jackson Immuno (West Grove, PA, USA)	Tissue Staining	3.33µg/ml
IgG (Fcγ)	Mouse	Goat	AF 647	Jackson Immuno (West Grove, PA, USA)	Tissue Staining Arrays	Tissue: 6.7µg/ml Arrays: 2µg/ml
IgG (Fcγ)	Mouse	Goat	HRP	Southern Biotech (Birmingham, AL, USA)	ELISA	1:3000
IgG1	Mouse	Goat	HRP	Southern Biotech (Birmingham, AL, USA)	ELISA	1:3000
IgG2a	Mouse	Goat	HRP	Southern Biotech (Birmingham, AL, USA)	ELISA	1:3000
IgG2b	Mouse	Goat	HRP	Southern Biotech (Birmingham, AL, USA)	ELISA	1:3000
IgG3	Mouse	Goat	HRP	Southern Biotech (Birmingham, AL, USA)	ELISA	1:3000
IgM (µ)	Mouse	Goat	TRITC	Southern Biotech (Birmingham, AL, USA)	Tissue Staining	6.7µg/ml
IgM (µ)	Mouse	Goat	AF 555	Jackson Immuno (West Grove, PA, USA)	Tissue Staining Arrays	Tissue: 6.7µg/ml Arrays: 2µg/ml
IgM (µ)	Mouse	Goat	AF 647	Jackson Immuno (West Grove, PA, USA)	Tissue Staining Arrays	Tissue: 6.7µg/ml Arrays: 2µg/ml
IgM (µ)	Human	Goat	AF 647	Jackson Immuno (West Grove, PA, USA)	Arrays	2µg/ml
IgG (H+L)	Rabbit	Goat	FITC	Life Technologies, Carslbad, CA, USA	Tissue Staining	3.33µg/ml
IgG (H+L)	Rabbit	Goat	TRITC	Life Technologies, Carslbad, CA, USA	Tissue Staining	3.33µg/ml
IgG (H+L)	Rat	Goat	FITC	Life Technologies, Carslbad, CA, USA	Tissue Staining	3.33µg/ml

binding to the post-synaptic nicotinic acetylcholine receptors on the muscle surface. Both of these toxins were purchased from Life Technologies, Carlsbad, CA, USA. Rabbit anti-S100 acted as a Schwann cell marker and was purchased from Dako, Santa Clara, CA, USA.

2.1.5 Tissue Culture Media

Roswell Park Memorial Institute (RPMI) 1640 media (Sigma Aldrich, St Louis, MO, USA) was used for growing all hybridoma cultures and frozen cell lines. RPMI 1640 with glutamax was used for long term culture to reduce the build up of ammonia and improve the long term growth of the cells. Media was normally

supplemented with 10% or 20% heat inactivated foetal calf serum (FCS) (Gibco Life Technologies, Carlsbad, CA, USA) and was marked as 10% or 20% accordingly. All media was supplemented with pen/strep (20U/ml, 20µg/ml) (Life Technologies, Carlsbad, CA, USA), fungin (10µg/ml) (San Diego, CA, USA) and gentamicin (50µg/ml) (Life Technologies, Carlsbad, CA, USA).

Hybridoma cells were originally cultured with 10% Opticlone (Santa Ana, CA, USA) but this product was discontinued. This was replaced with 5% HyMax (Antibody Research Corporation, St Charles, MO, USA). Media containing these feeder supplements were marked as "+FS".

Schwann cells were grown in two different media referred to as basic growth media (BGM) and Schwann cell growth media (SGM). The BGM consisted of high glucose DMEM + L-glutamine (Life Technologies, Carslbad, CA, USA), 10% horse serum (Life Technologies, Carslbad, CA, USA), 2ng/ml human heregulin-β1 (Sigma Aldrich, St Louis, MO, USA), 0.5µM forskolin (Sigma Aldrich, St Louis, MO, USA), pen/strep (20U/ml, 20µg/ml) (Life Technologies, Carlsbad, CA, USA), fungin (10µg/ml) (San Diego, CA, USA) and gentamicin (50µg/ml) (Life Technologies, Carlsbad, CA, USA).The SGM consisted of the same components as the BGM plus 10ng/ml human basic fibroblast growth factor (Sigma Aldrich, St Louis, MO, USA) and 20µg/ml bovine pituitary extract (Sigma Aldrich, St Louis, MO, USA).

2.2 Animals

Mice were all housed in the university central research facility under controlled conditions. These consisted of 12 hour light/dark cycles in temperature and humidity controlled rooms with food and water provided ad libitum. All immunisations were performed on 6-10 week old male and female mice weighing at least 15g unless otherwise stated. The weights of the mice were monitored throughout the immunisation cycles to ensure that the antigens were not having any adverse effects. Immunofluorescence studies were all carried out on 4-10 week old male and female mice.

Animals were euthanized with a rising concentration of CO_2 as per Home Office guidelines. Following cessation of breathing, death was ensured through a secondary measure, such as cervical dislocation or cardiac puncture.

2.2.1 Transgenic Mice

Different transgenic mice were used for active immunisations and tissue staining depending upon the requirements of the specific experiment. These mice and their uses are listed in Table 2.2.

Mice lacking the enzyme GalNAc transferase (GalNAc T-/-) only synthesise simple gangliosides and were a good candidate for the production of anti-GM1 complex antibodies (Chiavegatto et al., 2000). These were therefore used for all experiments involving immunisations against gangliosides complexes.

A knockout mouse lacking the enzyme cerebroside sulfotransferase (CST) was unable to synthesise sulfatide (Honke et al., 2002). These mice were used for immunisations with sulfatide, as it was hypothesised that they would be antigen naïve and, as a result, produce higher titres of antibody. The intention was to use these mice for immunisations involving ganglioside:sulfatide complexes.

A mouse known as GD3 S-/- lacked the enzyme GD3 synthase and was unable to synthesise any of the b series gangliosides (Handa et al., 2005). As a result of this modification, the mice overexpress the a-series ganglioside, especially GM1 and GD1a. These were therefore useful in determining the binding capabilities of anti-GM1 and anti-GD1a antibodies in tissue studies.

Litter mates that contained the normal repertoire of gangliosides or sulfatide were used as controls in both immunisations and tissue staining experiments.

2.2.2 Fluorescent Mice

Some lines of the GalNAc T-/- and GD3-/- mice also had green fluorescent protein (GFP) and cyan fluorescent protein (CFP) endogenously expressed in cells utilising the S100B (neural crest cells) and Thy1 (neuronal) promoters respectively (Kang et al., 2004; McGonigal et al., 2010; Rupp et al., 2012). This resulted in mice that had fluorescent axons and Schwann cells which allowed direct imaging of the tissue. These mice were used for immunofluorescent staining with antibodies and not for the immunisations themselves.

Table 2-2 – Use of Transgenic Mice Throughout Thesis

Genotype	Background	Fluorescence	Number	Experiment
GalNAc T-/-	C57Bl/6	N/A	23	Immunisations
GalNAc T+/+	C57Bl/6	N/A	24	Immunisations
GalNAc T-/-	C57Bl/6	N/A	10	*Ex vivo* preparations
GalNAc T+/+	C57Bl/6	N/A	10	*Ex vivo* preparations
GalNAc T-/-	C57Bl/6	Axon-CFP Glial-GFP	10	*Ex vivo* preparations
GalNAc T+/+	C57Bl/6	Axon-CFP Glial-GFP	17	*Ex vivo* preparations
GalNAc T-/-	C57Bl/6	N/A	2	Sciatic Nerve Sections
CST-/-	C57Bl/6	N/A	6	Immunisations
CST+/+	C57Bl/6	N/A	3	Immunisations
CST-/-	C57Bl/6	N/A	4	*Ex vivo* preparations
CST+/+	C57Bl/6	N/A	4	*Ex vivo* preparations
CST-/-	C57Bl/6	N/A	1	Schwann Cell Cultures
CST+/+	C57Bl/6	N/A	1	Schwann Cell Cultures
CST-/-	C57Bl/6	N/A	1	Sciatic Nerve Sections
CST+/+	C57Bl/6	N/A	2	Sciatic Nerve Sections
GD3 S-/-	C57Bl/6	Axon-CFP Glial-GFP	12	*Ex vivo* preparations
GD3 S+/+	C57Bl/6	Axon-CFP Glial-GFP	12	*Ex vivo* preparations
C57Bl/6	C57Bl/6	Axon-CFP Glial-GFP	2	*Ex vivo* preparations
C57Bl/6	C57Bl/6	N/A	2	*Ex vivo* preparations
B6CGTGNxDBA	C57Bl/6 + DBA	Axon-CFP Glial-GFP	3	Control Sera
B6CGTGNxDBA	C57Bl/6 + DBA	Axon-CFP Glial-GFP	3	Immunisations
DBA	DBA	N/A	3	Immunisations
CST-/-xGalNAcT-/-Thy1	C57Bl/6	N/A	1	Immunisations

2.2.3 Genotyping and Phenotyping

The mice were all bred and genotyped by a colleague prior to commencement of the experiments. Once the mice had been euthanized, additional tissue was retained in case unusual serological responses were detected. In this situation, the tissue was re-genotyped to confirm that it was from the correct strain.

Fluorescence was confirmed phenotypically by examination of ear punches removed from each mouse. The skin from the ear punches was split apart and

mounted on a slide with Citifluor and coverslipped. The sample was then excited at 474nm and 433nm using an epifluorescent microscope to identify GFP and CFP fluorescence respectively.

2.3 Human Serum Samples

Serum samples were collected from MMN, ALS, and CMT patients alongside healthy controls by the referral centre for ALS and neuromuscular diseases in Nice, France. Patients gave informed consent and the research was approved by the South Glasgow and Clyde Research Ethics Committee. Upon collection, blood was centrifuged at 400xg for 10 minutes. The serum was then aspirated, aliquoted into microtubes and frozen at -80°C prior to use.

2.4 Screening for Anti-Ganglioside Antibodies

2.4.1 Preparation of Lipid Stocks

Stock solutions of lipids were made up in a 1:1 (v/v) methanol:chloroform mixture, at 1-10mg/ml and stored at -20°C. GalC was more difficult to dissolve than the other lipids and was made up in a 1:2 (v/v) methanol:chloroform mixture at 1mg/ml. Working solutions were prepared for w:w arrays by further dissolving the lipids in 300µl of methanol to a concentration of 0.1mg/ml. Complexes were prepared by adding 150µl of each single lipid to a vial and mixing.

All the lipids were stored in glass micro vials (Chromacol, Waltham, MA, USA), which were compatible for use with the combinatorial glycoarray. Mol:mol arrays were prepared by dissolving the main target lipid in the array in 300µl of methanol to a concentration of 0.1mg/ml. All other lipids were prepared at an equivalent number of moles to the original lipid. The only exception was cholesterol, which was consistently made as 5 times the number of moles of other lipids to more closely resemble the proportion in cell membranes (Bowes et al., 2002).

2.4.2 ELISA

The traditional method for screening for anti-ganglioside antibodies is the enzyme linked immunosorbent assay (ELISA), which was performed according to the methods developed at the Southern General Hospital, Glasgow, UK (Willison et al., 1999). Working solutions of single glycosphingolipids were prepared at 2µg/ml in methanol. Complexes were created to the same final concentration by mixing equal volumes of the component lipids and then sonicating the mixture for 15 minutes. Negative wells consisted of methanol only.

Immulon 2 HB 96-well ELISA plates (Thermo Scientific, Waltham, MA, USA) were prepared by adding 100µl of glycosphingolipid to every odd row and 100µl of methanol to every even row so that each sample also had a negative control. These plates were then dried in a fume hood overnight. Plates were blocked by adding 200µl of 2% BSA/PBS to each well for 1 hour at 4 °C. The solution was tipped off and the plate tapped dry before the primary solution was added.

The primary solutions were prepared as follows: serum samples were prepared at a 1:100 dilution in 0.1% BSA/PBS; monoclonal antibodies were added at 10µg/ml in 0.1% BSA/PBS and tissue culture supernatant was added neat. 100µl of primary solution was added to both the glycosphingolipid and the control wells except for tissue culture supernatant which had only 50µl added to each well due to the limited volumes. The plates were incubated for either 4 hours at 4°C or overnight. They were then washed 4 times in an ELISA plate washer (RDX Dynex microtiter dispensing system, Chantilly, VA, USA) by cyclically adding 300µl of PBS to each well and tipping it off.

100µl of the appropriate secondary HRP conjugated antibody was then added at a 1:3000 dilution to each well and incubated at 4°C for 1 hour. The plates were washed on the ELISA plate washer as above. They were developed by adding 100µl of an o-phenylenediamine (OPD) solution to each well for 20 minutes at room temperature. This reaction produces a colour change from clear to orange/brown when antibody is detected. It was terminated through the addition of 50µl of 4N H_2SO_4 and then read on an ELISA plate reader (Sunrise, Tecan, Männedorf, Switzerland) at 492nm.

2.4.3 Quantitative ELISA

Quantitative ELISAs were performed to determine the concentration of IgM monoclonal antibodies in tissue culture supernatant. These were performed using kits provided by Bethyl Laboratories, Montgomery, TX, USA. The appropriate capture antibody was diluted 1 in 100 in coating buffer and 100µl was added to each well in the odd numbered rows of an ELISA plate (Immulon, Thermo Scientific, Waltham, MA, USA). The even numbered rows contained 100µl of coating buffer alone.

Once, the coating buffer was added the plates were incubated at room temperature for 1 hour. This was followed by 6 wash cycles in 0.05% Tween 20/PBS (Sigma Aldrich, St Louis, MO, USA). The wells of each plate were then blocked with 200µl of 2% BSA/PBS at room temperature for 1 hour followed by a wash cycle as described.

To establish the concentration of the antibodies a standard was prepared as per the manufacturer's instructions. This consisted of a serial dilution of a reference serum in 0.1% BSA to give specific concentrations. A 1:1 serial dilution was also prepared of the supernatant samples being tested and 100µl of each was added to both the antibody coated wells and the control wells at room temperature for 1 hour. This was followed by another wash cycle in 0.1% Tween 20. 100µl of the appropriate HRP conjugated secondary antibody was then added to each well at a dilution of 1:10000. The plates were then washed one last time before they were developed following the same method as per the standard ELISA.

The optical density (OD) readings of the standard were plotted against the corresponding log concentration to give a sigmoidal curve. Only sample ODs which fell between the steepest points in the curve were used to extrapolate the concentration of the antibody. Multiple OD readings of the unknown antibody were taken, and the corresponding concentrations of the standards at these OD values were then multiplied by the dilution factors of the unknown antibody. The final concentration of the unknown was calculated from the average of these readings.

2.5 Screening Development

2.5.1 Fluorescent Secondary Antibodies

Screening of the antibodies on arrays employed the use fluorescent secondary antibodies which had not been previously tested. The benefit of using fluorescent antibodies was that different fluorophores could be conjugated to different isotypes and used for screening simultaneously. An experiment was performed prior to their use to ensure the antibodies were not capable of binding to one another. If this occurred then artificial signals could be produced suggesting the presence of an antibody that was in fact not present. This was tested by printing the different secondary antibodies on slides using the microarray and then screening them against the opposing antibody. This was performed following the protocol in Section 2.5.4 except that the primary step was omitted. The result indicated that the secondary antibodies did not bind to one another and were suitable for use in screening.

2.5.2 Mouse Control Serum

The variability between print runs on the combinatorial glycoarray could occasionally be high depending upon a number of factors related to the normal operation of the equipment. To ensure that this variation was not skewing the sample results, a control serum was created which was applied during each experiment and used to normalise the data.

Three DBA mice were immunised with GM1 liposomes created as per the section 2.6. These mice were selected as they had previously been shown to respond well to immunisation and were readily available. The mice were injected with 100μg of liposome each three times a week. After six immunisations the mice were killed and their serum was collected and pooled.

This serum was screened against a standard grid of lipids at several different dilutions to establish the antibody response as per section 2.5.4. The optimal dilution was selected and used in each screening of mouse serum to normalise the data.

2.5.3 IgG Monoclonal Control

The mouse serum did not have a high IgG response so an in-house monoclonal antibody called DG2 was used to normalise IgG data. A serial dilution was created of the antibody as per the mouse control serum and the optimal antibody concentration was used to normalise all mouse IgG data.

2.5.4 Combinatorial Glycoarray

Slides were prepared by affixing a low fluorescence PVDF membrane (Millipore) to 26x75 mm slides (Thermo Scientific, Waltham, MA, USA) using a PhotoMount spray adhesive (3M, St Pauls, MN,USA). Excess membrane was removed using a scalpel blade and the bottom right hand corner was cut out to orientate the slides. Lipids were printed onto slides using an automatic TLC autosampler from CAMAG, Switzerland. The lipid vials were fitted with rubber caps which could be pierced by the autosampler and the vials were sonicated for 15 minutes prior to use. The slides were marked with a pencil and secured in a metal grid in the autosampler to ensure that they did not move during printing. The winCATS software (CAMAG, Muttenz, Switzerland) was used to create programs that printed 0.1µl of the lipids onto a predefined area of the slides. Once printing was complete, the slides were air dried for 10 minutes and a hydrophobic pen (Vector Laboratories, Peterborough, UK) was used to mark the boundaries of the array. The slides were then stored at -4°C overnight.

The protocol for probing the slides was adapted from the standard ELISA. All incubations were carried out in a humid chamber which consisted of a sealable slide holder box that contained tissue soaked in dH_2O. The slides were blocked in 100ml of 2% BSA/PBS at room temperature and placed on an orbital shaker set to 100rpm for 1 hour. The concentration of the primary sample differed depending upon the specific experiment but generally serum was applied at a 1:100 dilution and antibodies were applied at 10µg/ml. After blocking, excess liquid was tipped off and 300µl of the primary sample was applied to each slide at 4°C for 1 hour. The samples were then tipped off the slides and they were washed twice in 1% BSA/PBS on an orbital shaker set to 100rpm at room temperature for 15 minutes. The appropriate secondary antibodies were applied at the concentrations

described in section 2.1.2. 300µl was applied to each slide and these were incubated at 4°C for 1 hour. This was followed by two wash cycles in 1% BSA/PBS for 30 minutes, a wash cycle in PBS for 5 minutes and then a wash cycle in dH_20 for another 5 minutes. These all took place at room temperature on an orbital shaker set to 100rpm. The slides were then air dried at 4°C for approximately 30 minutes prior to imaging.

2.5.5 Lipid Microarray

The lipid microarray (Scienion, Berlin, Germany) was an alternative form of printing large arrays of lipids. The machine was capable of producing 20 slides, each consisting of 16 arrays, which could each be composed of up to 400 lipids targets. Slides were prepared for the microarray in the same way as the combinatorial glycoarray except that the hydrophobic pen was not used to mark the working area. Stock lipids were also prepared in a similar way but were made at a concentration of 200 µg/ml. The slides were locked in place in the microarray to prevent movement during printing. To limit sample loss through evaporation, each lipid was sonicated 4 minutes prior to use and 40µl was added to the machine. The microarray then collected the sample using a glass needle and printed 6 spots of 300 pl onto each array. A row of fluorescent markers, above and below each array, were also printed to allow for orientation of the slides and individual targets in the scanner. Once printing was complete the slides were stored at 4°C until required.

These slides were probed in the same way as the combinatorial glycoarray slides with some minor differences. A FAST Frame (Maine manufacturing, USA) was used to secure a 16 well incubation chamber to each slide which allowed different samples to be applied to each array. An IgG and an IgM monoclonal antibody were also added to each slide to act as positive controls. The incubation times and wash cycles were the same as the combinatorial glycoarray protocol except that after the primary incubation 100µl of 1% BSA/PBS was added to each well and aspirated 3 times to prevent transference of samples between wells.

2.5.6 Array Imaging

The arrays were imaged using either a PerkinElmer Scan Array Express (PerkinElmer, Waltham, MA, USA) or a Sensovation FLAIR scanner (Sensovation, Radolfzell, Germany). The former employed the use of a 633nm laser to excite the fluorescent secondary antibodies on each slide. Different photomultiplier tube (PMT) settings could be used to visually enhance the signals and the images produced could be optimised by manipulating their colour or black settings (this did not alter the raw data used for quantification). The images were quantified using the accompanying software which associated a number between 0 and 65535 with each pixel. Adaptive circles were used to identify each spot which had the local background subtracted from the median spot intensity. This value was taken as a representation of the binding capabilities of each antibody to the specific antigen contained within that spot.

The Sensovation scanner used high power LED illumination with a green/red channel which was capable of exciting at 549/647nm. Each channel had a corresponding emission filter to prevent bleed through of opposing signals however it was found that intense signals or prolonged exposure of the 647 channel were detectable in the 549 channel. In such cases the exposure time was reduced, for all arrays within the same experiment, to circumvent this issue. Four slides could be scanned at the same time and once processed were quantitated using the accompanying software which was similar to the other scanner. The images produced were in gray scale and had to be copied and modified using the PerkinElmer software to produce colour images.

2.6 Liposome Production

Liposomes were produced based upon the method previously described by Bowes et al, 2002, with some minor modifications. The liposomes were composed of a ganglioside, cholesterol, SM, DCP, and the complex partner in a 1:5:4:1:1 molar ratio although some GM1:GalC liposomes omitted SM. The concentration of the liposomes was based upon the ganglioside and was 1mg/ml for immunisations. Whole lipid extract (WLE) liposomes were produced by adding 5mg of WLE to the same proportion of chol, SM, DCP that were used with GM1 liposomes.

They were prepared by sonicating the stock lipids for 5 minutes before the different components were added together in a 15ml conical tube. The mixture was then sonicated for a further 5 minutes before the chloroform/methanol was dried off under a stream of nitrogen. For immunisations, 25mg of ovalbumin (Sigma Aldrich, St Louis, MO, USA) was added to 5ml of PBS and heated to 37°C. This was added to the dried lipid film and vortexed for 2 minutes and then sonicated for 15 minutes. A series of freeze/thaw cycles were then performed by plunging the conical tube into liquid nitrogen and then thawing it in a water bath set to 37°C. The liposomes were then centrifuged at 600xg for 20 minutes (Jouan, Saint-Herblain, France) after which the supernatant was removed and heated to 37°C. This was followed by extruding the solution through a 0.4µm membrane (Whatman, Little Chalfont, UK) in a hand held extruder (Avanti Polar Lipids, Alabaster, US) 11 times over a heat block set at 37°C. The extruded liposomes were then ultra-centrifuged using a Ti-70 rotor (Beckman Coulter, Brea, CA, USA) at 152300xg for 1 hour at 16°C. The supernatant was disposed of and the liposome pellet was resuspended in 1ml PBS and stored at 4°C for up to one week.

2.7 Immunisation Protocol

All mice selected for immunisations were bled at regular intervals, so that the immune response could be monitored. 100µl was collected from each mouse via venesection of the tail vein. Once collected it was allowed to clot at room temperature for 30 minutes after which the blood was centrifuged at 21000xg on a Hermle Z216MK microcentrifuge (Wehingen, Gemrany) at 4°C for 20 minutes. The serum was removed and aliquoted into microtubes and stored at -20°C.

2.7.1 Liposome Immunisation

Mice were bled prior to the start of the immunisation cycle (Day 0) before being primed with 100µl of ovalbumin suspended in aluminium hydroxide (Sigma Aldrich, St Louis, MO, USA) at a concentration of 0.6mg/ml. The mice were then bled and injected intraperitoneally (i.p.) with 100µl of liposomes once a week for three consecutive weeks (Day 7, 14, 21). This ensured that each mouse received 100µg of ganglioside liposomes in each injection. The blood collected

on the 4th time point was screened for anti-ganglioside antibodies so that mice could be selected for hybridoma fusion. All mice received 50µl intravenous injections (i.v.) of liposomes at 200µg/ml on days 25, 26 and 27. The mice were then culled on day 30 through asphyxiation with CO_2 after which the blood and tissue was harvested.

2.7.2 CFA/IFA Immunisation

Mice were immunised against whole lipid extract (WLE) by suspending the antigen in complete Freund's adjuvant (CFA) (Sigma Aldrich, St Louis, MO, USA) containing 1mg/ml heat killed *mycobacterium tuberculosis*. The WLE was first prepared by sonicating the stock (242mg/ml) for 5 minutes after which 50µl was transferred to a glass bijou. The chloroform that the WLE was stored in was dried off under a stream of nitrogen and the WLE was resuspended in 3ml of saline. It was then transferred to a glass syringe (Hamilton, Reno, NV, USA) and emulsified with 3ml of 0.5mg/ml CFA using a double hub needle. The resultant mixture was stored at 4°C overnight prior to use. Immunogens were created with incomplete Freund's adjuvant (IFA) following the same protocol except that the CFA was replaced with IFA.

The mice were bled prior to the beginning of the immunisation cycle as per the liposome immunisations. Each mouse was injected with 100µl of WLE/CFA subcutaneously so that they received approximately 2mg of WLE per injection. Each subsequent injection consisted of 100µl of WLE/IFA and was performed at week 2 and week 4.

2.8 Production of Hybridomas

Monoclonal antibodies were produced by fusing the splenocytes from the immunised mice with a myeloma cell line to create a hybridoma. This method employed the use of hypoxanthine/aminopterin/thymidine (HAT) media which blocks the *de novo* pathway of DNA synthesis forcing cells to use the salvage pathway. The myeloma cell line lacks the enzyme HGPRT and is unable to use the salvage pathway which results in the myeloma cells dying. The hybridoma cells will have regained this enzyme from the splenocytes and are able to

synthesise DNA and thus expand. This allows for the production of an immortal cell line that is capable of producing antibodies.

Following an immunisation cycle, mice were culled with a rising concentration of CO_2 as per UK Home Office guidelines. Cardiac puncture was then performed using a 26G needle and the blood retained for subsequent analysis as per section 2.5.4.

All tissue culture work was carried out in category 2 laminar flow hoods and followed good tissue culture practice. Unless otherwise stated, centrifugation was performed using a Sorvall biofuge primo centrifuge (Thermo Scientific, Waltham, MA, USA) and all cells were incubated in a Forma Series ii 3110 water jacketed CO_2 incubator (Thermo Scientific, Waltham, MA, USA). Cells were counted using a haemocytometer (Hausser Scientific, Horsham, PA, USA) and employed the use of 0.4% trypan blue (Life Technologies, Carlsbad, CA, USA) as a method of dye exclusion.

2.8.1 Spleen Harvest

Mice selected for hybridoma fusion were euthanized, pinned out in the supine position and sprayed with 70% ethanol. The skin was removed from the thorax and the peritoneal cavity was opened to expose the abdominal organs. The stomach was pulled aside to access the spleen which was then removed with sterile dissection scissors. The spleen was placed in a 50ml tube containing 20ml of sterile 20% RPMI and moved to a laminar flow hood.

A glass mortar and pestle were used to homogenise the spleen which was then filtered through a 70µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) into a new 50ml tube. 5ml of fresh 20% RPMI was washed through the strainer to maximise cell retention. The splenocytes were then centrifuged at x300g for 10 minutes using a sorvall biofuge primo centrifuge (Thermo Scientific, Waltham, MA, USA). They were then resuspended in 10ml of RPMI and counted using a haemocytometer.

2.8.2 Hybridoma Fusion

The mouse myeloma fusion partner, P3X63Ag8.653 (hereby referred to as 653s), were raised a week before the hybridoma fusion and were grown following the standard methods described in Section 2.9.1. They were expanded into several 150cm^2 vented flasks and were placed into the log phase of growth the day before the fusion to ensure that the cells were rapidly expanding. Following the preparation of the splenocytes, the 653s were loosened in their flasks by gentle agitation and centrifuged at 250xg for 10 minutes. They were then resuspended in 10ml of RPMI and counted using a haemocytometer.

The splenocytes and 653s were mixed in a 10:1 ratio and centrifuged to a common pellet at 250xg for 10 minutes. All the supernatant was removed from the tube and the pellet was loosened by gentle agitation. In a water bath set to 37°C, 1ml of polyethylene glycol (PEG) was added dropwise to the pellet over a 1 minute period with constant agitation, to allow fusion of the cells to create the hybridoma. This was followed by a further 90 seconds of agitation after which 2ml of RPMI was added dropwise over a period of 2 minutes. 20ml of RPMI was then added over a 5 minute period followed by centrifugation at x130g for 15 minutes.

The supernatant was removed and the pellet was resuspended in 60ml 20% RPMI +FS. 96 well plates were prepared by filling all the outer wells with 200µl of media without cells. The inner wells were then filled with 150µl of media containing the suspended cells and the plates were incubated at 37°C in 5% CO_2.

2.8.3 Maintenance and Screening of Hybridomas

After two weeks of incubation, 110µl of supernatant was removed from each well and replaced with 120µl of 20% RPMI +FS. The supernatant was screened for monoclonal antibodies on ELISA or microarray as per sections 2.4.2 and 2.5.5 respectively. Antibody positive wells were expanded by triturating the cells with 200µl of media three times and applying the suspension to wells in a 24 well plate. These were then topped up with 500µl of 20% RPMI +FS. After three days of growth all the media was removed from each well and replaced with 1ml of 20% RPMI +FS. The cells were incubated for a further 3 days after which the

supernatant was screened again for monoclonal antibodies following the method as above.

2.8.4 Cloning by Serial Dilution

Wells containing antibodies of interest were cloned by serial dilution to ensure that only one cell line was present. The cells were counted using a haemocytometer and the volume that contained 1200 cells was calculated. This was added to well A1 in a 96 well plate and topped up to 200µl with 20% RPMI +FS. The other wells were filled with 100µl of media and 100µl of the cell suspension in A1 was serially diluted 1:1 down column A. This was then topped up to 200µl with media and 100µl was then serially diluted 1:1 across all the columns. All the wells were then topped up to 200µl with 20% RPMI +FS. The plates were incubated at 37°C in 5% CO_2 for two weeks until colonies began to appear and the media changed colour. The wells were then screened using the ELISA method as per section 2.4.2. The smallest colony that was positive for antibodies was expanded into a 24 well plate as per section 2.8.3 and the cloning process was repeated for a total of three times.

2.8.5 Freezing Cells

At each stage of development the cells were frozen to ensure cell line retention. Following centrifugation the supernatant was removed and the pellet was resuspended at a density of 10^6 cells/ml in 20% RPMI + 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, MO, USA). This suspension was added to several 2ml freezer tubes which were then placed inside a Mr Frosty Freezer Container (ThermoScientific , Waltham, MA, USA) that contained isopropyl alcohol. This was frozen at -80°C for 24 hours and then moved to liquid nitrogen for long term storage.

2.8.6 Growth Curves

Once the hybridoma cloning process had been completed, growth curves were produced to determine the doubling time of each cell line (Figure 2.1). These were useful in establishing the growth rates of the cells, which would be

informative, in terms of cell health and viability, if they were raised again (Assanga, 2013). Any deviation from the normal growth curves could act as an indication of changes in the local environment and could be used to alter the growth conditions accordingly.

To produce these growth curves, hybridoma cells were counted using a haemocytometer and the volume required to produce 10^4 cells/ml was calculated. This volume was then added to 5ml of 10% RPMI +FS in a vented 25cm^2 flask, which was then incubated at 37°C in 5% CO_2. Every day, 20µl was taken from each flask and counted until the cell number plateaued. This data was plotted and the points on the steepest slope of the curve (log phase) were used to determine the cell doubling time (Figure 2.1).

These were 20.60 hours, 31.61 hours, 30.52 hours, 23.38 hours, 64.85 hours, 38.49 hours, 55.77 hours, 47.43 hours, 26.49 hours and 10.14 hours with the GAME-M1, GAME-M2, GAME-M3, GAME-M4, GAME-M5, GAME-M6, GAME-M7, GAME-G1, GAME-G2 and GAME-G3 hybridomas respectively.

Most of the hybridoma cells followed the standard shape of a growth curve; initially experienced a lag phase of growth, where the cell numbers either stalled or decreased, followed by the log phase where the cells grew at an exponential rate until they reached a plateau (Freshney, 2010). The only exception to this growth pattern was the hybridoma that produced GAME-M4. These cells experienced a rapid decrease in number after they reached their peak. During antibody production it was noted that this cell line was very sensitive to changes in the media, particularly the absence of feeder supplements. Unlike the other hybridoma cells that were successfully weaned off the supplement, this cell line died during each attempt.

This may help explain the rapid cell death experienced during the growth curve, as the cell line would most likely be sensitive to the increased levels of waste products produced by the growing cells. Despite these problems, the cell line was still able to produce antibodies although it was incapable of producing them long term.

Aside from the shapes of the growth curves, there were also differences in their doubling times. This may have been related to the resources they invest in producing antibodies. GAME-M7, for example, was only able to maintain cell

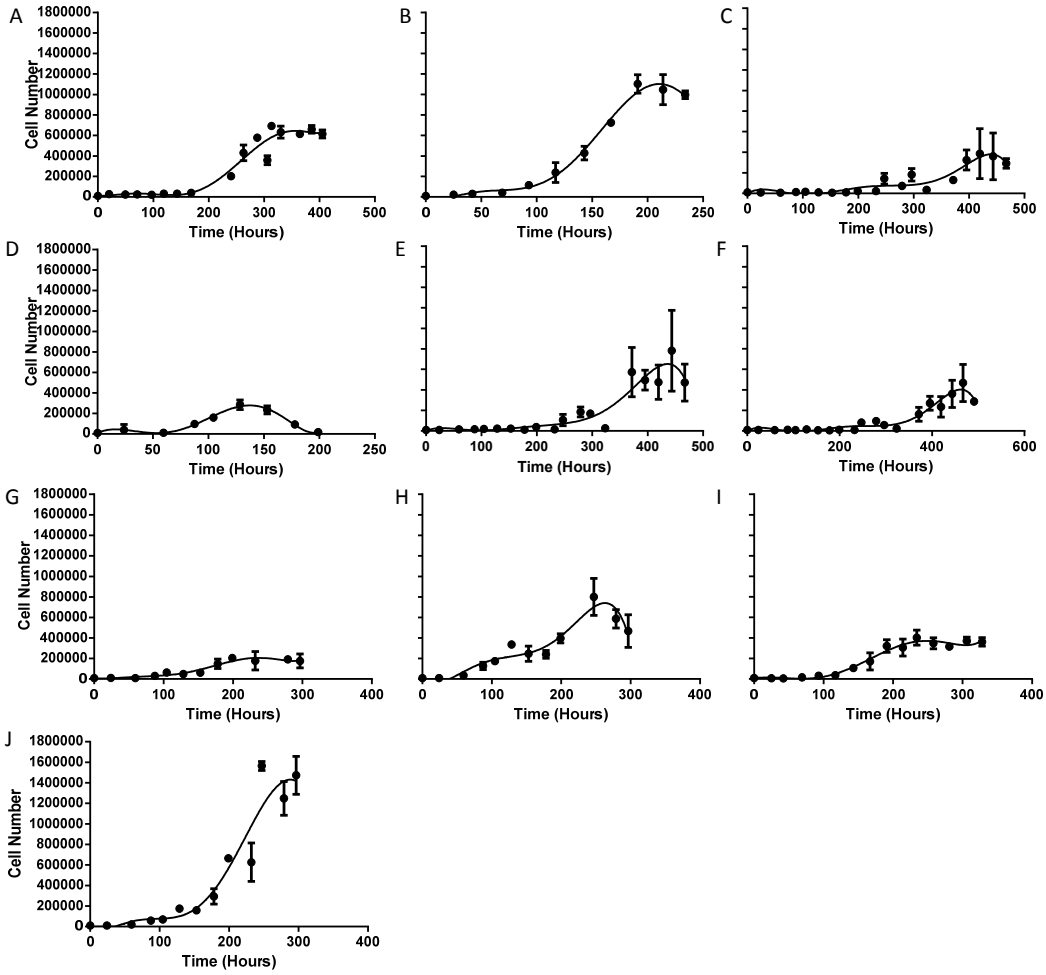


Figure 2.1 – Growth Curves of Anti-Glycolipid Antibodies

Cells were counted every day and a growth curve was plotted for each hybridoma cell line. The doubling time was calculated from the steepest point on each curve. These were as follows: A: GAME-M1: 20.60 hours; B: GAME:M2: 31.61 hours; C: GAME-M3: 30.52 hours; D: GAME-M4: 23.38 hours; E: GAME-M5: 64.85 hours; F: GAME-M6: 38.49 hours; G: GAME-M7: 55.77 hours; H: GAME:G1: 47.43 hours; I: GAME-G2: 26.49 hours; J: GAME-G3: 10.14 hours.

growth up to a maximum 200000 cells. Antibody production in this cell line was particularly high, however, suggesting that the reduced cell growth was allowing the hybridoma cells to focus resources on antibody production (E. Suzuki & Ollis, 1990).

2.9 Production of Monoclonal Antibodies

2.9.1 Production of Existing Hybridoma Cell Lines

Existing hybridoma cell lines were thawed from storage in liquid nitrogen, and centrifuged at 250xg for 10 minutes. The supernatant was disposed and the

pellet resuspended in 20% RPMI and centrifuged again. This was repeated a total of three times. The cells were grown in 15ml of 20% RPMI in 25cm^2 vented flasks until confluent after which they were expanded into bigger flasks containing 10% RPMI until there were eight 150cm^2 flasks containing 100ml of media each.

2.9.2 Collection of Antibody Supernatant

As the cells became confluent, 50ml of media was removed from each flask and replaced with 50ml of 10% RPMI. The media that was removed was centrifuged at 525xg for 10 minutes and the supernatant was retained and frozen at -20°C. This process was repeated until approximately 1L of antibody containing media was collected after which all the media was thawed at 4°C for approximately 16 hours. It was then pooled together and filtered through a 0.22µm sterile filter unit (Millipore, Billerica, MA, USA) under a constant vacuum.

2.9.3 Purification of IgM Antibodies

Attempts were made to purify the human IgM monoclonal antibodies using affinity purification columns (GE Healthcare, Little Chalfont, UK). The column operated on the principle of thiophilic adsorption to fractionate the IgM antibodies from the cell supernatant. The column was first washed through with 5ml of binding buffer (20mM Na3PO4/0.8M $(NH_4)_2SO_4$), elution buffer (20mM Na3PO4) and regeneration buffer (20mM Na3PO4 and 30% isopropanol) using a Watson Marlow 205S peristaltic pump (Watson Marlow Pumps, Falmout, UK). This was followed by equilibrating the column with another 5ml of binding buffer. The IgM antibody samples were prepared by slowly adding the same amount of ammonium sulphate that was in the binding buffer. This was followed by filtration through a 0.22µm sterile filter unit (Millipore, Billerica, MA, USA) using a vacuum pump.

The sample was pumped through the column using a peristaltic pump at a speed of 1ml/min. When there was less than 5ml of sample remaining the pump was stopped and 15ml of binding buffer was run through the column to remove any unbound IgM antibody. To remove the bound IgM fractions, 12ml of elution buffer was run through the column, followed by 5ml of regeneration buffer to remove any remaining antibody. The column was then re-equilibrating with 5ml

of binding buffer and was stored in 20% ethanol at 4°C to prevent bacterial growth. The elution was screened for antibody presence using a quantitative ELISA kit as per section 2.4.3.

2.9.4 Concentration of IgM Antibodies

The use of IgM purification columns proved unreliable in isolating antibodies from tissue culture media. A different method was therefore employed to allow for the production of sufficient stocks for analysis. This method involved running 250ml of filtered media through a 100kDa filter inside a Vivacell bench top concentrator (Sartorius, Göttingen, Germany). The concentrator was sealed and a pressure of 3 bars was applied to the container which was then placed on a shaker at 100rpm. The media was topped up and the flow through removed as required until all of the media had been concentrated to a final volume of between 5 and 20ml. The concentrate was then screened using quantitative ELISA as per section 2.4.3

2.9.5 Purification of IgG Antibodies

IgG antibodies were purified using HiTrap protein G affinity purification columns (GE Healthcare, Little Chalfont, UK). The filtered media containing IgG antibodies was first dialysed against binding buffer (0.2M Na_2HPO_4/0.2M NaH_2PO_4) overnight at 4°C. The column was then prepared by running 5ml of binding buffer through it using a peristaltic pump set to 50rpm. The media was placed on ice and run through the column at a rate of 50rpm with the flow through being collected in a 1l Duran bottle. The pump was stopped when less than 10ml of media was remaining and binding buffer was then run through the column and 10x 1ml wash aliquots were collected. Elution buffer (0.1M Glycine, pH 2.5) was subsequently run through the column followed by the collection of 10x1ml elution aliquots which each contained 40µl of TRIS-HCl (pH 9.5). The column was capable of being reused so binding buffer was again run through it to remove any residual elution buffer followed by the addition of 20% ethanol which was used to store it at 4°C.

The wash and elution aliquots were analysed using a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA, USA) that had been set up

to read absorbance at a wavelength of 280nm. The software was capable of translating this absorbance into a protein concentration in mg/ml. The spectrophotometer was initialised using a sample of RNAse free water to act as a control. 2µl of each sample was then applied and each reading was noted.

An example of the spectrophotometer read out is shown with the antibody, GAME-G3, in Figure 2.2. The initial wash fractions indicated that high concentrations of protein were present in the samples; however, this was due to the presence of FCS in the cell culture supernatant, which skewed the results. High concentrations of antibody were found in elution fractions 3-7 from the first run and 4-5 from the second run. These were subsequently pooled to create antibody stocks.

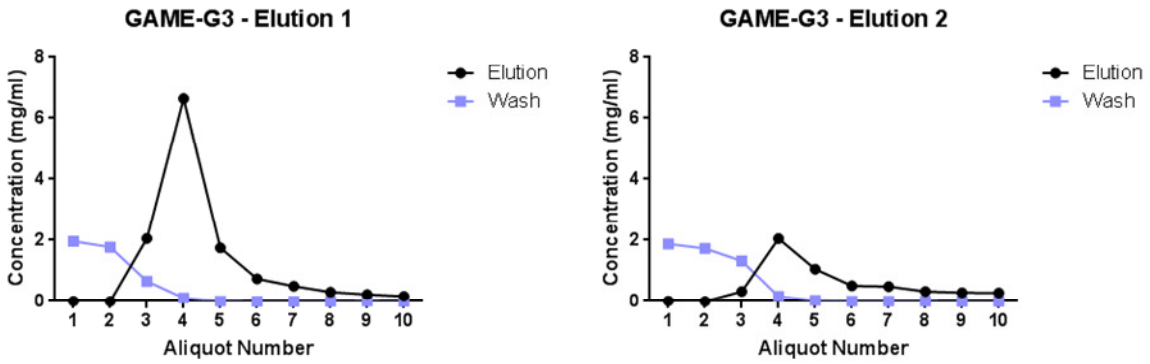


Figure 2.2 –GAME-G3 Elution Fractions

GAME-G3 supernatant was run through a commercial protein G column. The high protein concentration in the wash fractions was due to the presence of FCS. The highest antibody concentration was found in elution fractions 3-7 from the first run and 4-5 from the second run.

Elution fractions that had high values were pooled together and desalted using PD-10 desalting columns (GE Healthcare, Little Chalfont, UK). 25ml of PBS was first run through the columns and disposed, followed by the samples which were topped up to 2.5ml with PBS. The flow through was retained to ensure that it was protein free and an additional 3.5ml of PBS was added to the column and collected in a bijou. Both of these elutions were analysed on the spectrophotometer for IgG concentration.

2.9.6 Isotyping

Isotyping of the IgG sub-classes was performed by ELISA using secondary antibodies that were specific for the different antibody isotypes. The ELISAs were carried out as per Section 2.4.2 with some minor modifications. Briefly, the primary consisted of 100µl of the monoclonal antibody at 10µg/ml in 0.1% BSA/PBS, which was added to each well of the plate. The secondary consisted of IgG1, IgG2a, IgG2b and IgG3 antibodies at a 1:3000 dilution, which were added to rows A+B, C+D, E+F, and G+H respectively. These secondary antibodies were serially diluted 1:1 along the rows. The wash and development steps of the protocol were all performed as standard.

Using this method GAME-G1, GAME-G2 and GAME-G3 were all determined to be of the IgG3 subclass.

2.10 Isoelectric Focussing

The monoclonality of the antibodies was determined by isoelectric focussing (IEF) which separates the antibodies according to their isoelectric points. This work was carried out in conjunction with laboratory staff at the Southern General Hospital, Glasgow, UK.

A glass plate was cleaned with 70% ethanol and 5 drops of 50% methanol/dH_2O was applied to the surface. The hybrophobic side of a pre cut sheet of GelBond film (Lonza, Basel, Switzerland) was then aligned with the edge of the glass plate and evenly pressed to remove air bubbles. A second plate containing a spacer was then placed on top and secured before a boiling glycerol sorbitol gel was applied. This was prepared by dissolving 24g of sorbitol in a mixture of 20ml of glycerol and 180ml of dH_2O followed by the addition of 1ml of pharmalyte 3-10 and 0.25ml of pharmalyte 8-10.5 (GE Healthcare, Little Chalfont, UK). The gel was left to set at room temperature for 1 hour before the glass was removed. It was then stored at 4°C in a humid chamber for up to 1 week.

The multiphor ii electrophoresis system (GE Healthcare, Little Chalfont, UK) was used for the IEF portion of the experiment and was continually cooled through a constant flow of cold water. Five drops of 50% methanol/dH_2O was applied to

the cooling plate and the gel was aligned with its edge to seal it in position. A strip of fixed Ilford Pan F Plus 50 dx 135-36 film (Calumet Photographic Ltd, Leith, UK) was applied 2.5cm from the anodic edge of the gel and was used to load 2.5µl of each sample. IgG and IgM antibodies were applied at 100µg/ml but IgM samples were first reduced by incubating them in 2% mecaptoethanol at room temperature for 30 minutes. The anode and cathode IEF electrode strips (Healthcare, Little Chalfont, UK) were soaked in 0.05M H_2SO_4 and 1M NaOH respectively and then applied to the gel to give an approximate interelectrode distance of 7cm. The glass plate containing the electrodes was then placed on top and the electrode wires were aligned with the IEF electrode strips. The voltage was set to 1200V at 20W and integrated over time to give a total run interval of 1000 volts/hour. The gel took 20 minutes to complete the IEF run after which it was removed from the plate using tweezers.

The gel was placed on a plastic plate and blotted with HYBOND C nitrocellulose membrane (NCM) (GE Healthcare, Little Chalfont, UK) to remove excess moisture. A new sheet of NCM was then applied carefully onto the gel and covered with several sheets of filter paper and another plastic plate. This was weighed down by a 3kg weight for 30 minutes to ensure transference of the protein from the gel to the NCM.

Once this had been completed the NCM was probed with secondary antibodies to detect the isoelectric points of the monoclonal antibodies. All the steps took part on a rocking platform shaker set to 30 rpm. The NCM was first blocked with 2% Marvel (Premier Foods PLC, St Albans, UK) for 30 minutes followed by an incubation with 50ml of either goat anti-mouse IgM or goat anti-mouse IgG (1:1000) in 0.2% Marvel for 30 minutes. The NCM was then washed three times in saline for 5 minutes each. 50ml of HRP conjugated rabbit anti-goat Ig was then applied (1:1000) in 0.2% Marvel for 30 minutes followed by a repeat of the wash cycles. The membrane was developed by incubating it with a 3-amino-9-ethyl carbazole (AEC) solution in the dark for 15 minutes. Once bands appeared the NCM was washed with water several times and dried overnight by placing it between several sheets of blotting paper with a 3kg weight on top.

2.11 Thin layer chromatography

The components of WLE were determined by thin layer chromatography (TLC) to establish what antigens were present in the immunisation bolus. Immuno-overlays were also performed using TLC to screen the serum and the monoclonal antibodies to determine their targets.

2.11.1 TLC Resorcinol Staining

Lipids were prepared as per section 2.4.1 and WLE was prepared at 5mg/ml in methanol. Resorcinol was made 24 hours before use by first dissolving 200mg of resorcinol (Sigma Aldrich, St Louis, MO, USA) in 10ml dH_2O and adding it to 80ml of 13M HCl containing 250µl of 0.1M CU_2SO_4. This was then topped up to 100ml with dH_2O.

A square of filter paper was attached to the back of a glass TLC tank (CAMAG, Muttenz, Switzerland) which was then filled with a solvent solution composed of 50ml chloroform, 42ml methanol and 11ml 0.2% $CaCl_2$.The rim of the tank was sealed with petroleum jelly and the lid was attached. The tank was then left to equilibrate at room temperature for 3 hours.

HPTLC Silical gel 60 plates (Merck KGaA, Darmstadt, Germany) were loosely covered in foil and heated to 80-100°C for 1 hour prior to printing. The plates were then removed and placed in the TLC autosampler (CAMAG, Muttenz, Switzerland) which was fitted with a 100µl needle. The accompanying software was used to apply 30µl bands of each lipid (100ug/ml) or WLE (5mg/ml) to the plate. A maximum of 10 individual bands could be applied to each plate for standard TLC or 5 duplicate bands could be applied to each half for overlay TLC. Once printing was completed the plate was allowed to dry at room temperature for 15 minutes.

When the tank was equilibrated, the plate was stood vertically against the filter paper inside the tank and the lid was replaced. The solvent solution moved up the plate and in doing so displaced the lipids according to their molecular weight. This was allowed to proceed for 45 minutes at room temperature.

The plate was then removed and dried flat for 10 minutes at room temperature until it no longer appeared transparent. It was then stood vertically against the outside of the tank and sprayed with resorcinol until the whole plate was covered. A second plate which had micro tube lids affixed to the corners was then laid on top of the plate and both were covered with foil and placed on the bottom shelf of an oven at approximately 90°C for 10 minutes. This allowed for the development of the lipid migration pattern.

2.11.2 TLC Immuno-overlay

Once the TLC had been performed, a diamond pen was used to split the plate into two halves. One half was sprayed with resorcinol and developed as described whilst the other half was dipped into a solution of 0.2% polyisobutlymethacrylate (PIBM) in n-hexane for 1 minute. It was then air dried inside a fume hood at room temperature for 30 minutes followed by a blocking step with 2% BSA/PBS for 16 hours. Subsequently, 3ml of a monoclonal antibody (10µg/ml) or serum (1:100) was applied to the plate, which was then covered with a sheet of parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) and incubated at 4°C for 2 hours. The plate was washed three times in 100ml of 1% BSA/PBS for 5 minutes on a shaker set to 100rpm. 3ml of HRP conjugated goat anti-mouse IgM (1:3000) was then applied to the plate, which was again covered with a sheet of parafilm and incubated at 4°C for 1 hour. The wash cycle was repeated as previously described and the plate was developed using the OPD method as per the ELISA protocol in Section 2.4.2. Stop solution was applied for 10 seconds and the plate was air dried for 20 minutes.

2.12 Growth and Immunofluorescent Staining of Primary Cells

2.12.1 Schwann Cell Culture

Schwann cells were cultured based upon the protocol laid out by Honkanen et al., (Honkanen et al., 2007). The day before the culture, plates were prepared by placing sterile coverslips in each well of a flat bottomed 24 well plate. 500µl of 10µg/ml of poly-l-lysine (Sigma Aldrich, St Louis, MO, USA) in dH_2O was applied to each well and the plate was incubated at 37°C in 5% CO_2 for 1 hour. The wells were then washed twice in dH_2O and allowed to dry overnight.

The following day neonatal mouse pups at P7-P8 were culled with an injection of 100µl of sodium pentobarbital and decapitated to ensure death. The mice were pinned in the prone position and sprayed with 70% ethanol before the skin was removed around the legs. Dissection scissors were used to remove the overlying muscles and both sciatic nerves were isolated. These were stored on ice in DMEM containing 10% horse serum for no more than 1 hour. The nerves were moved to a laminar flow hood and chopped using a sterile scalpel blade in a petri dish. They were then centrifuged at 290xg for 2 minutes and the supernatant was removed. The nerve fragments were then resuspended in a mixture of 3ml of 0.6U/ml dispase and 100U/ml collagenase and incubated for 45 minutes at 37°C in 5% CO_2. This was followed by two washes in DMEM and trituration of the samples through a 23G needle in 300µl of BGM. 75µl of this cell suspension was applied to each coverslip and the plates were incubated at 37°C in 5% CO_2 for 1 hour. The wells were then flooded with an additional 700µl of BGM and incubated for 24 hours.

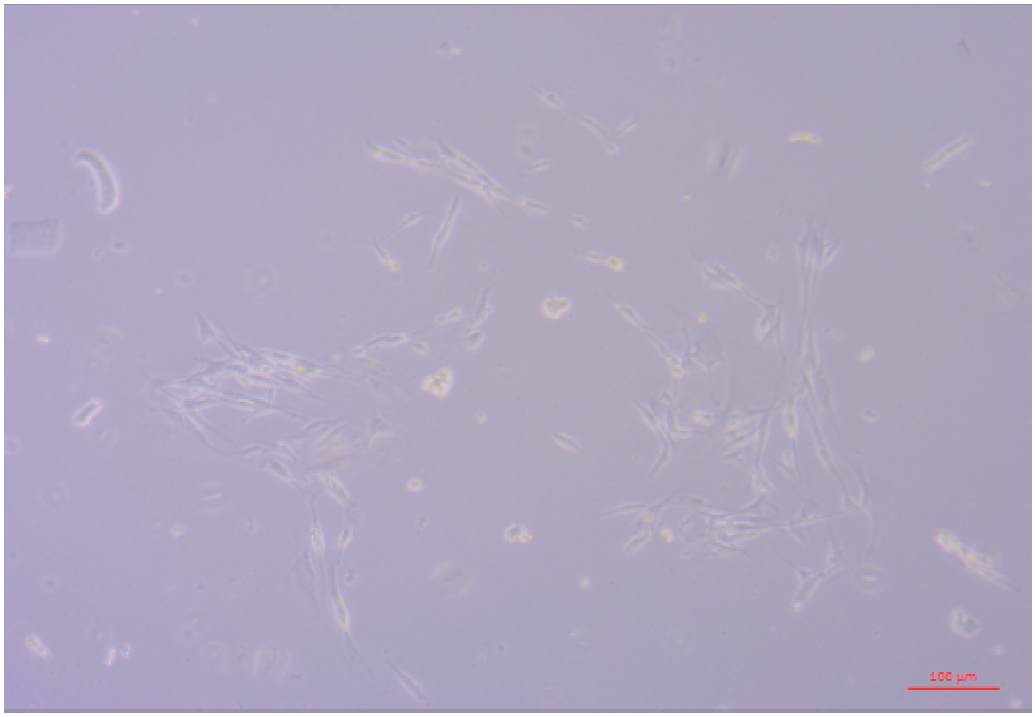


Figure 2.3 - Schwann Cells

Light microscopy of Schwann cell growing for 6 days

The next day the cells were supplemented with 10µM cytosine arabinoside (ARAC) and incubated for a further 48 hours followed by replacement with ARAC free BGM. They were then incubated for another 24 hours after which the media

was removed and replaced with 700µl Schwann cell growth media. The cells were incubated for another 4 days until they had a morphology resembling an elongated spindle shape as those in Figure 2.3.

2.12.2 Schwann Cell Immunofluorescence

After eight days of growth the Schwann cells were probed with anti-sulfatide antibodies. Following each stage of the staining protocol, the wells were washed twice with PBS to remove excess reagents. The plates were incubated on ice and 500µl of the appropriate antibody was added to each well at 10µg/ml for 1 hour. The cells were then fixed with 4% PFA for 20 minutes followed by a 5 minute incubation with 0.1% Triton X-100. 500µl of 5% heat inactivated normal goat serum (NGS) in PBS was then added to each well and the plates were incubated at room temperature for 30 minutes. 500µl of rabbit anti S-100 (Dako, Santa Clara, CA, USA) was then added in PBS at 40µg/ml and the plates were incubated overnight at 4°C.

The following day, 500µl of an appropriate secondary antibody conjugated to FITC (3.33mg/ml) was added to each well alongside TRITC-conjugated anti rabbit IgG (3.33mg/ml). The plates were incubated at room temperature for 30 minutes followed by 3x five minute washes in PBS. The coverslips were then removed from each well, mounted in DAPI (Life Technologies, Carslbad, CA, USA) and sealed with nail varnish onto a microscope slide. The slides were stored at 4°C prior to imaging.

2.12.3 Oligodendrocyte Progenitor Cells

Rat derived oigodendrocyte progenitor cells (OPCs) were grown by a colleague within the institute according to established methods (Lindsay et al., 2013). These were grown on poly-l-lysine coated coverslips for 10 days prior to immunofluorescent staining.

2.12.3.1 Immunofluorescence

The staining protocol for the OPCs was similar to that used for staining Schwann cells with a few changes. The primary antibody was applied with an immature

OPC marker, NG2 (Millipore, Billerica, MA, USA), at a concentration of 5µg/ml. After permeabilisation, anti-S100 was not added but instead an in-house monoclonal antibody (AA3), which binds proteolipid protein (PLP), was applied at a concentration of 10µg/ml. All other stages of the protocol remained the same.

2.13 Muscle and Nerve Preparations

The binding behaviours of the monoclonal antibodies in tissue were determined through application of the antibodies to muscles and nerves taken from naive mice. These tissues could be studied as either ex vivo preparations, where the tissue was kept alive in a physiological solution, or as frozen sections. All mice were culled by a rising concentration of CO_2 as per Home Office guidelines. Upon cessation of breathing, death was confirmed by cervical dislocation.

2.13.1 Triangularis Sterni

The mouse was pinned in the supine position and the skin was retracted around the thoracic cavity. The lower abdominal organs were pulled away from the rib cage and the diaphragm was removed. The heart and lungs were carefully removed and the rib cage was detached from the spinal cord. It was then pinned pectoral side up in a Sylgard (Dow Corning, Midland, MI, USA) lined petri dish that contained oxygenated Ringer's solution.

The overlying muscles were removed using a dissecting microscope and the ribs were peeled back to reveal the triangularis sterni (TS). This was repeated to create four windows which contained the TS surrounded by ribs. These were then individually cut out and pinned under tension in separate wells of a Sylgard lined 24 well plate containing Ringer's solution.

Once all the TS sections were prepared, the solutions were removed and the antibodies were applied at a concentration of 100µg/ml with α-bungarotoxin (2µg/ml) for 1 hour at 4°C. This was followed by three washes in Ringer's solution and fixation with 4% PFA for 20 minutes at room temperature. The tissues were then washed for 10 minutes in PBS, 10 minutes in 0.1M glycine (pH 7.5), and 10 minutes in PBS. The appropriate secondary antibodies were then

applied overnight at 4°C. The following day, the tissues were washed 3 times in PBS for 5 minutes each. Finally, each TS was completely removed from the supporting ribs and tissue and mounted on adhesive slides (Tissue Tek, Sakura, Netherlands) with Citifluor AF1 (Citifluor, Fanshaw, UK) or DAPI (Life Technologies, Carslbad, CA, USA.) Coverslips were applied to slides and sealed with nail varnish followed by storage at 4°C, prior to imaging.

2.13.1.1 Permeabilisation to Detect Uptake of Antibody

Tissue was permeabilised to determine if any antibody had been internalised during the initial incubation. Once the original tissue has been imaged, the coverslip was removed and the TS was washed several times in PBS. It was then incubated in 0.5% Triton X-100 for 30 minutes at room temperature. This was followed by 3 washes in PBS and the application of the secondary antibody as previously described. The tissue was again mounted in the same manner as before.

2.13.1.2 S100 Staining

In mice that were not S100-GFP fluorescent an anti-S100 antibody was used to identify glia. After the permeabilisation step, rabbit anti- S100 was applied at 20µg/ml for 1 hour at 4°C. The tissue was then washed three times and the secondary antibody was applied with an appropriate fluorophore conjugated goat-anti rabbit IgG as per the standard protocol.

2.13.1.3 Neuraminidase Treatment

The enzyme neuraminidase from *Clostridium perfringens* (Sigma Aldrich, St Louis, MO, USA), which cleaves the terminal sialic acid from gangliosides, was applied to tissue to examine how changing the ganglioside environment affected antibody binding behaviour. Once the TS preparations were pinned out in a 24 well plate, 300µl of neuraminidase was applied at 10U/ml in Ringer's solution to half the wells whilst the control wells received Ringer's solution alone. The plate was incubated at 37°C for 2 hours followed by three washes in Ringer's solution.

The rest of the experiment followed the standard protocol as laid out in Section 2.13.1.

2.13.1.4 Complement Mediated Cell Injury

To establish whether certain antibodies were pathogenic, it was necessary to apply normal human sera (NHS) to tissue, to determine if the antibodies were capable of activating the complement cascade. Mice with CFP positive axons were always used for these experiments and their TS were prepared as described previously. They were incubated with 100µg/ml of antibody and FITC conjugated-BTx for 2 hours at 32°C, followed by 30 minutes at 4°C and finally 10 mins at room temperature. The control underwent the same protocol except that no antibody was added. Following incubation, the solutions were removed and the tissue was washed 3 times with Ringer's solution. 40% NHS in Ringer's solution was then applied for 1 hour at room temperature followed by three washes. Ethidium homodimer-1 (EthD-1) was then applied at a 1:500 dilution in Ringer's solution for 1 hour at room temperature followed by 3 further washes. The TS was then fixed and an appropriate secondary antibody was applied as per Section 2.13.1. It was then mounted onto a microscope slide as standard.

2.13.1.5 Terminal Myelinating Schwann Cell Characterisation

Anti-sulfatide monoclonal antibodies were found to stain the terminal myelinating Schwann cells. As this staining was unique, it was thought that the cell may be different from the surrounding Schwann cells so it was characterised against a series of Schwann cell markers.

Following incubation of anti-sulfatide antibodies with TS, the tissue was fixed as standard and then permeabilised with methanol for 10 minutes at -20°C. The tissue was then washed 3 times in PBS before a Schwann cell maker was added at an appropriate concentration. These were: anti-MBP (Millipore, Billerica, MA, USA) at 2µg/ml; anti-MAG (Millipore, Billerica, MA, USA) at 50µg/ml or anti-GFAP (Dako, Santa Clara, CA, USA) at 5.8µg/ml. The tissues were incubated with these makers overnight followed by three further washes in PBS. The appropriate secondary antibodies were then applied to the tissue for six hours at

room temperature, after which it was mounted on adhesive slides with DAPI (Life Technologies, Carslbad, CA, USA) as per Section 2.13.1.

2.13.2 Sciatic Nerve

Adult mice were culled with a rising concentration of CO_2 as per Home Office guidelines. The sciatic nerves were removed following the same procedure as for the Schwann cell culture discussed in Section 2.12.1. The nerves were frozen at -80°C until required for cryosectioning.

2.13.2.1 Sciatic Nerve Sectioning

The nerves were embedded transversely in optical cutting temperature cryostat sectioning medium (OCT) in preparation for cutting. 8µm sections were cut using a Bright OFT cryostat (Bright Instrument Co Ltd, Huntingdon, UK) and applied to 24x70mm adhesive slides (Tissue Tek, Sakura, Netherlands). The slides were then stored at -20°C prior to staining.

2.13.2.2 Sciatic Nerve Immunofluorescence

A hydrophobic pen (Vector Laboratories, Peterborough, UK) was first used to mark around the tissue sections on each slide to prevent dissemination of solutions prior to antibody application. The slides were then blocked in 3% NGS in PBS for 1 hour at 4°C followed by application of 300µl of the primary antibody (10µg/ml) for 2 hours at the same temperature. The slides were then washed three times in PBS followed by application of a suitable secondary antibody (3.33µg/ml) for 1 hour at 4°C. The slides were then washed three times with PBS and a myelin marker called Fluoromyelin (Life Technologies, Carslbad, CA, USA) was applied at a 1:400 dilution for 12 minutes at room temperature. The slides were again washed three times in PBS and mounted in Citifluor AF1 as previously described.

2.14 Microscopy

2.14.1 Zeiss AxioImager Z1 ApoTome Microscope

Representative images were taken on an AxioImage Z1 ApoTome microscope (Carl Zeiss, Oberkochen,Germany) which employs the use of epifluorescence. As the microscope contained five different channels, it allowed for the imaging of up to five different secondary antibodies or dyes which could be compiled into one image. All images were taken at x63 magnification unless otherwise stated.

2.14.1.1 Cell Counts

Antibody binding to cells was quantified by manually counting the number of bound cells around each NMJ. The total number of bound cells was expressed as a percentage of the total number of NMJs.

2.14.1.2 EthD-1 Positive Cell Counts

EthD-1 stains the nuclei of dead cells and was used as an indication of complement activation by anti-sulfatide antibodies. Immunofluorescent studies using the new anti-sulfatide antibodies established that the terminal myelinating Schwann cell is present on a swelling of the CFP-positive axon at the terminal hemi node near the NMJ. This was used as a reference as to the location of the cell in the control tissue, which lacked antibody staining. 50 NMJs were imaged for each tissue and the number of EthD-1 positive cells in this location were counted. The total number of EthD-1 positive cells was then expressed as a percentage of the total number of NMJs counted.

2.14.2 Confocal Microscopy

For quantitative purposes, images were taken upon a Zeiss LSM 5 Pascal confocal microscope or a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). Confocal microscopes employ the use of lasers to excite the secondary antibodies at defined wavelengths of light. Fine optical z-slices through the tissue can be achieved due to the exclusion of all out of focus light.

All images were taken at a magnification of 40x with oil immersion and the same settings were used throughout the quantification of an antibodies binding.

This was performed by first orientating the tissue by eye and then taking a snap image. Approximately one hundred and fifty NMJs were imaged for each tissue section which, once collected, were quantified using an in-house macro. This worked on the principal of identifying the BTx staining and any overlying secondary antibody staining. The intensity of antibody binding was then given as a value between 0 and 255 arbitrary units (AU). These values were used to create box and whisker plots but statistical analysis was only performed on the median intensity value of all the NMJs for each mouse.

2.15 Statistical Analysis

All graphs were generated using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and statistical analysis, where appropriate, was performed using the same software with p values <0.05 deemed as significant. Array results were expressed as either individual values with the median marked by a line or as bar graphs displaying the mean ± standard error of mean (SEM). Immunofluorescence studies were graphed using either bar graphs or Tukey box and whisker plots. The statistical tests performed on individual experiments are given in their figure legends.

2.15.1 Coefficient of Variation

The intra-assay and inter-assay variation was calculated between runs using the mouse control serum. In large arrays every 12^{th} combinatorial glycoarray slide was used as a control slide. The intra-assay variation for each lipid antigen was determined by comparing all the control slides within the one experiment. This was calculated by multiplying the mean of the SD by 100 and then dividing it by the total mean of the duplicates. The inter-assay variation was determined by comparing multiple control slides from different experiments using the same calculation.

2.15.2 Heat Map Analysis

Raw intensity values from the human array studies were used to generate heat maps using MultiExperiment Viewer software (Dana-Farber Cancer Institute, Boston, MA, USA).. These maps underwent hierarchical clustering (HCL) and Pearson correlation with average linkage clustering. Raw values were displayed using a rainbow colour scheme, whereas complex signals that had individual values subtracted were displayed using a green-black-red colour scheme.

3 ARRAY SCREENING OF HUMAN MONOCLONAL ANTIBODIES

3.1 Introduction

As already discussed, the exact pathogenic mechanisms involved in MMN are still unclear. Evidence from clinical studies provide strong support that the condition is immune mediated, as it is commonly associated with anti-GM1 antibodies and responds well to immune modulating treatments (Lawson & Arnold, 2014); however, this has yet to be definitively proven.

In an attempt to address whether the immune system was in fact responsible for disease pathogenesis, the Willison group cloned anti-GM1 antibodies from MMN and GBS patients over 20 years ago (Paterson et al., 1995; Willison et al., 1994). These antibodies were originally screened on ELISA against single ganglioside antigens and were found to bind GM1 and its structural analogues, GA1 and GD1b, to varying degrees.

Subsequent studies attempted to establish the binding patterns of the antibodies in both human and mouse tissue (Greenshields et al., 2009; O'Hanlon et al., 1996, 1998; Willison et al., 1997). They found that the antibodies were capable of binding to a wide range of neuronal structures, but were unable to adequately explain the specific injury experienced by MMN patients. The original conclusions drawn from these studies suggested that single antigen distribution alone could not account for disease pathogenesis. However, they did not address the effect of the lipid raft environment on ganglioside presentation or the pathophysiological vulnerability of different target sites to immunological attack.

The recent advancements in neuropathy research regarding the roles of ganglioside complexes led to the desire to re-examine these antibodies and their target antigens. It was hypothesised that different antigen combinations may modify the binding abilities of these antibodies, which may better explain their binding patterns in tissue and their roles in the pathogenesis of the disease. This chapter therefore probed the human monoclonal antibodies against a series of

ganglioside complexes, printed using the combinatorial glycoarray, to better understand their significance in the pathogenesis of MMN.

3.2 Results

3.2.1 Human Monoclonal Antibodies

The monoclonal antibodies were originally produced from the PBMCs of four MMN patients and one GBS patient. These were immortalised with EBV and then fused with a mouse myeloma cell line to allow for long term culture. As mentioned, the antibodies were screened upon ELISA and were found to bind to GM1, GA1 and GD1b, with the exception of SM1 which did not bind the latter ganglioside. Table 3.1 indicates the names and original reactivities of the antibodies to the different target antigens.

Table 3-1 – ELISA Binding Properties of Human Monoclonal Antibodies

			Reciprocal of Ab Conc that gave ½ max binding		
Antibody	**Isotype**	**Disease**	**GM1**	**GA1**	**GD1b**
BO1	IgMλ	MMN	1200	11500	250
BO3	IgMλ	MMN	1500	7250	50
BR1	IgMκ	MMN	1530	1680	680
DO1	IgMλ	GBS (AMAN)	5500	4300	5700
SM1	IgMλ	MMN	6500	50	0
WO1	IgMλ	MMN	9100	14280	1700

The original binding values of the human monoclonal antibodies as determined by ELISA. (Paterson et al., 1995)

3.2.2 Antibody Production and Quality Screening

The antibody producing cells were thawed from storage in liquid nitrogen and grown over a period of several weeks. Samples of supernatant from each cell line were tested periodically to determine if the cells were still producing antibody. Several different stocks of BO3, BR1 and SM1 were raised but all were poor antibody producers and eventually stopped producing antibody altogether. This resulted in the low availability of these three antibodies, particularly BO3 and BR1.

Attempts were made to purify the antibodies following the collection of supernatant from the various hybridoma cell lines. Originally this was attempted by running the supernatant though a commercial IgM affinity purification column, which was expected to bind to and isolate the antibody. Starting material, wash and elution fractions were all tested using quantitative ELISA but it was found that none of the antibodies bound to the columns.

This was somewhat expected as IgM antibodies are notoriously difficult to purify (Gautam & Loh, 2011). To overcome this problem, an alternative method was employed in which the supernatant was run through a 100kDa membrane to concentrate the antibodies. This method did not purify the antibodies but did allow for the production of sufficient stocks for subsequent characterisation studies.

Some antibodies, particularly BO3, had very low concentrations even after this process. This resulted in the antibodies all being screened at 2µg/ml to allow for a fair comparison of the binding capabilities of all six antibodies.

3.2.3 Combinatorial Glycoarray Screening

Screening studies commonly use complexes in 1:1 w:w configurations; however, these do not take into consideration the differences in the molar ratios, which will vary substantially depending upon the molecular weights of the individual lipids. This may have a significant impact upon the comparability of the different complexes to one another but this effect has yet to be examined experimentally (Galban-Horcajo et al., 2013). To ensure that this was not being overlooked in this study, the antibodies were screened against lipids printed in both 1:1 w:w and 1:1 mol:mol arrangements. All experiments were performed using the combinatorial glycoarray as per Section 2.4.5. Signals were given in arbitrary units of fluorescence for both the single and complex epitopes. To best represent cis-enhancement and cis-inhibition the signals of the single constituent lipids were both subtracted from the dimer complex signal and then graphed. Negative values indicated cis-inhibition while positive values indicated cis-enhancement. Due to the non parametric nature of the data and the limited number of independent experiments, statistical analysis was not performed.

3.2.3.1 BO1

Under these conditions, BO1 did not appear to bind particularly well to the single ganglioside species in either array configuration (Table 3.2 and Figure 3.1). Signals could be detected to GM1 and associated complexes if the scanners PMT or the antibodies concentration was increased but the signals were still relatively weak (data not shown).

The only antigens that did produce strong signals were GA1:Chol, GA1:GalC and GA1:sulfatide. In the w:w configuration, they displayed respective values of 7056, 14714 and 8501 FI units, whilst in the mol:mol configuration they displayed values of 35517, 631 and 4184 FI units.

Comparisons of the different array configurations found that there were particularly large differences between the same complexes. This was most apparent with GA1:Chol, which in the mol:mol configuration had a signal that was approximately 28000 FI units higher than the w:w signal.

These differences appeared to be related to the amount of Chol that was present in the complexes. GA1:Chol w:w was the equivalent of 1:3.23 mol:mol so the complex contained a lower proportion of Chol than the other configuration. This resulted in lower binding signals, suggesting that BO1 preferentially binds to gangliosides in a Chol rich microenvironment.

Similarly, when GA1 was in complex with GalC and sulfatide, it produced higher signals with the w:w lipids, which had the equivalent molar ratios of 1:1.5 and 1:1.4 respectively. This suggested that increased number of accessory lipids, such as GalC or sulfatide, also improved antibody binding.

3.2.3.2 BO3

BO3 was derived from the same patient as BO1 but showed a stronger binding pattern to GM1 and GM1 complexes at the same concentration. It bound the single GM1 antigen with median signals of 13385 and 11777 FI units in the w:w and mol:mol arrays respectively.(Table 3.3 and Figure 3.2) Both configurations

Table 3-2 – Median Binding Values of BO1 to Ganglioside Complexes

	GM1	GA1	Chol	GalC	Sulfatide	GA1:Chol	GA1:GalC	GA1:Sulfatide
w:w	989	1352	26	75	77	7056	14714	8501
mol:mol	617	1708	0	51	0	35517	631	4184

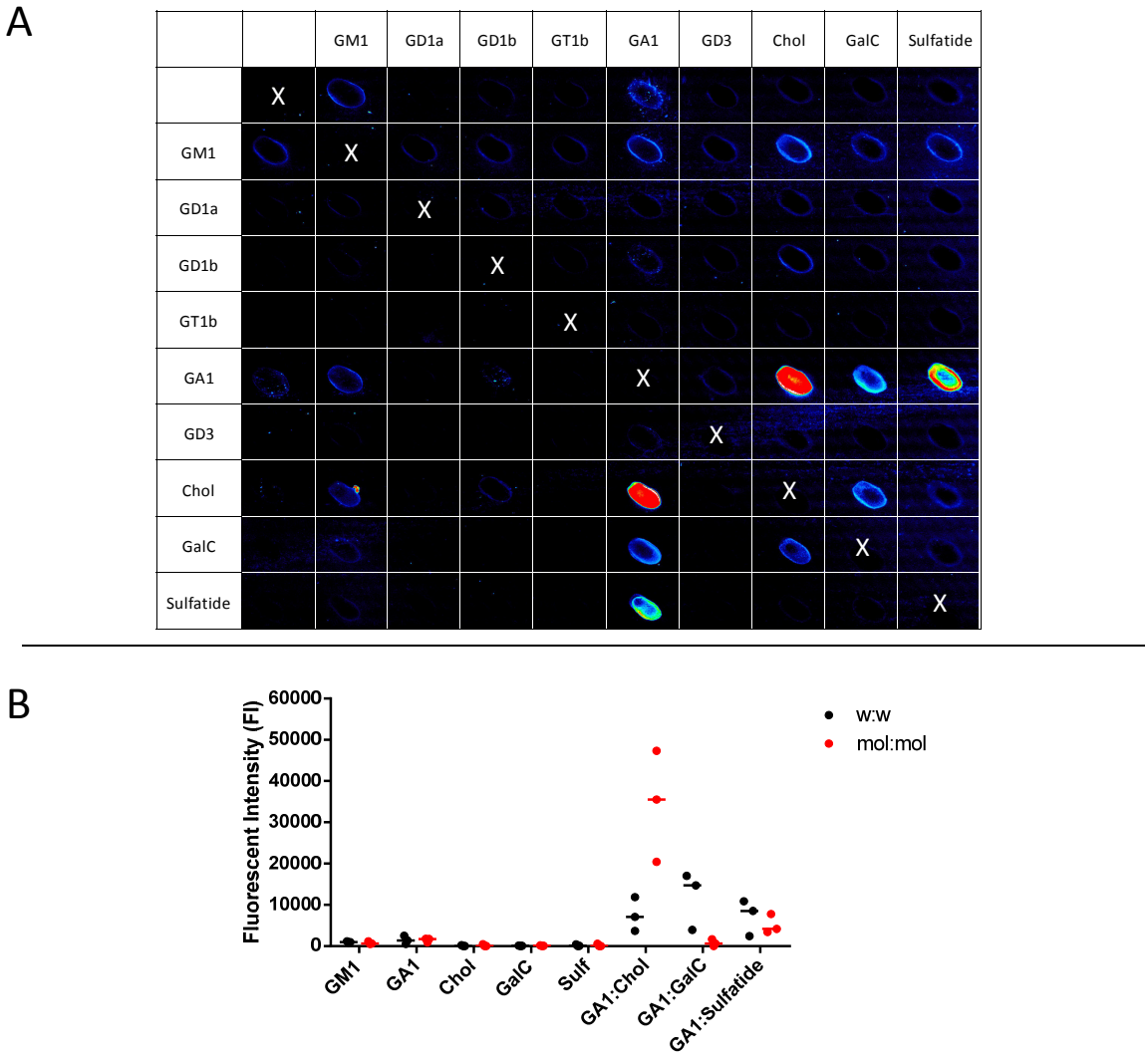


Figure 3.1 – BO1 Characterisation against Ganglioside Complexes

BO1 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by BO1 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. BO1 had weak signals to all single ganglioside antigens but was strongly enhanced with GA1:Chol, GA1:GalC and GA1:sulfatide in the w:w array and GA1:Chol and GA1:sulfatide in the mol:mol array.

showed slight enhancement when GM1 was in complex with Chol, whilst all other GM1 complexes were neutral or inhibitory.

BO3 also bound to the single GA1 antigen displaying median signals of 4077 and 7115 FI units in the w:w and mol:mol arrays. GA1:sulfatide complexes enhanced antibody binding in both array configurations, displaying approximate median signals of 11000 FI units. Complexes containing Chol similarly enhanced BO3 binding in the mol:mol array, whilst in the w:w array they had no effect.

There was also binding to the single GD1b antigen, which displayed median signals of approximately 6000 FI units in both array configurations. Unlike GM1 and GA1, however, there was no enhanced antibody binding with GD1b complexes.

A common inhibitory signal was seen with GM1, GA1 and GD1b when the gangliosides were in complex with either GD1a or GT1b. Unlike other lipids, which attenuate binding when in complexes, GD1a and GT1b completely abolished the binding signals altogether, giving approximate raw readings of 0 FI units.

Table 3-3 – Median Binding Values of BO3 to Ganglioside Complexes

		-	GM1	GD1a	GD1b	GT1b	GA1	GD3	Chol	GalC	Sulfatide
w:w	**GM1**	13385	-	-12820	-14363	-13385	-13337	-12628	1560	-3295	883
mol:mol	**GM1**	11777	-	-10317	-13319	-11582	-11247	-10619	6146	-5330	-1642
w:w	**GA1**	4077	-11247	-4066	-9456	-4077	-	-1643	11485	-3044	11868
mol:mol	**GA1**	7115	-13337	-5422	-7068	-6758	-	-5920	-5872	-4812	11515
w:w	**GD1b**	6262	-14363	-5545	-	-6262	-7068	-5773	-5113	-4824	-1652
mol:mol	**GD1b**	6121	-13319	-5535	-	-6121	-9456	-6031	-4715	-3803	-1216

A comparison of both array configurations showed results similar to those seen with BO1 binding. The only major differences were with complexes containing Chol, which enhanced BO3 binding more in the mol:mol arrays. As described with BO1 binding, this could be attributed to the increased concentration of Chol producing an enhanced signal.

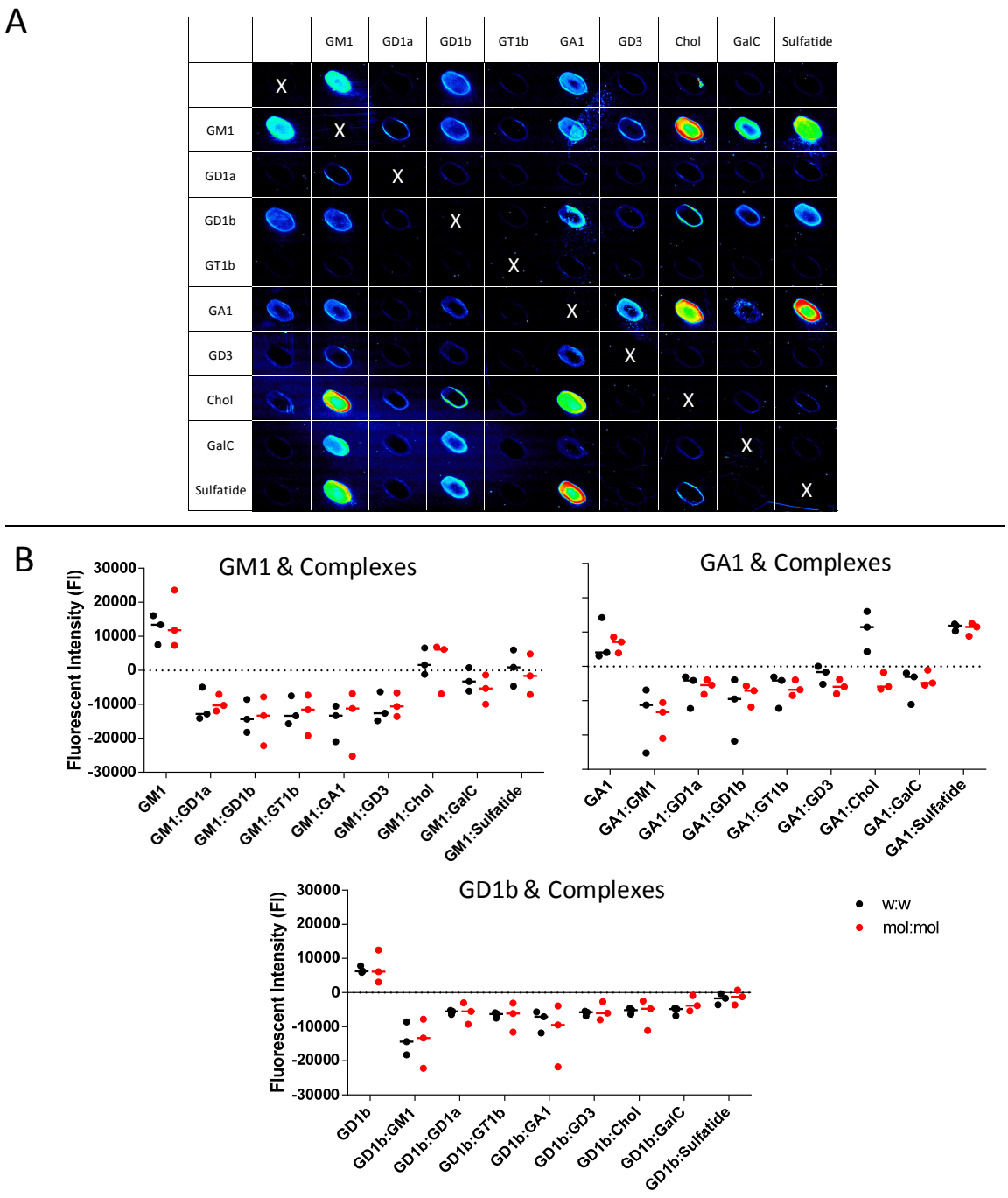


Figure 3.2 - BO3 Characterisation against Ganglioside Complexes

BO3 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by BO3 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. BO3 bound to the single GM1, GA1 and GD1b antigens. It was enhanced by GM1:Chol, GA1:Chol and GA1:sulfatide complexes.

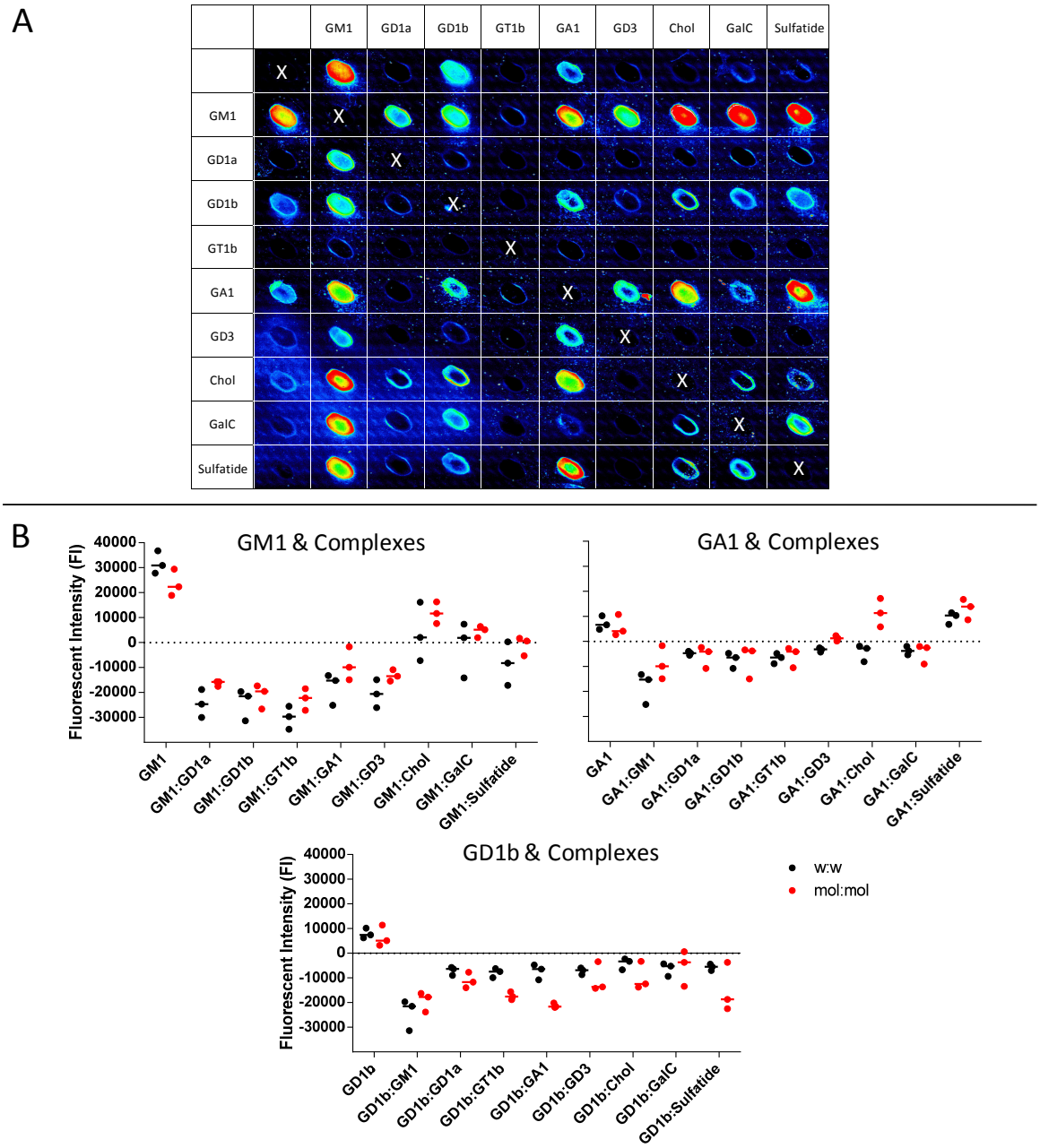


Figure 3.3 – BR1 Characterisation against Ganglioside Complexes

BR1 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody.B: Graphs indicate the fluorescent intensity values produced by BR1 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. BR1 showed a strong response to GM1 and GM1 in complex with Chol, GalC and sulfatide. It also bound GA1 and GA1 sulfatide in both array configurations and GA1-Chol in the mol:mol array. The antibody bound to GD1b but was inhibited by all associated complexes.

3.2.3.3 BR1

BR1 showed a much stronger GM1 signal than either BO1 or BO3 under the same conditions (Table 3.4 and Figure 3.3). In the w:w array the single antigen displayed a median signal of 30931 FI units and showed enhancement when in complex with Chol and GalC. A similar result was shown in the mol:mol array, with the single GM1 antigen displaying a median signal of 22344 FI units, which was also enhanced by Chol and GalC. As with BO3, the antibody binding signal was almost completely abolished when GM1 was in complex with GT1b and GD1a and to a lesser degree when it was in complex with GD3.

Table 3-4 – Median Binding Values of BR1 to Ganglioside Complexes

		-	GM1	GD1a	GD1b	GT1b	GA1	GD3	Chol	GalC	Sulfatide
w:w	GM1	30931	-	-24709	-21532	-29705	-15250	-20632	2042	1869	-8246
mol:mol	GM1	22344	-	-15756	-19534	-22223	-9918	-13483	11640	5186	631
w:w	GA1	6627	-15250	-4706	-6421	-6434	-	-3145	-2765	-3777	10387
mol:mol	GA1	4114	-9918	-4114	-3782	-4114	-	1253	11348	-2491	13928
w:w	GD1b	7429	-21532	-6374	-	-7429	-6421	-6931	-3286	-5204	-5519
mol:mol	GD1b	5105	-17794	-11733	-	-17576	-21609	-13644	-12453	-3687	-18674

This was similarly found with GA1, where BR1 binding was inhibited in the presence of GD1a or GT1b. The antibody bound the single GA1 antigen in both arrays with low signals and was only enhanced in the w:w array when GA1 was in complex with sulfatide, whilst in the mol:mol array there was an enhancement with both sulfatide and Chol.

The GD1b signals for both arrays were comparable to the signals found with GA1 binding. Unlike with GA1 though, all complexes with GD1b were inhibitory in both array configurations.

The largest difference between arrays was again with GM1:Chol and GA1:Chol, which produced higher binding signals in the mol:mol configuration. As previously stated, the higher amount of cholesterol present in the mol:mol array appeared to enhance the binding of the antibody to gangliosides.

3.2.3.4 DO1

DO1 was the only antibody derived from a GBS patient (Table 3.5 and Figure 3.4). In the w:w array, it bound GM1 with a median signal of 9614 FI units, whilst

in the mol:mol array it had a median signal of 15378 FI units. Unlike the other antibodies, DO1 was not enhanced when GM1 was in complex with either Chol or sulfatide.

The antibody bound GA1 in the w:w array and mol:mol array with median signals of 17493 and 7629 FI units respectively. An enhancement was seen with GA1:sulfatide in the mol:mol array but this was not replicated in the w:w array. As with the other antibodies, there was a large enhancement when GA1 was in complex with cholesterol in the mol:mol array, displaying a median signal of 34010 FI units. This was higher than both the single antigen and the other complexes including GA1-sulfatide. This same enhancement was not seen in the w:w array.

Table 3-5 – Median Binding Values of DO1 to Ganglioside Complexes

		-	GM1	GD1a	GD1b	GT1b	GA1	GD3	Chol	GalC	Sulfatide
w:w	**GM1**	9614	-	-7577	-12499	-8923	-8260	-5425	-7469	-5512	-11284
mol:mol	**GM1**	15378	-	-8991	-17794	-14946	-12033	-6060	-6118	-11755	-9859
w:w	**GA1**	17493	-8260	-8521	-28428	-17006	-	-9102	-3668	-11580	-515
mol:mol	**GA1**	7269	-12033	-7269	-21609	-7269	-	-3169	31717	-5368	8258
w:w	**GD1b**	25572	-12498.5	-19057	-	-23984	-28428	-19794	-11445	-16216	-13344
mol:mol	**GD1b**	18731	-17794	-11733	-	-17576	-21609	-13644	-12453	-3686.5	-18674

DO1 also bound GD1b displaying median signals of 25572 and 18731 FI units in the w:w and mol:mol arrays respectively. As with the other antibodies, this binding was inhibited when GD1b was in complex with any other lipid.

Complexes containing GD1a or GT1b were again inhibitory with DO1 binding, as described with BO3 and BR1. A similar inhibition was shown with GD3 to a lesser degree in which complexes containing the ganglioside consistently displayed raw signals of 5000 FI units or less.

Comparisons of the binding fingerprints of DO1 between arrays configurations showed higher binding to GA1:Chol and GA1:sulfatide in the mol:mol array compared to the w:w array. This again suggested that the higher cholesterol content in the GA1-Chol complex enhanced the binding capability of the antibody. The increased binding to GA1:sulfatide was unexpected as the mol:mol array contained a smaller amount of sulfatide than the w:w array. It was possible that DO1 preferentially bound to the complex with less sulfatide but, as the differences between the arrays were small, no definitive conclusions could be drawn from this data.

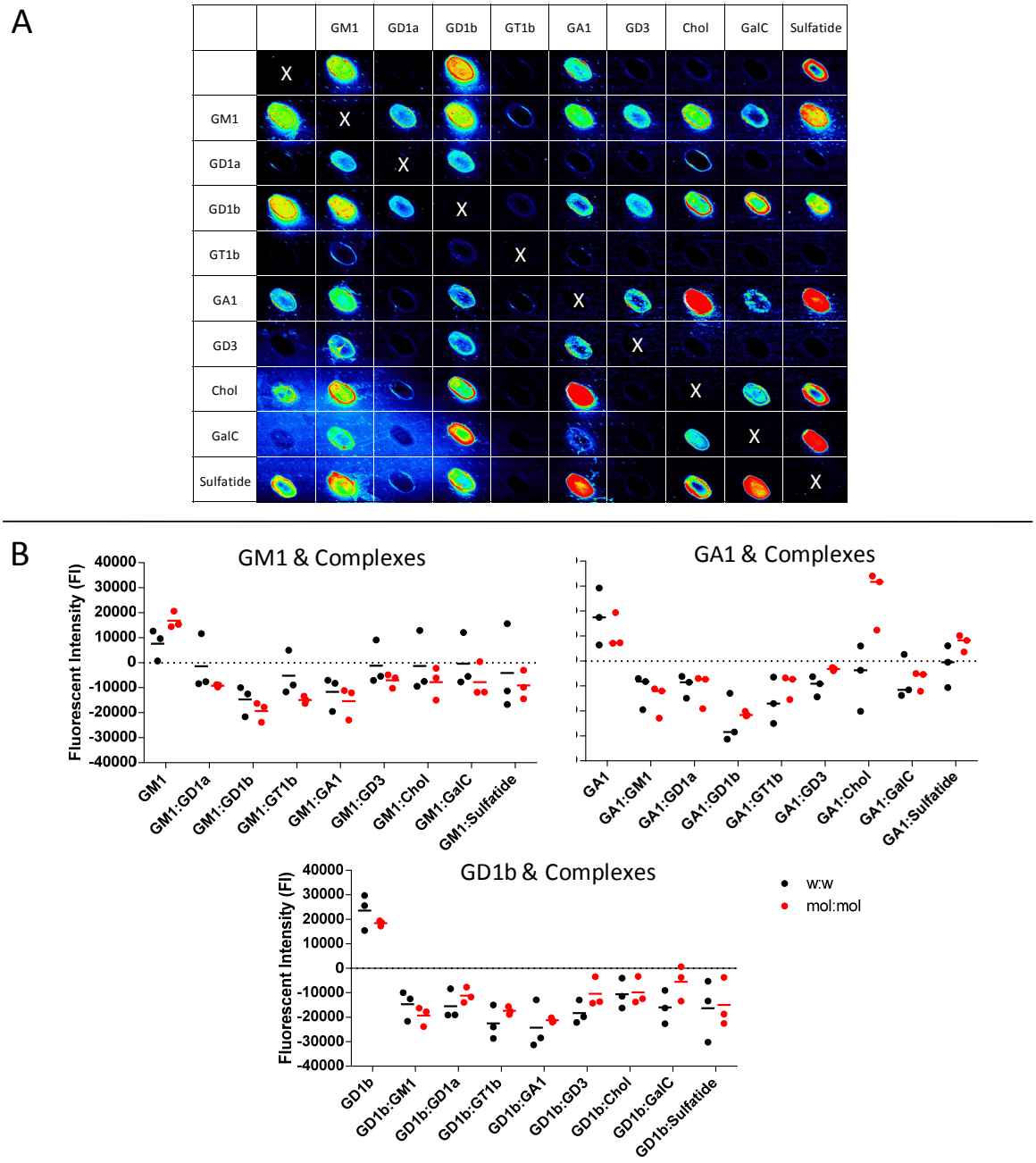


Figure 3.4 - DO1 Characterisation against Ganglioside Complexes

DO1 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by DO1 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. DO1 bound to GM1 but was not enhanced by any GM1 complexes. It also bound GA1 and GA1 sulfatide in both array types and GA1-Chol in the mol:mol array. The antibody bound to GD1b but was inhibited associated complexes.

Table 3-6 - Median Binding Values of SM1 to Ganglioside Complexes

		-	GM1	GD1a	GD1b	GT1b	GA1	GD3	Chol	GalC	Sulfatide
w:w	GM1	17421	-	-15479	-13582	-15957	-17705	-11201	-2096	-4780	-1877
mol:mol	GM1	24372	-	-20470	-22665	-23328	-15468	-19067	-1186	-5859	-6009
w:w	GA1	20884	-17705	-20745	-20559	-20764	-	-20717	-16845	-24809	-9292
mol:mol	GA1	12829	-15468	-12659	-5374	-12741	-	-10277	-2501	-15632	-1735

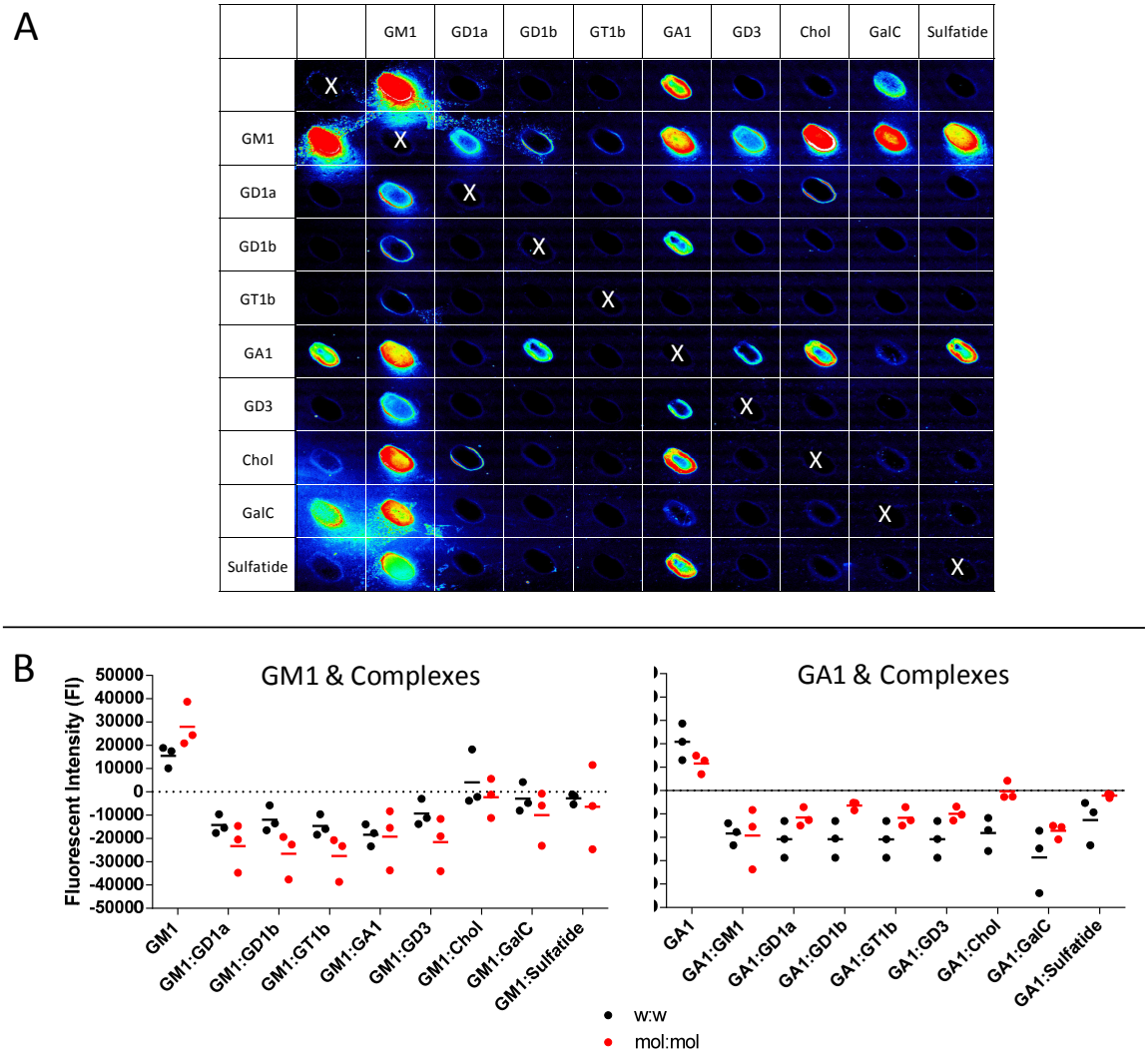


Figure 3.5 - SM1 Characterisation against Ganglioside Complexes

SM1 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by SM1 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. SM1 bound to GM1 in both array configurations but was not enhanced by GM1 complexes. The antibody similarly bound the single GA1 antigen but was not enhanced by any GA1 complexes.

3.2.3.5 SM1

SM1 bound well to GM1 in both array configurations displaying median signals of 17421 and 24372 FI units in the w:w and mol:mol arrays respectively (Table 3.6 and Figure 3.5). Neither array showed a median cis-enhancement with GM1 complexes, although both had at least one repeat where a complex containing Chol, GalC or sulfatide was enhanced.

A similar result was seen with GA1, where the single antigen produced a high signal in both arrays that was not enhanced by any GA1-complexes. SM1 also differed from BO3, BR1 and DO1 as it did not bind the single GD1b epitope at all.

A comparison between array configurations did not show any clear differences. It should be noted, however, that although GA1:Chol did not produce an enhancement, it did have a higher signal in the mol:mol array compared to the w:w array, which is similar to the results seen with the other antibodies.

3.2.3.6 WO1

Under these conditions, WO1 showed a poor response to GM1, displaying FI medians of 925 and 6235 in the w:w and mol:mol arrays respectively (Table 3.7 and Figure 3.6). Little enhancement was seen in either array configuration, with most complexes showing similarly low signals as those of the single antigen. The only enhanced signal was found with GM1:Chol in the mol:mol array, which was higher than the single antigen and the other complexes.

Table 3-7 - Median Binding Values of WO1 to Ganglioside Complexes

		-	GM1	GD1a	GD1b	GT1b	GA1	GD3	Chol	GalC	Sulfatide
w:w	**GM1**	925	-	430	1391	-365	-18103	981	915	578	3212
mol:mol	**GM1**	6235	-	-3198	-4605	-5245	-19863	-3434	12070	-2601	-398
w:w	**GA1**	25543	-18103	-19814	-20118	-25423	-	-21925	17421	28150	-12839
mol:mol	**GA1**	19925	-19863	-19914	-11094	-19925	-	-16848	40803	-1105	-1657
w:w	**GD1b**	1961	1390.5	-1961	-	-1961	-20118	-1961	11998	-3136	-1059
mol:mol	**GD1b**	1566	-4605	-1424	-	-1566	-11094	-1307	7542	-1605	-865

The antibody produced a much stronger response to GA1 than GM1, displaying FI medians of 25543 and 19925 in the w:w and mol:mol array respectively. In the w:w arrays this signal was enhanced when GA1 was in complex with Chol and GalC. In the mol:mol array, the only enhancement was with GA1:Chol.

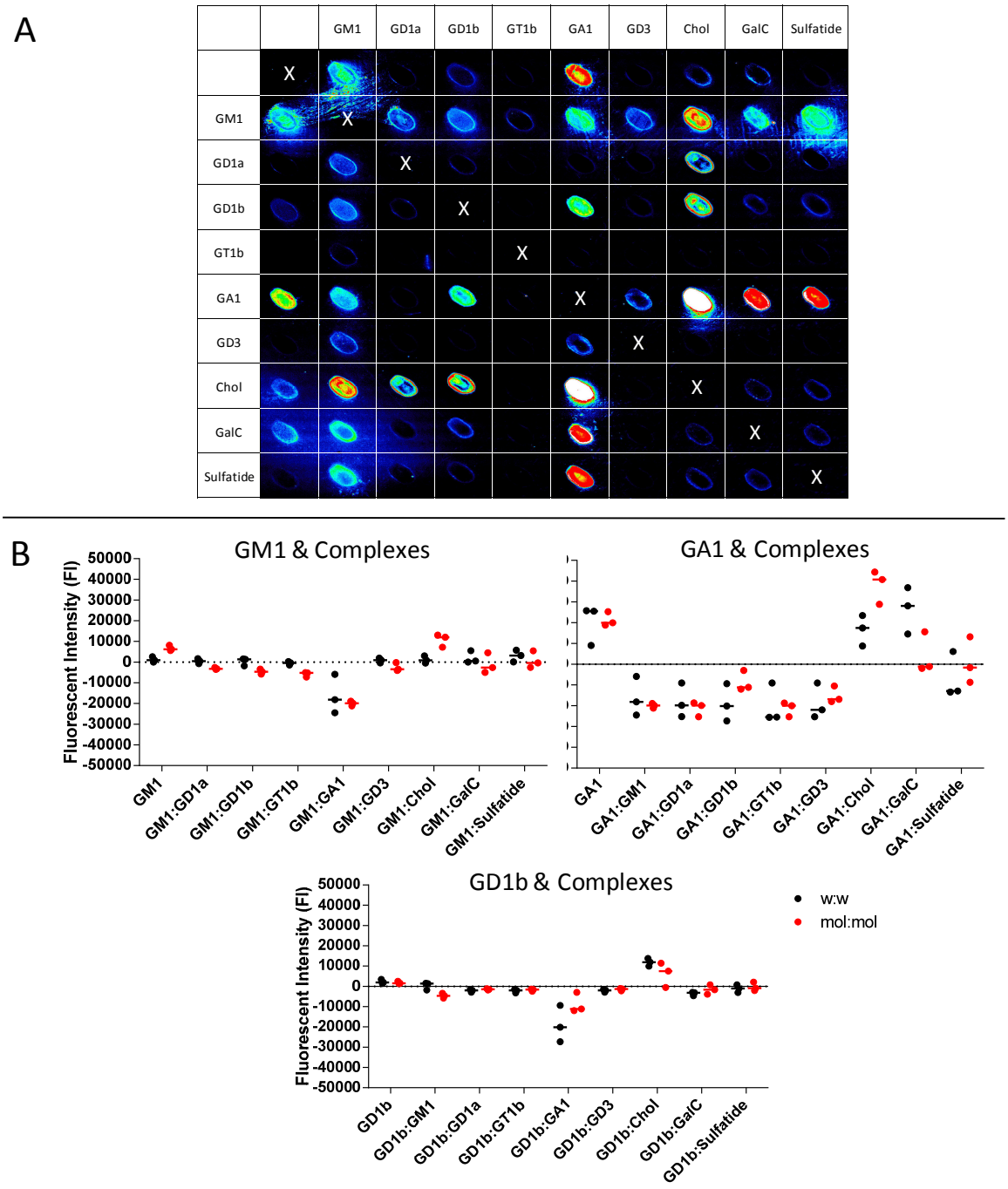


Figure 3.6 – WO1 Characterisation against Ganglioside Complexes

WO1 (2µg/ml) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. A: A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by WO1 to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. WO1 bound poorly to GM1 but showed a good signal to the single GA1 epitope and GA1-Chol in both array configurations. It also had poor GD1b reactivity but shown enhancement with the GD1b-Chol complex.

There was little binding to the single GD1b epitope but unlike the other antibodies, WO1 was enhanced when GD1b was in complex with Chol in both the w:w and mol:mol arrays.

A comparison between the two array configurations showed a larger enhancement to GM1:Chol and GA1:Chol in the mol:mol array compared to the w:w array, which was consistent with the results of the other antibodies. In contrast, the w:w array showed a larger enhancement with GA1:GalC and GD1b:Chol which was unique to WO1 alone.

3.2.3.7 Galactocerebroside and Sulfatide Binding

GalC and sulfatide are closely associated with lipid rafts and are major components of the myelin sheath. Many anti-carbohydrate antibodies have been found to cross react to these lipids due to the presence of specific binding sub domains (Alving, 1986).

The single species and complexes composed of both glycolipids are also targets of auto-antibodies in their own right and were included in the array to determine if the human monoclonal antibodies were cross reactive with their structures (Table 3.8 and Figure 3.7).

At 2µg/ml BO1 and CTx showed little or no binding to either GalC, sulfatide or GalC:sulfatide complexes, whilst WO1 showed a small signal to the single GalC species in both array configurations. BO3 did not bind the single species in either array but showed enhancement to the GalC:sulfatide complex in the w:w array displaying a median signal of 8937 FI units. This enhancement was not replicated in the mol:mol array. A similar result was seen with BR1 which shown little reactivity to the single antigens but was enhanced with the GalC:sulfatide complex displaying values of 5733 and 13831 FI units in the mol:mol and w:w arrays respectively.

DO1 did not show any binding to the single GalC epitope but did bind to sulfatide displaying an approximate median signal of 11000 FI units in both array configurations. This signal was enhanced with the GalC:sulfatide complex displaying median signals of 15791 and 6910 FI units in the mol:mol array and w:w arrays respectively.

Table 3-8 – Median Binding Values of Human Monoclonal Antibodies to Sulfatide and GalC Complexes

		BO1	BO3	BR1	DO1	SM1	WO1	CTx
w:w	GalC	75	134	1204	91	6407	1975	0
mol:mol	GalC	51	67	306	7	6598	1547	147
w:w	Sulfatide	77	4	1865	11245	10	42	428
mol:mol	Sulfatide	0	25	113	11739	0	5	749
w:w	GalC:Sulfatide	121	8937	13831	6910	-6252	-1742	-163
mol:mol	GalC:Sulfatide	-34	290	5733	15791	-6598	-1393	-700

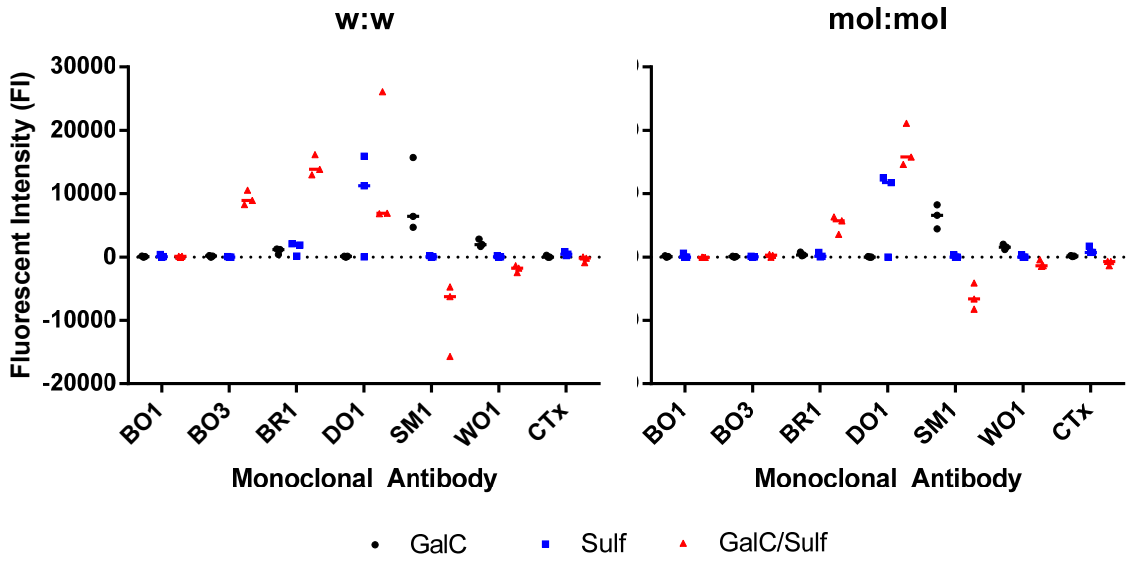


Figure 3.7 – GalC:sulfatide Complexes

All 6 human monoclonals and CTx were screened against GalC (black), sulfatide (blue) and GalC:sulfatide complexes (red) which were printed in either 1:1 w:w or 1:1 mol:mol configurations. Each spot represents the mean of each repeat and the complex value represents the complex signal minus both the single constituent values. In the w:w array BR1 and DO1 bound to the single sulfatide antigen, whilst SM1, BR1 and DO1 bound to the single GalC antigen. The binding signals of BO3, BR1, and DO1 were enhanced with GalC:sulfatide complexes but SM1 was attenuated by GalC:sulfatide complexes. In the mol:mol array SM1 and WO1 bound to GalC, DO1 bound to sulfatide and BR1 and DO1 bound to GalC:sulfatide complexes. The SM1 binding signal was attenuated by GalC:sulfatide complexes.

SM1 differed from the other antibodies as it bound to the single GalC species in both array configurations, while showing little or no binding to the single sulfatide antigen. The GalC binding signal was completely attenuated in the presence of sulfatide (GalC:sulfatide complex).

3.2.3.8 Cholera Toxin

Cholera toxin (CTx) is commonly used as a marker of GM1 but is known to bind to other gangliosides including GA1, GD1a, GM2, GD1b and GT1b (Kuziemko et al., 1996). When these antibodies were first cloned they were screened against human tissue and characterised as either being CTx like or CTx unlike in their binding behaviour (O'Hanlon et al., 1998).

Cholera toxin conjugated to Alexa-fluor 647 was therefore screened on the combinatorial glycoarray at 2µg/ml to establish its binding pattern and determine any similarities between it and the human monoclonal antibodies (Table 3.9 and Figure 3.8). As a result of the intense signals seen with CTx, the slides were scanned at a lower PMT (reduced amplification of the fluorescent signal) to allow for the full binding fingerprint to be elucidated.

In both the array configurations CTx bound GM1 and GA1 with similar median values of approximately 10000 FI units. In the w:w array, GD1b also displayed a similar value whereas in the mol:mol array there was very little binding to the single GD1b antigen, which contained a higher proportion of ganglioside. This reduction in the binding signal may be attributed to a change in density, which affects the ability of CTx to bind.

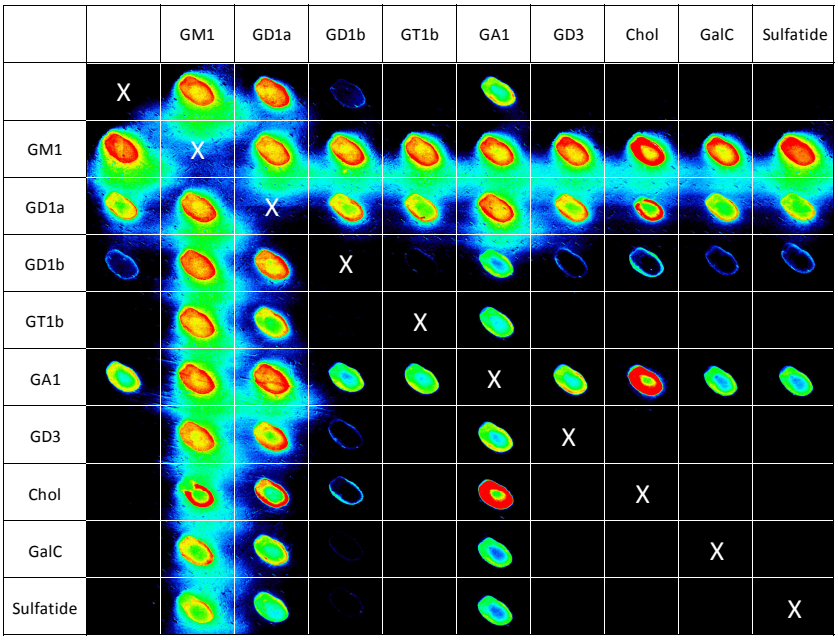
There was little or no inhibition of the binding signals with complexes except with GD1b, which was inhibited when in complex with any of the other lipids. The only enhancing complex was GA1:Chol, which was higher than the single antigens in the mol:mol array. This enhancement was not seen in the w:w array.

Cholera toxin differed from the human monoclonal antibodies as it bound to complexes containing GD1a as well as the single GD1a antigen displaying medians of approximately 10000 FI units. Under these conditions it did not bind GT1b even though this had previously been reported (Kuziemko et al., 1996). To ensure the validity of the stock of GT1b, it was tested against an in house mouse antibody targeting the ganglioside and was shown to bind successfully. The absence of CTx binding on the combinatorial glycoarray was therefore surprising but may have been related to differences in lipid concentration or density in the array format.

Table 3-9 - Median Binding Values of Cholera Toxin to Ganglioside Complexes

	GM1	GD1a	GD1b	GA1	GA1:Chol
w:w	8836	9238	7290	14076	-1803
mol:mol	11511	8503	1003	13425	16203

A



B

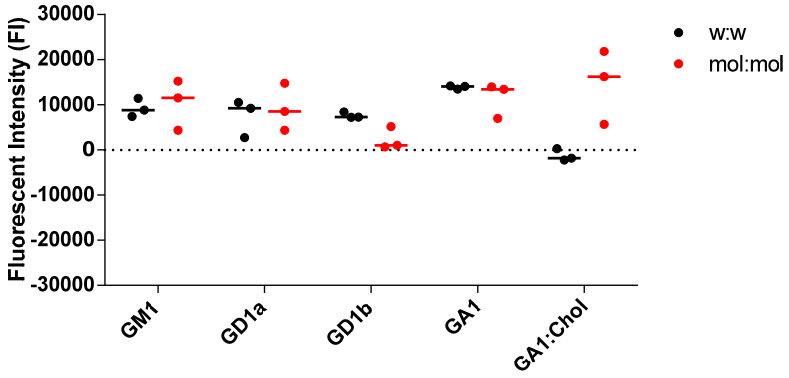


Figure 3.8 - Cholera Toxin Binding Profile

CTx (2µg/ml)) was screened against a panel of lipids with complexes in a 1:1 w:w or 1:1 mol:mol ratio, with the exception of cholesterol which was in a 1:5 mol:mol ratio. **A:** A representative blot indicates the binding pattern of the antibody. B: Graphs indicate the fluorescent intensity values produced by CTx to different antigens in w:w (black) and mol:mol (red) configurations. Complex signals were calculated by subtraction of both constituent single lipid values from the dimer complex signal. Each dot indicates the mean of each repeat. In the w:w array CTx bound with similar values to GM1, GD1a, GD1b and GA1 with little or no complex enhancement or inhibition. In the mol:mol array CTx similarly bound to GM1, GD1a and GA1 but not GD1b. There was little complex enhancement except for GA1-Chol.

Overall, there was no apparent difference between the CTx like (BO3, SM1, WO1) and the CTx unlike (BO1, BR1, DO1) antibodies, nor was there anything unusual about the CTx binding fingerprint. The differences between the tissue binding properties of the two groups must therefore be related to the presence of other lipids or proteins in the natural lipid raft environment, which were not examined in this study.

3.3 Discussion

The use of the combinatorial glycoarray allowed for a more robust screening of the human monoclonal antibodies than had previously been performed. By incorporating different ganglioside and glycolipid complexes, it was possible to establish how the presence of different lipids could influence the binding behaviour of antibodies, which is more akin to the lipid membrane environment.

As reported previously, the presence of GD1a in complexes inhibits the binding of GM1 antibodies in mouse peripheral nerve (Greenshields et al., 2009).This was also found to be true with all six human monoclonal antibodies which had FI values of 0 when screened against any complexes containing GD1a. Greenshields et al hypothesised that this could be caused by the interaction between the glycosphingolipid hydroxyl groups in the gangliosides, which would act as hydrogen donors between the molecules. This interaction would result in a change in the conformation of GM1 which would become cryptically hidden from the immune system.

A similar inhibitory effect was seen in this experiment, when antibodies were probed against complexes that contained GD1a and GT1b. Both GD1a and GT1b differ from the other gangliosides screened as they share a sialic acid on their terminal galactose. As sialic acids contain several hydroxyl groups it is plausible that these interact with the GM1/GA1/GD1b molecules to mask their antibody binding epitope through steric hindrance.

In contrast, the presence of non ganglioside accessory lipids appeared to enhance the binding signals of the various antibodies. This was shown through the comparisons between the mol:mol and w:w arrays, which indicated that antibody binding improved in varying degrees when higher amounts of Chol, GalC or sulfatide were present in complexes. As these are all found in lipid rafts it

could be assumed that their presence more closely imitates the natural cellular environment, which improves the ability of the antibodies to bind.

Recent work has shown that the presence of these lipids can modify the ganglioside head group orientation and thus enhance or inhibit their presentation to the immune system. This has been shown in particular with cholesterol, which is almost entirely buried in the plasma membrane except for a hydroxyl group which protrudes into the exoplasm. This hydroxyl group can interact with glycosphingolipids to form complexes, which have been shown to enhance the binding of certain toxins, proteins and recently anti-ganglioside antibodies (Fantini & Yaho, 2013; Galban-Horcajo et al., 2014; Mahfoud et al., 2010).

Alternatively, it is possible that the presence of greater number of accessory lipids improves the spacing between the gangliosides. When screened as single antigens, it is often assumed that the gangliosides exist as single standalone epitopes that are free to interact with antibodies. The different hydroxyl groups within the various molecules, however, are likely to interact with one another and thus change the presentation of the head groups. This has been explored previously in work that suggested that the presence of different lipids such as DPPC alters the phase behaviour of GM1 to form condensed domains which modifies molecular packing (Frey et al., 2008). Other work has also suggested that cholesterol can interfere with interactions between headgroups of other lipids by acting as a spacer molecule (Kucerka et al., 2007). With respect to this work, it is possible that the presence of accessory lipids may interfere with interactions between ganglioside head groups and thus open up them up to interactions with antibodies.

Aside from gangliosides, it was also noted that many of the antibodies cross reacted to sulfatide and GalC. Previous research has shown that anti-ganglioside antibodies commonly bind to these glycolipids in solid phase assays, due to the presence of specific binding domains (Alving & Richards, 1977; Townson, Greenshields, et al., 2007). This phenomenon may not be relevant in disease, however, as the antibodies are unable to bind these glycolipids if they have been incorporated into plasma membranes.

Examination of the glycoarray blots from this experiment may offer an explanation for this behaviour, as the antibodies were unable to bind either GalC or sulfatide when they were in complex with most other lipids. It would therefore be highly improbable that the antibodies would bind to these antigens *in vivo*, as the glycolipids would be unlikely to be found naturally in standalone formations.

Although this chapter provided a fresh insight into the binding behaviours of the human monoclonal antibodies, it also raised questions about a previously unexplored aspect of neuroimmunology research. It was noted, through the use of the two different array configurations, that the concentration of lipids in complexes could drastically alter the binding signals of the antibodies.

Current screening methods, however, only employ complexes in 1:1 w:w configurations, raising the possibility that they are unable to detect low levels of particular antibodies. To address this issue, the binding behaviours of the antibodies would need to be assessed against complexes containing a range of different lipid concentrations, which is a concept that is further explored in Chapter 4.

4 ACCESSORY LIPID COMPLEXES

4.1 Introduction

Clinical assays acts as supportive tools in the diagnosis of inflammatory neuropathies with ELISA acting as the current gold standard. The established method for ELISA involves coating plates with single gangliosides to identify specific anti-ganglioside antibodies (Willison et al., 1999). The drawback of this method is that gangliosides would not be naturally found in standalone formations in the plasma membrane. They would be clustered with other lipids and proteins in liquid ordered (lipid rafts) and liquid disordered domains, which would alter their presentation and thus their ability to bind antibodies (Hammond et al., 2005; Sonnino et al., 2007).

Although the exact mechanism of disease has not been elucidated in GBS or MMN, research has suggested that antibodies are able to exert pathogenic effects via specific binding to lipid rafts (Ueda et al., 2010). These rafts contain higher proportions of certain lipid containing molecules such as gangliosides, Chol and SM compared to the liquid disordered phase, which contain high amounts of other lipids such as PC (Brown & Rose, 1992; Pike et al., 2002). There are also other lipids that are enriched in rafts but their presence is dependent upon the function and state of the cells in which they are contained. GalC and sulfatide for example, are enriched within the lipid rafts of myelin where they aid in the transportation of myelin proteins, whereas PS is enriched in the rafts of apoptotic or necrotic cells, which aids in their clearance by macrophages (DeBruin et al., 2005; Simons et al., 2000).

The cis-interactions of these raft lipids with gangliosides have been explored from an immunological viewpoint by autoimmune neuropathy researchers (Galban-Horcajo et al., 2013; Rinaldi et al., 2013). They identified a subset of antibodies in patient sera, which were either enhanced in the presence of these ganglioside:lipid complexes or were only capable of binding in their presence. This led to the hypothesis that the antibodies were binding to a neo-epitope, which is a unique structural epitope formed by components of both lipids.

These neo-epitopes would possibly account for the discrepancy between the presence of gangliosides in a large number of tissues and the limited injury experienced in autoimmune neuropathies (Gong et al., 2002; Svennerholm et al., 1992, 1994). For example in MMN, in which patients frequently have anti-GM1 antibodies, it could be postulated that pathogenic antibodies would bind exclusively to complexes consisting of GM1 and those lipids that are enriched within myelin such as GalC or sulfatide (Norton & Cammer, 1984).This would explain why other GM1 enriched tissues remain unaffected in the disease as the absence of the other lipids would prevent formation of the complex.

Whilst this hypothesis is valid, it is reliant upon the formation of neo-epitopes, which is a concept that has never been demonstrated at a molecular level (Harschnitz et al., 2014). In contrast, the concept of conformational modulation, in which lipids in the microenvironment influence the presentation of the ganglioside, has much more compelling evidence, having been demonstrated both experimentally as well as in modelling studies (Fantini & Yaho, 2013; Lingwood et al., 2011). The distinguishing feature in this theory is that the lipids are not incorporated into the binding epitope themselves but are merely responsible for its exposure on the gangliosides. As such, the lipids in this process are referred to as accessory lipids.

By altering the orientation of the ganglioside, these accessory lipids would directly influence the concealment or exposure of different binding epitopes, which would affect the binding capabilities of different anti-ganglioside antibodies (P. Lopez et al., 2006). This suggests that the composition of the ganglioside microenvironment, not only influences which antibodies can bind, but also which tissues are vulnerable to immune mediated injury.

A prime example of this was shown in Chapter 3 where the human monoclonal antibodies were shown to bind preferentially to ganglioside complexes containing Chol, GalC or sulfatide. These lipids are enriched in myelinating cells, which would suggest that the antibodies arose to preferentially target ganglioside epitopes that are uniquely presented in myelin rich tissues. This suggestion corresponded well with the known source of the antibodies, which were MMN patients. As MMN is a demyelinating condition, it is cogent to assume

that patient antibodies would bind and injure the myelin sheath, whilst leaving other tissues unaffected (Krämer et al., 1999; Lee, 2001).

In addition to their presence, another aspect relating to accessory lipids was found during the experiments in Chapter 3. Comparisons of the different array configurations indicated that the antibodies appeared to bind preferentially to complexes containing higher proportions of accessory lipids.

This was an interesting finding as our previous research has tended to focus on screening antibodies against complexes in uniform ratios (Galban-Horcajo et al., 2013; Rinaldi et al., 2013). The drawback of screening sera in this fashion is that it does not take into account that the concentrations of lipids will presumably differ in rafts in relation to their function and location. This will likely have an impact upon how they interact with gangliosides and thus how certain epitopes will be presented for antibody binding. This would also have ramifications in the effectiveness of sera studies, which may be overlooking the detection of certain antibodies that can only bind to complexes containing higher proportions of accessory lipids.

To further address the roles that these lipids have in complexes, an experiment was devised to examine the binding of the human monoclonal antibodies to ganglioside complexes containing a variety of raft and non raft lipids in increasing proportions. These included Chol, GalC, sulfatide, PS, SM, and PC. It was hypothesised that increased binding signals would be detected against complexes containing higher proportions of raft rich lipids common to the myelin sheath, whereas non raft lipids would be expected to have only a minimal effect.

4.2 Results

4.2.1 Accessory Lipid Arrays

All experiments were performed using the combinatorial glycoarray as per Section 2.4.5. Each array consisted of single lipid targets and complexes composed of the gangliosides and lipids in molar ratios. These were all prepared as per Section 2.3.1. The number of moles of ganglioside remained constant throughout the experiment and were 6.39×10^{-12}, 7.96×10^{-12} and 5.47×10^{-12} for

GM1, GA1 and GD1b respectively. The 1:1 complexes consisted of the same number of moles of accessory lipids as the gangliosides and these doubled in each corresponding complex to produce ratios of 1:2. 1:4, 1:8, 1:16 and 1:32. Complexes were not printed at higher ratios as the excess lipid was found to interfere with the ability of the TLC printer to accurately dispense the lipid solution.

To best represent cis-enhancement and cis-inhibition the signals of the single constituent lipids were both subtracted from the dimer complex signal and then graphed. Negative values indicated cis-inhibition while positive values indicated cis-enhancement. The experiments were performed five times and analysed using a Kruskal-Wallis test followed by Dunn's multiple comparisons.

4.2.1.1 Chol Complexes

The different array configurations in Chapter 3 indicated that the human monoclonal antibodies bound better to ganglioside complexes that contained a higher proportion of Chol. As Chol is the most abundant lipid in mammalian cell membranes (Bloch, 1991), it was hypothesised that further increasing its proportion in complexes would lead to the detection of even higher binding signals.

This was confirmed to varying degrees within this experiment; with some antibodies producing substantially higher binding signals, whilst others showed more subdued increases (Figure 4.1). In general, the largest binding signal enhancements of each antibody were found to be against their main target antigen as originally established by ELISA (Table 3.1).

An example of this was the antibody BO1, which had previously been shown by ELISA and combinatorial glycoarray to primarily target GA1 and GA1 complexes (Willison et al., 1994). In this experiment, BO1 followed a similar pattern and produced considerably higher binding signals against GA1:Chol complexes compared to those composed of GM1 or GD1b.

Based upon their target antigens, the antibodies were found to follow a general pattern, where increasing the proportion of Chol in complexes led to an increase

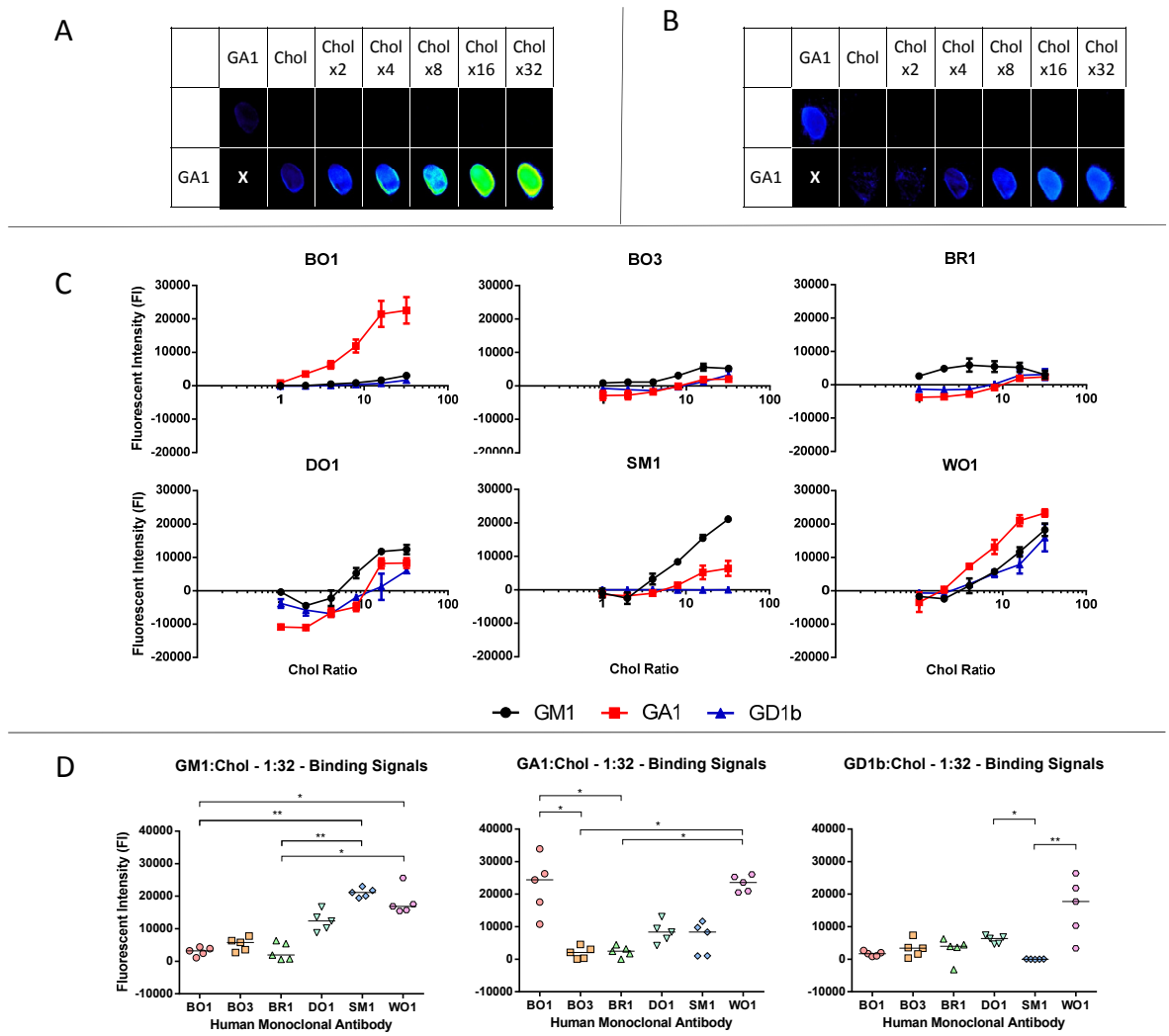


Figure 4.1 – Binding of Human Monoclonal Antibodies to Chol Complexes

The human monoclonal antibodies were screened against ganglioside:Chol complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of all the antibodies tended to increase as the proportion of Chol increased. At a 1:32 ratio, BO1, SM1 and WO1 produced the highest binding signals and were significantly higher than both BO3 and BR1 against different ganglioside:Chol complexes. DO1 was slightly enhanced with Chol ratios but to a lesser degree than the other antibodies (*=$p<0.05$), (**=$p<0.01$), (***=$p<0.001$).

in binding signal. For the majority of antibodies, this resulted in a ganglioside:Chol ratio of 1:32 being deemed optimal for antibody detection. The only exception to this was BR1, which was found to produce a peak binding signal for GM1:Chol complexes at a ratio of 1:8 that decreased in each subsequent complex.

Examination of the binding signals at a 1:32 ratio indicated that the antibodies fell into two broad categories: those that were greatly enhanced and those that were to a lesser degree. Comparisons between the antibodies respective binding signals indicated that BO1, SM1 and WO1 showed the largest levels of enhancement, which were found to be significantly higher than those signals produced by BO3 and BR1 (Kruskal-Wallis test, Dunn's multiple comparisons, $P<0.05$). These differences varied between complexes depending upon which ganglioside was present, but the BO3 and BR1 signals tended to remain low regardless of the composition of the complex. Other differences between antibodies, such as the statistically higher difference between WO1 and DO1 compared to SM1 in the GD1b complexes, were attributed to the variability in ganglioside target antigens between antibodies.

The only exception to this broad categorisation was DO1, which showed intermediate levels of enhancement with most complexes. The differences in the derivation of the antibodies may have accounted for this as DO1 was derived from an AMAN patient, whereas the other antibodies were derived from patients suffering from MMN. Differences in epitope specificity would therefore be expected but there is not enough evidence from this data to draw any solid conclusions in regards to its binding behaviour.

4.2.1.2 GalC Complexes

As with Chol, the data in Chapter 3 showed that higher binding signals were detected against complexes containing higher proportions of GalC. As GalC is the most abundant glycolipid in the myelin sheath (Coetzee et al., 1996), it was hypothesised that further increasing its proportion in complexes would lead to the detection of even higher binding signals.

This hypothesis appeared to correlate well with the results from this experiment; however, the variation between antibodies was particularly high (Figure 4.2). In general, the binding signals followed the same pattern produced by the Chol complexes, with increased proportions of GalC producing increased binding signals. Again, the only exception to this was BR1, which produced an optimal signal with GM1:Chol complexes at a ratio of 1:8 that decreased in each subsequent complex.

Unlike Chol complexes however, the increases in binding signals were not solely focussed on the main target antigen. Similar levels of enhancement were detected with all ganglioside:GalC complexes although the target antigen tended to produce the highest overall signal.

The two broad categories described with the Chol complexes were similarly produced in this experiment; however the differences between the two groups were much more distinctive with GalC. A ratio of 1:32 was again optimal for producing the largest enhancements for the majority of antibodies. Statistical analysis between the antibodies at this ratio showed that the binding signals of BO1, SM1 and WO1 were significantly higher than BO3, BR1 and DO1 against a variety of complex compositions (Kruskal-Wallis test, Dunn's multiple comparisons, $P<0.05$). The latter group of antibodies were found to produce consistently low binding signals regardless of the ratio of GalC present in the complex, which suggested that they bind to their targets independent of the modulating effect of GalC.

4.2.1.3 Sulfatide Complexes

In Chapter 3, sulfatide followed the same pattern as both Chol and GalC with complexes containing higher proportions producing higher binding signals. sulfatide is closely related to GalC and is similarly found in abundance in the myelin sheath (Coetzee et al., 1996). It was therefore hypothesised that it would produce a similar pattern to that seen with the GalC ratios, in that complexes containing increased proportions would result in increased binding signals.

The data, however, did not fully support this hypothesis (Figure 4.3). Most antibodies did show an initial increase in their binding signals as the proportion

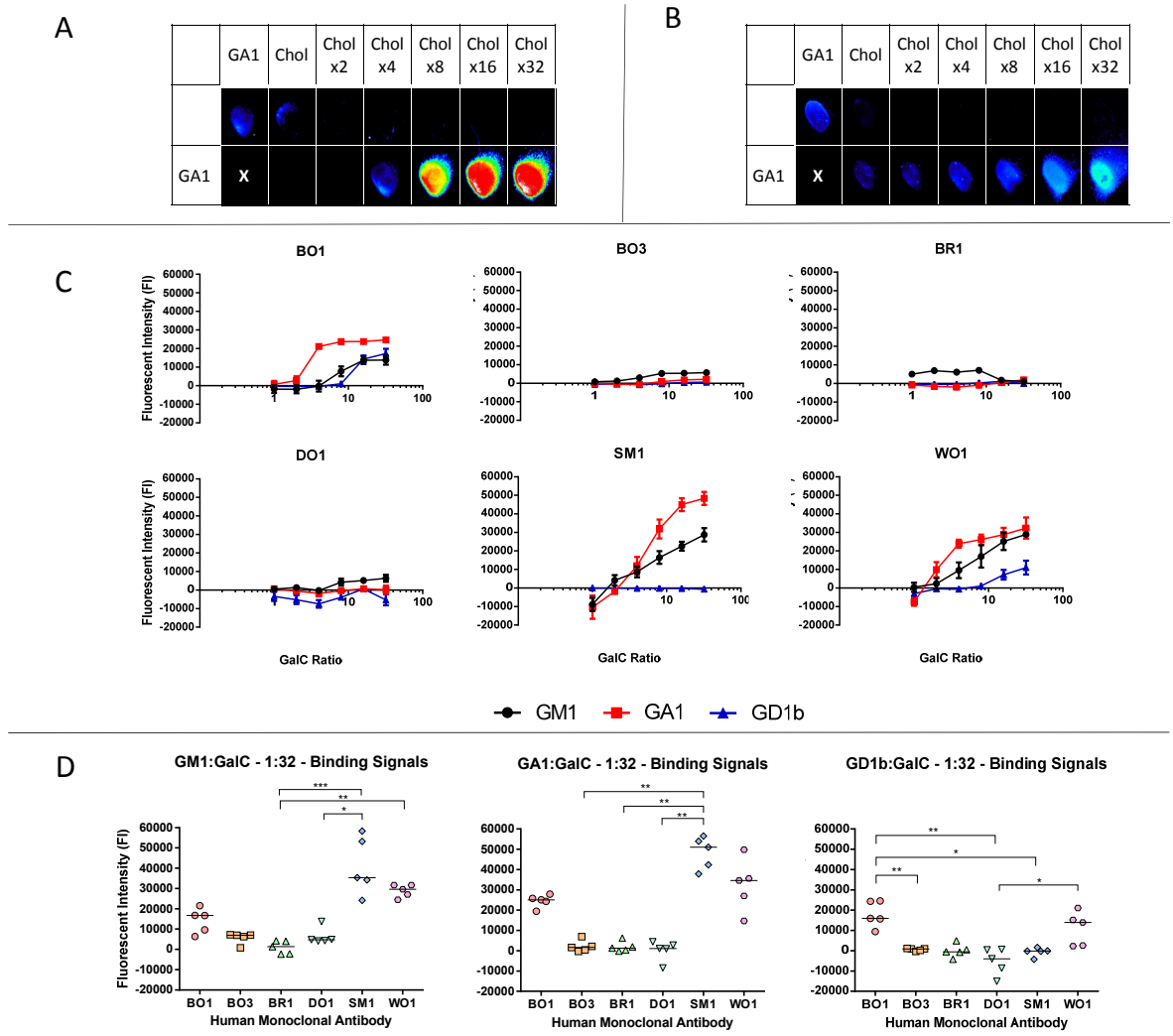


Figure 4.2 - Binding of Human Monoclonal Antibodies to GalC Complexes

The human monoclonal antibodies were screened against ganglioside:GalC complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of all the antibodies tended to increase as the proportion of GalC increased. At a 1:32 ratio, BO1, SM1 and WO1 produced the highest binding signals and were significantly higher than BO3, BR1 and DO1 against different ganglioside:GalC complexes (*=$p<0.05$), (**=$p<0.01$), (***=$p<0.001$).

of sulfatide increased; however, this appeared to peak at a ratio of 1:4 for GA1 complexes and 1:16 for GM1 and GD1b complexes, where the majority of the signals began to decrease again. Certain antibodies, such as BR1 and DO1, also bound to the single sulfatide antigens at higher concentrations, which further reduced their net binding signals.

The optimal ratios were used to compare the binding signals of the different antibodies. The two categories, which had been described previously for both Chol and GalC, were not replicated with the sulfatide complexes. The antibodies instead tended to have similar binding signals, which only varied in regards to their target antigens. The only exception to this was DO1 which did not appear to be enhanced by sulfatide at any ratio.

As mentioned previously, DO1 was derived from an AMAN patient whilst the other antibodies were derived from MMN patients. This may account for the differences in binding signal as AMAN is not a demyelinating disease and would, therefore, be unlikely to generate antibodies that would target gangliosides in environments enriched with myelin lipids.

4.2.1.4 PS Complexes

Ganglioside:PS complexes were not part of the original characterisation of the human monoclonal antibodies in Chapter 3, but were included within this experiment due to their previous association with antibodies in GBS (Rinaldi et al., 2013). As these complexes would be expressed on the membranes of necrotic or apoptotic cells (Hoffmann et al., 2001), it was hypothesised that they may be potential targets during ongoing neuropathy. Assuming that PS would be abundantly expressed on these cells due to pathological changes, it was also proposed that increasing the proportion of PS in the complex may lead to the detection of higher binding signals, due to increased interactions between the lipids and ganglioside headgroups.

The data appeared to correspond well with this hypothesis, as complexes containing higher amount of PS were found to produce higher binding signals (Figure 4.4). For the majority of antibodies, similar signals were produced for

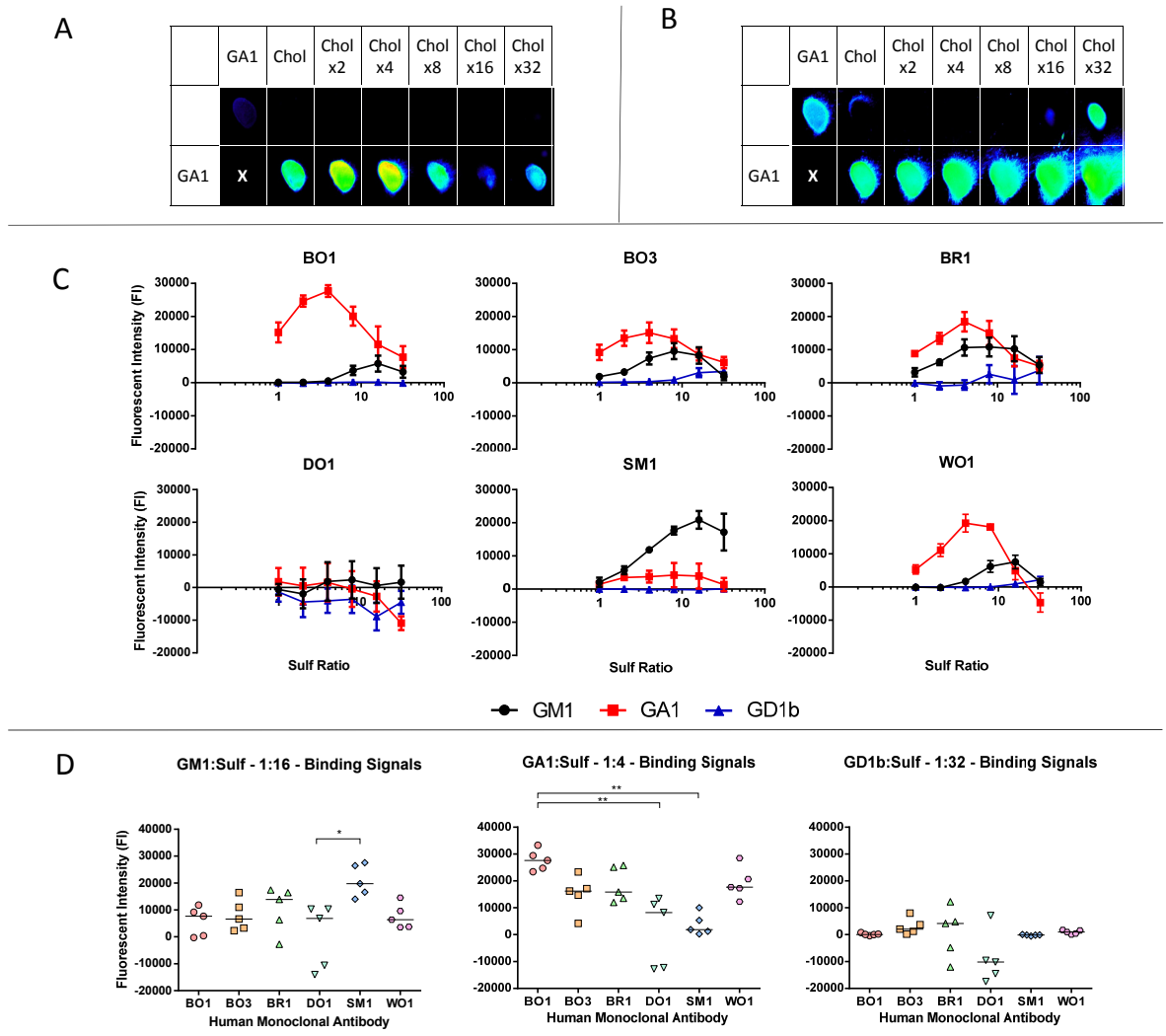


Figure 4.3 – Binding of Human Monoclonal Antibodies to Sulfatide Complexes

The human monoclonal antibodies were screened against ganglioside:sulfatide complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of all the antibodies except DO1 tended to increase as the proportion of sulfatide increased up to a point where binding began to decrease again. At high ratios some binding was detected to the single sulfatide. At a 1:16 ratio, SM1 was found to be significantly higher than DO1 in GM1:sulfatide complexes. In GA1:sulfatide complexes at a ratio of 1:4 BO1 was found to be significantly higher than DO1 and SM1 (*=$p<0.05$), (**=$p<0.01$), (***=$p<0.001$).

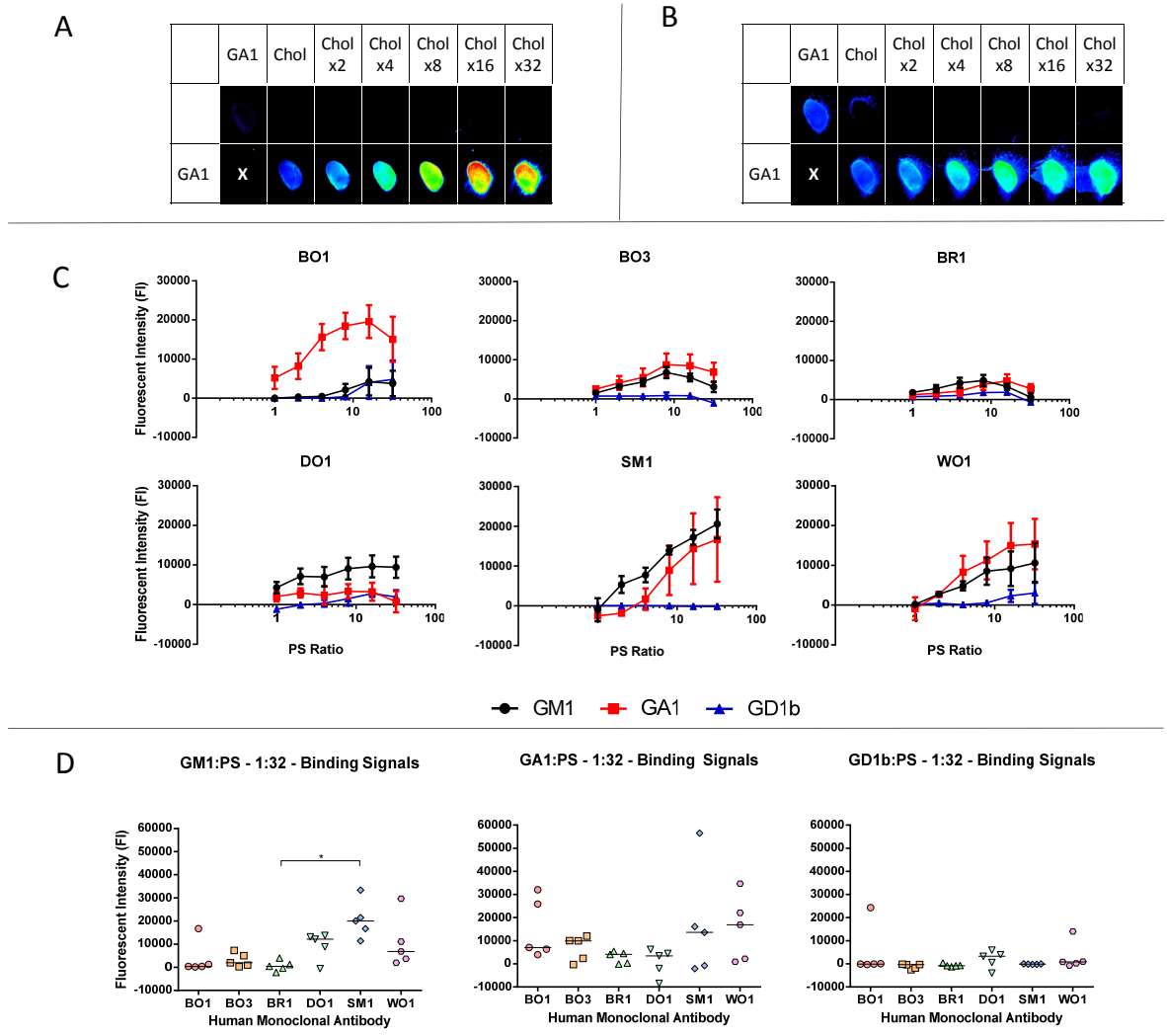


Figure 4.4 - Binding of Human Monoclonal Antibodies to PS Complexes

The human monoclonal antibodies were screened against ganglioside:PS complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of all the antibodies tended to increase as the proportion of PS increased up to an optimal ratio of between 1:16 and 1:32. No discernible differences were detected between the antibodies except with BR1 which indicated very little enhancement. The only significant difference was between SM1 and BR1 in the GM1:PS complexes. (*=$p<0.05$), (**=$p<0.01$), (***=$p<0.001$).

both GM1 and GA1 complexes; however, BO1 and DO1 were found to only produce high signals against one type of ganglioside complex.

The optimal ratio for binding varied between antibodies with some preferring a ratio of 1:16, whilst others preferred a ratio of 1:32. For comparison purposes, the latter was selected as it was found to produce the highest signals for the most antibodies. Unfortunately, the variation between experimental repeats was found to be particularly high with PS complexes, which resulted in the detection of few statistically significant differences between antibodies.

Regardless of these limitations, BO1, SM1 and WO1 were still found to produce the highest binding signals, although there was less of a clear definition between these antibodies and the others. BO3 and DO1 produced reasonably high signals, particularly against complexes containing higher proportions of PS, whilst BR1 was found to produce minimal increases in binding signals that, again, peaked at a ratio of 1:8.

4.2.1.5 SM Complexes

Alongside Chol, SM is one of the most abundant raft lipids in mammalian cell membranes (Brown & Rose, 1992). It is found in particularly high quantities in the myelin sheath of the PNS (Morell & Quarles, 1999), which suggested that it may have roles in influencing the presentation of gangliosides within these tissues. This led to the hypothesis that the human monoclonal antibodies would produce higher binding signals against ganglioside:SM complexes compared to those produced against the single glycolipids. As SM is highly enriched within myelinating cells, it was also hypothesised that complexes containing a higher proportion would produce higher binding signals compared to those containing a lower proportion.

This was confirmed with half the antibodies; however, the other half had a drastically different response (Figure 4.5). The binding signals of BO1, SM1 and WO1, as hypothesised, increased as the proportion of SM in the ganglioside complexes increased. BO1 and WO1 were only enhanced by complexes containing GA1 whilst SM1 was enhanced by complexes containing either GA1 or GM1. In contrast, the binding signals of BO3, BR1 and DO1 were inhibited by all

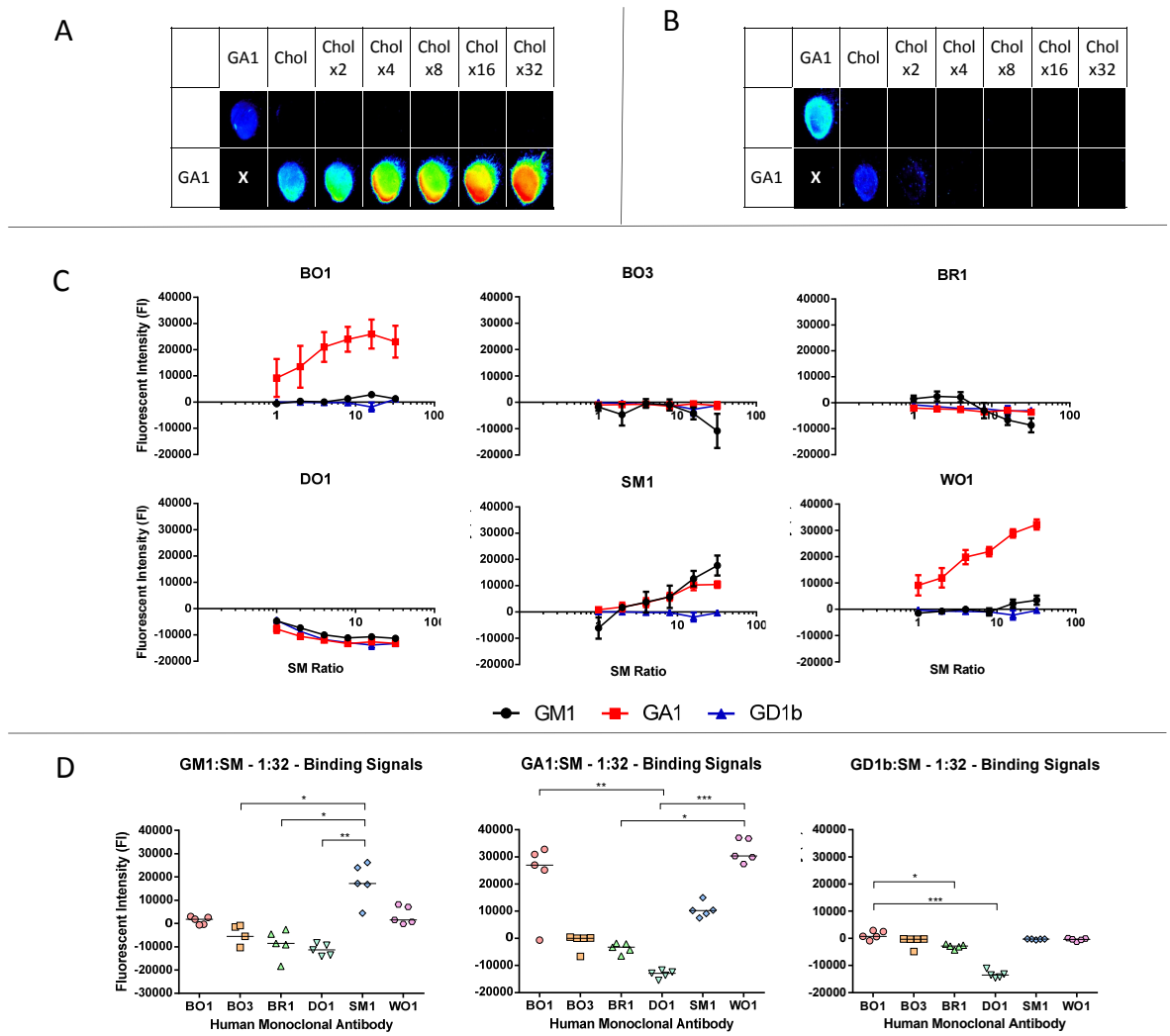


Figure 4.5 - Binding of Human Monoclonal Antibodies to SM Complexes

The human monoclonal antibodies were screened against ganglioside:SM complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of BO1, SM1 and WO1 increased as the proportion of SM increased up to an optimal ratio of 1:32. At this ratio they were significantly higher than BO3, BR1 and DO1. SM was found to inhibit the binding of these antibodies and was found to completely abolish the binding altogether (*=p<0.05), (**=p<0.01), (***=p<0.001).

complexes containing SM in low proportions and were completely abolished by complexes containing high proportions.

For those antibodies that were enhanced, the optimal ratio for binding was 1:32. This was used for comparisons between the antibody groups to establish any significant differences. As expected BO1, SM1 and WO1 were found to be significantly higher than BO3, BR1 and DO1 (Kruskal-Wallis test, Dunn's multiple comparisons, $P<0.05$). These differences varied depending upon the target antigens but, as stated, the binding signals for the latter group were always substantially lower.

4.2.1.6 PC Complexes

Phosphatidylcholine is the most common phospholipid in mammalian cell membranes (Ohvo-rekila et al., 2002). Unlike the other lipids used in this experiment, however, it is not commonly associated with lipid rafts but is instead found primarily in the liquid disordered partition of the plasma membrane (Pike et al., 2002). Based upon this information, it was hypothesised that ganglioside:PC complexes would have little influence on the binding capabilities of the human monoclonal antibodies, which would produce the same binding signals as seen with the single glycolipids.

Surprisingly, this was not found to be the case (Figure 4.6). All the antibodies showed enhanced binding signals against these complexes to varying degrees, with the usual group of BO1, SM1 and WO1 showing the most enhancements as described previously. Unlike the other ganglioside:accessory lipid complexes though, binding was somewhat restricted against PC. An example of this was BO1 and SM1, which produced strong enhancements against complexes containing their respective target antigens but no enhancements whatsoever against complexes containing other gangliosides.

In respect to their target antigens, the binding signals of the antibodies were found to increase as the proportion of PC increased. However, these enhancements tended to peak at a ratio of 1:8 where the majority of the antibody signals dropped off again. The antibodies fell into their previously

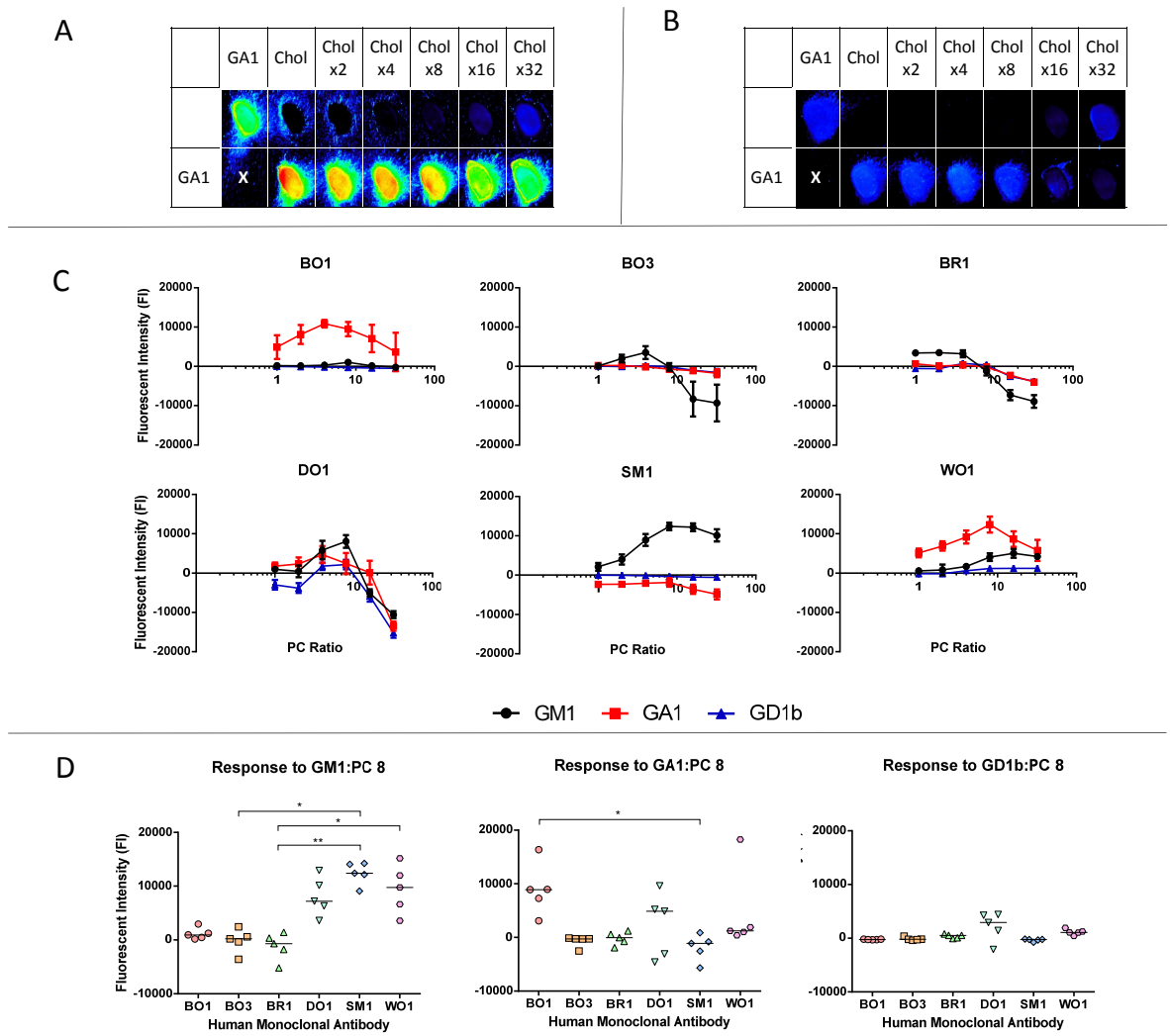


Figure 4.6 - Binding of Human Monoclonal Antibodies to PC Complexes

The human monoclonal antibodies were screened against ganglioside:PC complexes at various molar ratios. Representative blots indicate examples of concentration dependent (A) and concentration independent binding (B). Fluorescent intensity values for ganglioside complex enhancement or inhibition were calculated by subtraction of both constituent single lipid mean values from the mean complex signal. Each spot represents the mean of the repeat experiments. Positive signals indicate enhancement whilst negative signals denote inhibition (C). The relative binding signals were compared between each antibody and analysed using a Kruskal-Wallis test with Dunn's multiple comparisons (n=5) (D). The binding signals of most antibodies increased up to a ratio of 1:8 where they dropped off again. Comparisons at this ratio indicated that SM1 and WO1 produced significantly higher binding signals compared to BO3 and DO1. Binding to the single PC antigen was weakly detected in high ratios for all antibodies. (*=$p<0.05$), (**=$p<0.01$), (***=$p<0.001$).

described categories with statistical analysis between them indicating that the binding signals of SM1 and WO1 were significantly higher than both BO3 and BR1 (Kruskal-Wallis test, Dunn's multiple comparisons, $P<0.05$). DO1, as shown previously with Chol, produced an intermediate level of binding and did not fall strictly into either category.

4.2.2 Summary

The data from these experiments demonstrated that ganglioside:accessory lipid complexes were capable of enhancing the binding signals of monoclonal antibodies. Furthermore, they also established that by increasing the proportions of accessory lipids in these complexes, substantially higher binding signals could be achieved by certain antibodies, whilst others only obtained marginal improvements or inhibitions.

Examination of the individual antibodies found that they tended to fall into the same categories, regardless of which accessory lipid was present. Although it was not ideal to compare different antibodies to one another, previous research and in-house work had established that the antibodies had similar binding affinities and avidities (O'Hanlon et al., 1996; Willison et al., 1997). The differences between them in this experiment could therefore be assumed to be caused by the influence of the accessory lipids.

Based upon this assumption, it could be postulated that the antibodies that were enhanced (concentration dependent) preferentially bound to gangliosides that had undergone significant headgroup modification. In contrast, those antibodies which bound poorly to complexes containing high proportions of accessory lipids (concentration independent) could be assumed to bind preferentially to ganglioside headgroups in their relatively unmodified state.

4.3 Discussion

The roles of accessory lipids in neuroimmunology research are only now beginning to be appreciated. Even though they were first examined as far back as 1997 (Pestronk et al., 1997), it is only in the past few years that researchers

have begun to intensify their use in clinical assays (Galban-Horcajo et al., 2013; Nobile-Orazio et al., 2010).

Most of this work has focussed on GM1:GalC complexes, which several studies have indicated enhance the detection of antibodies in the sera of MMN patients (Nobile-Orazio & Gallia, 2013). The exact mechanism by which the complex achieves this is disputed; with some researchers believing that the antibodies are binding to a neo-epitope, formed by the combination of the lipids (Galban-Horcajo et al., 2013; Willison & Goodyear, 2013), whilst others believe that GalC is modulating the conformation of the GM1 headgroup, to reveal a hidden epitope (Harschnitz et al., 2014; Nobile-Orazio et al., 2013).

Evidence appears to favour the latter theory, with several different studies indicating that accessory lipids such as Chol can modify the orientation of GM1, to improve or impede the binding of proteins or toxins (Fantini & Yaho, 2013; Lingwood et al., 2011). Assuming that the same principle applies to antibody-epitope presentation, it could be proposed that different accessory lipids could alter the binding abilities of anti-ganglioside antibodies.

This was explored within this chapter by probing human monoclonal antibodies against a series of ganglioside:accessory lipid complexes. In line with the original hypothesis, all complexes containing low proportions of accessory lipids were found to enhance the binding signals of the antibodies. The levels of enhancement varied between accessory lipids, however, with complexes containing those found enriched in the rafts of the myelin sheath producing the highest binding signals, whilst those found in the liquid disordered partition of the membrane produced the lowest. In general, GalC was found to produce the most enhancement, followed by SM, sulfatide, Chol, PS and PC.

The exact mechanism by which the accessory lipids improved antibody binding was not established in these experiments; however, evidence from other fields supports the theory that the lipids interact with ganglioside headgroups to modify macromolecular access to binding sites. This has been shown most prominently by Alzheimer's researchers, who have investigated the interactions between GM1 and Chol and the effects that they have upon ligand binding (Lin et al., 2008; Lingwood & Simons, 2010; Lingwood et al., 2011). The research found that the hydroxyl group of Chol forms hydrogen bonds with the glycosidic

linkage between the galactose and ceramide molecules of GM1 (Fantini & Yaho, 2013; Manna & Mukhopadhyay, 2013). This produces a change in the orientation of the GM1 headgroup so that it shifts from perpendicular to parallel to the plasma membrane, resulting in the attenuation of the cytotoxicity of β-amyloid plaques.

As this glycosidic linkage is common to all three of the gangliosides screened within this experiment (GM1, GA1 and GD1b), it could be assumed that Chol would similarly modify their orientations to lie parallel to the membrane as well. This cannot be stated for certain however, as the influence of the gangliosides sialic acid group on its orientation is unknown.

Whilst this research has focussed on Chol, it would be reasonable to assume that other accessory lipids would also be able to interact with gangliosides in a similar manner. The structure of Chol is unique, however, as it is almost entirely buried within the plasma membrane, except for the hydroxyl group which protrudes into the exoplasm (Lodish et al., 2000). As such, this group is the only part of Chol that is able to form hydrogen bonds with GM1.

By contrast, only the ceramide or glycerol portions of the other accessory lipids are embedded in the plasma membrane, leaving their headgroups free to interact with gangliosides on the extracellular surface (Lee, 2001). These headgroups contain a variety of different functional groups that form a hydrogen bonding network with gangliosides (Mombelli et al., 2003; Stoffel & Bosio, 1997). The extent of these networks is dependent upon which accessory lipids are present. For example, GalC is capable of forming a large number of hydrogen bonds with gangliosides, due to the increased presence of a series of acceptor oxygen groups and donator hydroxyl and amine groups (Hall et al., 2010). Conversely, SM only has two donor groups, whilst PC has none.

It is unknown to what extent these hydrogen bonding networks influence the orientation of the gangliosides; however, as the accessory lipids are comparatively smaller, it could be assumed that they act to pull the ganglioside headgroups into an orientation parallel to the membrane, in a similar manner as Chol. The degree of this pull will presumably be determined by the functional groups of the accessory lipids and may explain the variation in the binding signals observed with the human monoclonal antibodies.

As GalC contains a large number of hydrogen bond donors and acceptors, it would presumably have a larger influence on the tilt of the ganglioside headgroup, leading to the preferential exposure of certain epitopes. This may explain why the binding signals of the human monoclonal antibodies were particularly high to ganglioside:GalC complexes.

In comparison, PC has only a limited ability to form hydrogen bonds with gangliosides, which may result in the reduced tilt of its headgroup. This may have led to a less optimal exposure of certain epitopes, which explains the reduced biding signals detected to complexes containing PC compared to those containing other accessory lipids.

In addition to conformational modulation, it is also possible that the accessory lipids act to disrupt the intra- and inter-molecular bonds that form between different gangliosides (Frey & Lee, 2013). This has been established in modelling studies, which have shown that lipids such as DPPC can act as spacer molecules to reduce electrostatic repulsion between the sialic acid groups of gangliosides (Patel & Balaji, 2008). As a result, the lipids reduce the influence that the gangliosides have upon one another's orientation, which may improve the presentation of certain epitopes.

Alongside 1:1 mol:mol complexes, this chapter also explored the impact that complexes containing different proportions of accessory lipids had upon the binding signals of the human monoclonal antibodies. As lipid rafts contain high concentrations of accessory lipids (Pike, 2003; Sezgin et al., 2012), it was hypothesised that complexes containing higher proportions would be more representative of the endogenous rafts and would, therefore, produce higher binding signals.

The results correlated well with this hypothesis; however, there were distinct differences in the levels of enhancement between antibodies. The binding signals of BO1, SM1 and WO1increased substantially as the proportion of accessory lipids increased, whilst the others only experienced marginal improvements or inhibitions depending upon the accessory lipid present.

The antibodies were therefore categorised as being either concentration dependent (BO1, SM1, WO1) or concentration independent (BO3 and BR1) in

their ability to bind to complexes containing high concentrations of accessory lipids. The only exception to this broad classification was DO1, which tended to have low or intermediate binding signals. This may be attributed to the derivation of DO1, which was created from an AMAN patient. As AMAN is not a demyelinating condition (J. W. Griffin et al., 1995), it would be unlikely that antibodies would bind to the same epitopes as those derived from patients suffering from MMN. This may explain the differences in the binding pattern between DO1 and the other antibodies but there is not enough evidence from these experiments to draw any solid conclusions.

In terms of the conformational modulation theory, it could be proposed that the concentration dependent and concentration independent antibodies bound to different epitopes on the ganglioside headgroups. Increasing the number of accessory lipids in complexes would presumably lead to the formation of larger hydrogen bonding networks. This would result in a more pronounced tilt in the headgroups so that they were lying almost completely parallel to the plasma membrane. As the concentration dependent antibodies had higher binding signals to these complexes, it could be surmised that they bound to epitopes that were optimally presented when the gangliosides were in this configuration.

In contrast, the concentration independent antibodies may bind to an epitope that is preferentially presented when the ganglioside headgroup is lying perpendicular to the membrane. The initial binding enhancements experienced by these antibodies to complexes containing low proportions of accessory lipids, may be the result of the gangliosides condensing into organised domains (Hall et al., 2010). This leads to an initial increase in the binding signals due to improved epitope presentation; however, with certain accessory lipids, such as SM, this improvement is lost when the concentration increases further and the gangliosides orientation is changed. The epitope will then become inaccessible leading to a reduction in the binding signal.

For the majority of the accessory lipids (Chol, GalC, PS and SM), the binding signals of the concentration dependent antibodies increased as the proportion of lipids in the complexes increased. These signals eventually reached a plateau, where the epitope was presumably as optimally presented as possible. PC and sulfatide did not follow the same pattern as these lipids but were instead found

to produce an increase up to a point, followed by a drop off in the binding signal.

With regards to PC, it was hypothesised that the decrease in binding was due to the presentation of the gangliosides within the lipid environment. Previous studies have established that gangliosides suspended in artificial POPC containing membrane bilayers are scattered; whereas those contained within Chol/SM bilayers are condensed into highly organised domains (Mori et al., 2012). Assuming that this same effect occurs with PC complexes, it could be proposed that the increased concentration of the lipid results in a larger degree of scattering of the ganglioside. In terms of antibody binding, this may lead to a reduction in the availability of certain epitopes on the headgroup, reducing the binding ability of the human monoclonal antibodies.

The decreases in the binding signals associated with sulfatide enriched complexes were more difficult to explain. Sulfatide is almost identical to GalC except that it contains a sulfate group attached to the 3rd carbon of its galactose ring (Coet et al., 1998). It was therefore expected to form similar hydrogen bonding networks as GalC, which would result in similar levels of antibody enhancement.

This was not found experimentally, however, as the complexes were only capable of producing an increase in enhancement up to a ratio of 1:4, followed by a rapid decrease in binding in each subsequent complex. This decrease was unexpected but may have been related to the sulfate group, which gives the molecule a negative charge.

When the concentration of sulfatide is high, the sulfate group may have a detrimental effect on epitope presentation; however, little is known about the molecular interactions between sulfatide and gangliosides. Modelling studies would need to be performed to substantiate whether this group was responsible for the relative decrease in antibody binding or if it was another aspect of the molecule that was producing this result.

Aside from the effect of the lipids concentration, sulfatide was also unique in its ability to elicit antibody enhancement. Unlike the other accessory lipids, which only produced enhancements with concentration dependent antibodies, sulfatide

complexes were able to produce them with all 5 antibodies derived from MMN patients. In comparison, DO1, which was derived from an AMAN patient showed no enhancement with sulfatide complexes whatsoever.

This suggests that the interactions between gangliosides and sulfatide may expose an epitope, which is specifically targeted in MMN. As such, it may be of interest in future studies to examine this complex further and to ascertain whether it is also a target in the serum.

Whilst the array data was useful in determining the effects that different accessory lipid concentrations had upon antibody binding, it did not establish whether these findings translated into differences in their tissue binding ability. To address this, the original paper, in which the antibodies were first screened against human tissue, was re-examined (O'Hanlon et al., 1998).

The concentration dependent and concentration independent antibodies were directly compared to ascertain whether they bound to different tissues. The only difference between the two groups appeared to be with cross-striations of the diaphragm to which the concentration independent antibodies were solely capable of binding. Unfortunately, this was not particularly informative as little is known about the lipid composition of these cross-striations or their relevance in disease.

Examination of the other tissues was inconclusive in establishing distinct differences between the two antibody groups; however, the nature by which the tissue was collected may have affected the ganglioside distribution within these tissues, which would impact the validity of the binding data. As the tissue was collected from amputated limbs, it was immediately snap-frozen or fixed in either an ethanol solution or 4% PFA. Both these fixation methods have previously been shown to drastically alter the distribution of gangliosides within plasma membranes (Schwarz & Futerman, 1997), to the extent that gangliosides appear on cells that do not express them endogenously.

As such, the human monoclonal antibodies may not bind to these tissues as they would *in vivo*, which may affect the interpretation of the results. To overcome these problems, it would be ideal to screen the antibodies against unfixed

tissue; however the logistics of collecting live human tissue may make this an unrealistic possibility.

Examination of the binding capabilities of the antibodies in mouse tissue is similarly difficult, as they are all incapable of binding gangliosides in the presence of GD1a (Chapter 3). Binding studies in these tissues would therefore be impractical, as GD1a is widely distributed throughout the mouse nervous system (Gong et al., 2002), and can only be removed by converting it to GM1 through neuraminidase treatment. Whilst this would allow binding to take place (Greenshields et al., 2009), it would also be counterintuitive for the intentions of this study, which require the membrane to resemble its natural state.

Despite the difficulties in correlating the array data with the tissue binding studies, the experiments still provided a novel insight into how the composition of ganglioside complexes affects the binding abilities of monoclonal antibodies. The emergence of two distinct antibody groups was particularly interesting, as it suggested that the complexes used in standard assays may be insufficiently designed for the optimal detection of all anti-ganglioside antibodies.

To determine if this was true, a new clinical array was created consisting of complexes that contained high concentrations of accessory lipids. These arrays were probed with MMN patient sera to determine whether the new complexes increased antibody detection compared to those in current use. The results of this study are discussed in the subsequent chapter.

5 SCREENING MMN PATIENT SERA AGAINST ACCESSORY LIPID COMPLEXES

5.1 Introduction

The work carried out using the human monoclonal antibodies in the previous chapters demonstrated the important roles that a ganglioside's microenvironment plays in the presentation of different binding epitopes. Through the use of different ganglioside complexes, it was shown that it was not just the content of the microenvironment that influenced the presentation of epitopes but the concentration of the lipids that were contained within it.

Whilst some antibodies were found to bind avidly to complexes containing high concentrations of accessory lipids, others were found to experience no change in their binding behaviour. It was therefore hypothesised that certain antibodies were concentration dependent and bound to ganglioside epitopes that were optimally presented in lipid rich environments. In contrast, the other antibodies were considered to be concentration independent as they were unaffected by changes in the lipids concentration and presumably bound to epitopes that were already optimally presented.

The suggestion that two classes of anti-ganglioside antibodies existed, raised questions about the effectiveness of current screening methods, which employ complexes in 1:1 w:w formations. Whilst these may effectively detect low levels of the concentration independent antibodies, they may not be able to detect low levels of the concentration dependent antibodies that preferentially bind gangliosides in lipid rich microenvironments.

To address this possibility, a new array was designed that incorporated the ganglioside:accessory lipid ratios that gave the highest binding signals for the concentration dependent antibodies. These were: 1:32; 1:32; 1:4; 1:32; 1:32: and 1:8 for Chol, GalC, sulfatide, PS, SM, and PC respectively. Alongside these new complexes, the new arrays also included the normal 1:1 complexes as well as the single constituent lipids of both.

Table 5-1 - Example of Array Layout for Screening Human Sera against Accessory Lipid Complexes

GM1		GA1		GD1b		M	GM1		GA1		GD1b	
Chol A	GM1:Chol 1:1	Chol B	GA1:Chol 1:1	Chol C	GD1b:Chol 1:1	M	PS A	GM1:PS 1:1	PS B	GA1:PS 1:1	PS C	GD1b:PS 1:1
Chol 32 A	GM1:Chol 1:32	Chol 32 B	GA1:Chol 1:32	Chol 32 C	GD1b:Chol 1:32	M	PS 32 A	GM1:PS 1:32	PS 32 B	GA1:PS 1:32	PS 32 C	GD1b:PS 1:32
GalC A	GM1:GalC 1:1	GalC B	GA1:GalC 1:1	GalC C	GD1b:GalC 1:1	M	SM A	GM1:SM 1:1	SM B	GA1:SM 1:1	SM C	GD1b:SM 1:1
GalC 32 A	GM1:GalC 1:32	GalC 32 B	GA1:GalC 1:32	GalC 32 C	GD1b:GalC 1:32	M	SM 32 A	GM1:SM 1:32	SM 32 B	GA1:SM 1:32	SM 32 C	GD1b:SM 1:32
Sulf A	GM1:Sulf 1:1	Sulf B	GA1:Sulf 1:1	Sulf C	GD1b:Sulf 1:1	M	PC A	GM1:PC 1:1	PC B	GA1:PC 1:1	PC C	GD1b:PC 1:1
Sulf 4 A	GM1:Sulf 1:4	Sulf 4 B	GA1:Sulf 1:4	Sulf 4 C	GD1b:Sulf 1:4	M	PC 8 A	GM1:PC 1:8	PC 8 B	GA1:PC 1:8	PC 8 C	GD1b:PC 1:8

As a consequence of the inclusion of these extra complexes, duplicate spots had to be removed from the arrays, due to space restrictions (Table 5.1). All other settings remained the same and the arrays were printed using the combinatorial glycoarray following the standard protocol.

5.2 Results

The arrays were probed with sera collected from a clinical centre in Marseille, France. These consisted of samples from MMN patients (n=20), other neurological diseases (OND) (n=40) and healthy controls (n=20). The ONDs comprised of samples from amyotrophic lateral sclerosis (ALS, n=20) and Charcot-Marie-Tooth (CMT, n=20) patients, which acted as controls for acquired and hereditary diseases respectively.

Following screening, the array data for all the MMN and control samples underwent cluster analysis and were plotted in a heatmap using a rainbow scale of intensity (Figure 5.1). At first glance, there was no immediate indication of an antigen that was specifically targeted by the MMN sera compared to the controls. However, a minority of sera were found to have noticeably higher values against GM1 and GA1 with a variety of complexes.

The 95th percentile for the controls (n=60) was calculated for each glycolipid target and was used for the threshold of positivity. These results were then applied to the MMN sera data, with values above this threshold being considered as true positives.

Having established the cut off values for the array data, the sensitivities of each antigen could be calculated, assuming a specificity of 95% (Table 5.2). From this data, it could be seen that complexes which contained higher proportions of accessory lipids had higher sensitivities. In general, they increased the sensitivity by 10% compared to the standard complexes, but some increased it even

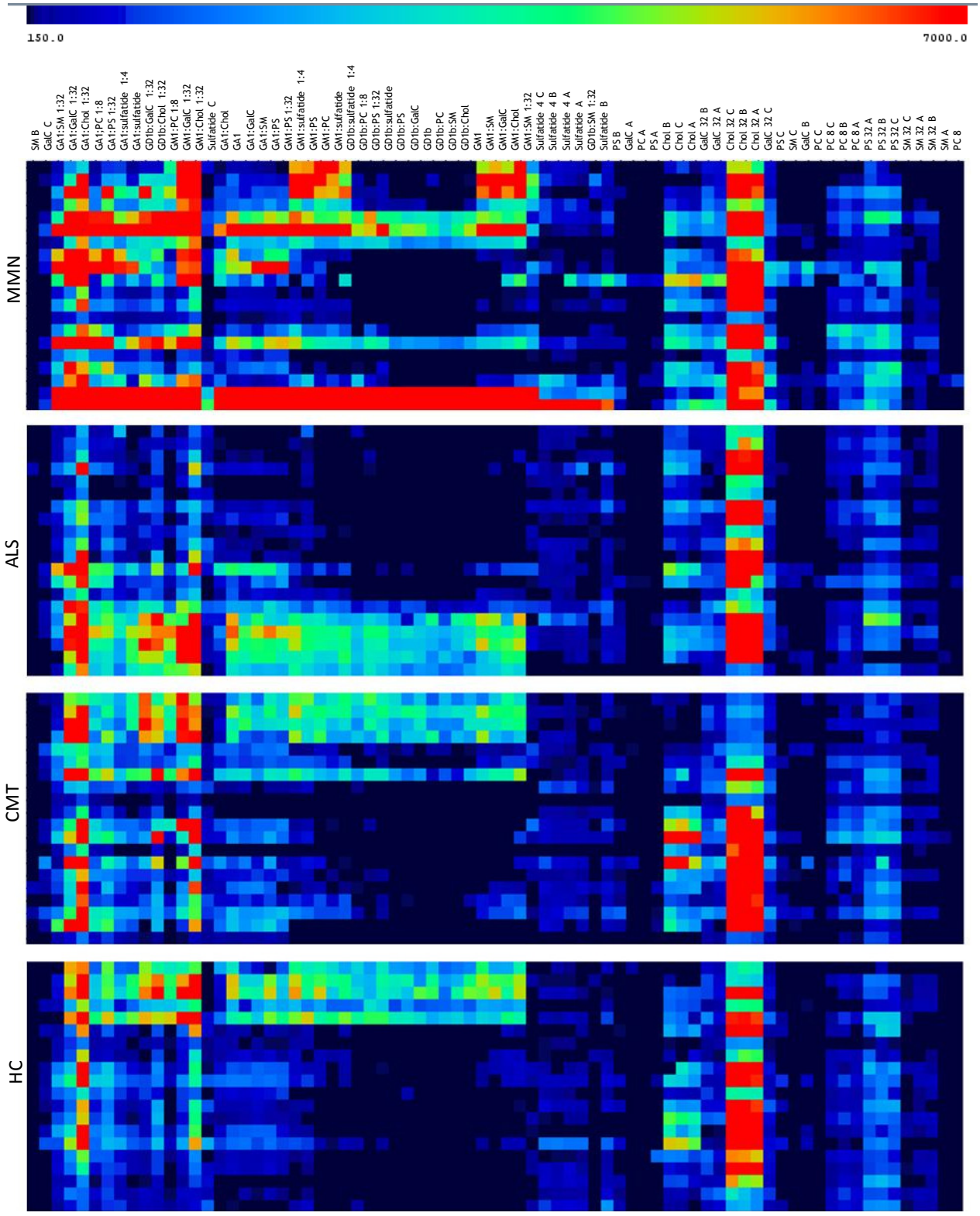


Figure 5.1 - Heat Map of Patient Antibody Binding

Sera from MMN, ALS, CMT patients and healthy controls (HC) were screened against an different lipids and complexes. The relative binding intensities of the IgM antibodies to the various gangliosides and complexes are indicated by the colours of the bands. These follow a rainbow scale, with red indicating the strongest binding, blue indicating low binding and black indicating a negative signal. The signals have been sorted by disease and have been ordered by hierarchical clustering with Pearson correlation.

further, with GA1:sulfatide 1:4; GA1:PS 1:32 and GM1:SM 1:32 displaying sensitivities that were 15-25% higher.

Further analysis was performed upon the array data to ensure that the increased sensitivities were not a result of increased binding to the single constituent lipids. As such, a second heat map was created that consisted of the complex binding signals with the individual constituents subtracted (Figure 5.2). This indicated whether the antibodies were enhanced or inhibited, which allowed for more accurate comparisons between the different groups.

Examination of these results indicated that most of the complexes were inhibitory for antibody binding. The only exceptions to this were GM1:GalC 1:1 and ganglioside:GalC 1:32 complexes. These were enhanced with all the sera but GM1:GalC 1:1, GM1:GalC 1:32 and GA1:GalC 1:32 were found to be significantly higher in the MMN sera compared to the both the ALS samples and the healthy

Table 5-2 – Sensitivity Values for GM1 and GA1 Complexes

	Sensitivity	1:1 Ratios	Sensitivity	Inc Ratios	Sensitivity
GM1	30%	GM1:Chol	30%	GM1:Chol 1:32	40%
GA1	25%	GA1:Chol	35%	GA1:Chol 1:32	35%
GD1b	15%	GM1:GalC	30%	GM1:GalC 1:32	40%
		GA1:GalC	30%	GA1:GalC 1:32	40%
		GM1:sulfatide	35%	GM1:sulfatide 1:4	35%
		GA1:sulfatide	30%	GA1:sulfatide 1:4	45%
		GM1:PS	35%	GM1:PS 1:32	35%
		GA1:PS	30%	GA1:PS 1:32	45%
		GM1:SM	35%	GM1:SM 1:32	60%
		GA1:SM	25%	GA1:SM 1:32	40%
		GM1:PC	35%	GM1:PC 1:8	30%
		GA1:PC	35%	GA1:PC 1:8	45%

The sensitivity values for GM1, GA1, GD1b, GM1 and GA1 complexes were calculated assuming a specificity of 95%. This was determined for each spot using the binding signals from the combined controls (n=60). Green indicates those lipids which had sensitivities higher than both the single lipid and the standard 1:1 mol:mol complex.

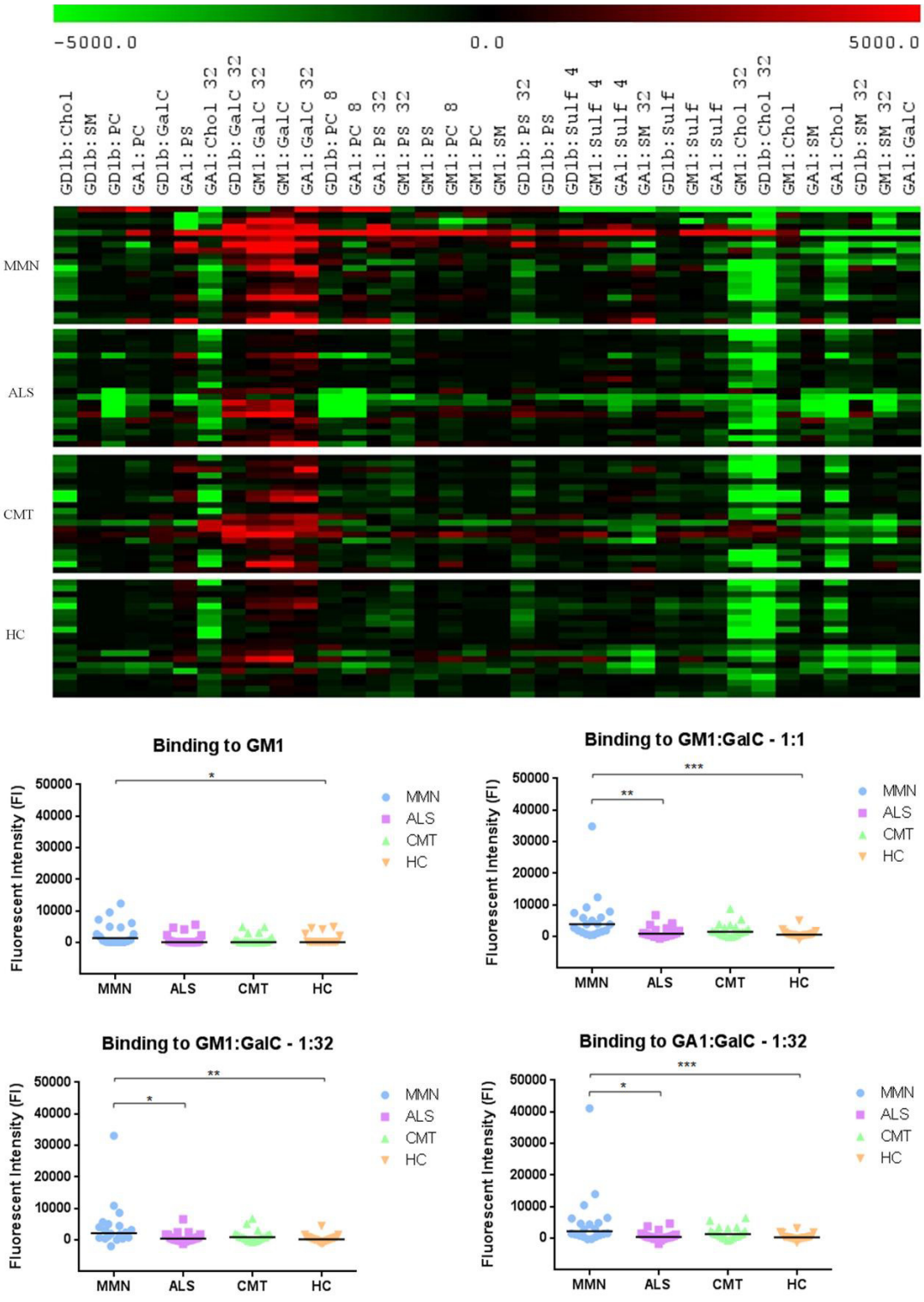


Figure 5.2 - Comparison of the Enhancements and Inhibitions of Human Sera

A heatmap was created of the human sera data following subtraction of single constituent lipids from complexes. Red indicates enhancements whilst green indicates inhibition. The data showed that GM1:GalC 1:1, GM1:GalC 1:32, and GA1:GalC 1:32 enhance the binding signals of the different sera. This enhancement is significantly higher in the MMN patients compared to the ALS and HC samples and was found to be higher than the enhancement with GM1 alone (Kruskal-Wallis Test, Dunn's Multiple Comparisons, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

controls (Kruskal Wallis, Dunn's multiple comparisons, $P<0.05$). There was no significant difference, however, between the MMN and CMT samples, although the MMN samples did have a higher median signal.

To determine if GalC was enhancing the binding signals of the antibodies beyond the single GM1 species, a side by side comparison was performed between the different antigens. This data indicated that, with GM1, the MMN sera were significantly higher than the healthy controls (Kruskal Wallis, Dunn's multiple comparisons, $P<0.05$). In comparison, with GM1:GalC, the MMN sera were significantly higher than both the healthy controls and ALS ($P<0.05$), which suggested that the presence of GalC enhanced antibody detection. However, there was no difference between the GM1:GalC 1:1 and GM1:GalC 1:32 ratios, which suggested that the increased proportion of GalC in the complex was not producing any additional enhancement.

Taken together, this data suggested that complexes containing increased proportions of accessory lipids do not improve antibody detection in MMN sera. Although the sensitivity of these complexes was increased compared to the standard configurations, this was offset by the increased sensitivities of the single accessory lipid antigens. As such, only complexes containing GalC were found to improve antibody detection compared to the single ganglioside target. This was in line with previous research, suggesting that the new array design did not provide any benefit compared to the standard assays that are already in use.

5.3 Discussion

The emergence of concentration dependent and concentration independent antibodies in the previous chapter raised questions about the effectiveness of current clinical assays. As these assays employ complexes in 1:1 w:w ratios, they may be inadvertently omitting the detection of antibodies that preferentially bind to complexes containing higher proportions of accessory lipids, giving the impression that they are not present.

To address this possibility, an experiment was performed in which a cohort of patient sera was screened against a newly designed array. This array was composed of the ganglioside:accessory lipid complexes that produced the most

enhancement for the concentration dependent antibodies, alongside the normal 1:1 mol:mol complexes and the single constituents of both.

In line with previous research, complexes containing GalC were found to enhance antibody detection compared to the single ganglioside antigens (Galban-Horcajo et al., 2013; Nobile-Orazio & Gallia, 2013). This was true for both the GM1:GalC 1:1 complexes as well as those composed of GalC in higher proportions. There was no difference between these complexes, however, suggesting that increasing the proportion of GalC had no additional benefit in detecting serum antibodies compared to those complexes in the standard configuration.

All other ganglioside:accessory lipid complexes were found to be ineffective in enhancing the detection of antibodies compared to the single antigens. The new complexes did have higher sensitivities; however, this was attributed to an increase in non-specific antibody binding rather than the increased detection of anti-ganglioside antibodies.

Overall, these results suggested that the performance of the new array was relatively poor, as the new complexes were not found to provide any additional enhancements compared to those complexes in the standard configuration. Whilst this was possibly due to the absence of concentration dependent antibodies in the serum, it was also possible that the lack of binding was due to issues with the samples or the array configuration itself.

In regards to the serum samples, it was reasonable to assume that their polyclonal nature may have occluded the detection of certain complex enhancements (Mahon et al., 2014; Wine et al., 2013). The presence of antibodies targeting the single accessory lipids would lead to the attenuation of the complex signal giving the impression that it was absent. This was most prominent in this experiment with anti-Chol antibodies, which were present in almost every patient in both the disease and control groups. Although the monoclonal antibodies were significantly enhanced with ganglioside:Chol complexes, in the serum study the complexes appeared to have no effect due to these anti-Chol antibodies. Unfortunately, as these are commonly found in the general population (Alving et al., 1989), it would be unlikely that complexes containing Chol would be useful in detecting anti-ganglioside antibodies.

The quality of the samples may also have affected the experimental results, due to degradation following freeze-thaw cycles. The effect of these cycles on anti-glycolipid antibodies is unclear, as some studies suggest that they have no effect, whilst others have shown degradation after as few as three cycles (Brey et al., 1994; Siebert et al., 2013). Unfortunately, these samples were taken from another study so their quality could not be guaranteed. It would therefore be ideal to repeat this experiment using freshly collected samples.

The relatively low levels of anti-GM1 antibodies in these samples may also be another reason to repeat the experiments. In most studies these antibodies are detected in up to 80% of MMN patients but in this cohort they were only detected in 30% of patients, which is substantially lower (Cats, Jacobs, et al., 2010; Nobile-Orazio & Gallia, 2013; Nobile-Orazio, 2001). This may be a valid result; however, it would be worthwhile to repeat this experiment on a different cohort of sera in the future to definitively determine whether different ratios of accessory lipid complexes are able to enhance antibody detection in MMN.

Aside from the serum samples, it was also possible that the configuration of the complexes was preventing the accurate detection of anti-ganglioside antibodies. Recent work within the Willison laboratory has established that GM1:GalC complexes between the ratios of 1:1 and 1:5 w:w are optimal for detecting anti-GM1 antibodies (Delmont et al., 2015). Complexes that contain higher proportions of GalC experience a reduction in specificity, which makes them less suitable for detecting antibodies and rules out their use in clinical assays.

This same problem may have occurred with the complexes used in this study. Although they were optimal for enhancing the binding signals of monoclonal antibodies, they may not be as well suited to detecting serum antibodies due to the aforementioned issues with polyclonality. Future work may therefore wish to analyse these complexes at the ranges used in the original monoclonal antibody characterisation, to fully establish their optimal configuration for use in clinical assays.

Overall, the results from the accessory lipid studies have indicated that the composition of ganglioside complexes, in terms of both the presence and concentration of accessory lipids, has a large influence on the presentation of different epitopes, which by extension affects antibody binding. These findings

also suggest that antibodies may be much more diverse than they appear when screened on a standard assay such as ELISA. When probed in a more biologically relevant manner, the differences become clearer and the antibodies appear to fall into different classes. This may translated into differences in pathogenicity; however, this study was unable to verify this hypothesis.

Although the findings of the previous chapter were not replicated in the serological studies performed here, future work may wish to more closely examine the effect that the local microenvironment has upon ganglioside presentation. Utilising assays that are more biologically representative will lead to vast improvements in the accuracy of antibody detection, which will ultimately increase their usefulness in diagnosing autoimmune neuropathies.

6 IMMUNISATIONS WITH GANGLIOSIDE COMPLEXES

6.1 Introduction

The presence of high titres of anti-ganglioside antibodies in patients are a distinguishable feature in autoimmune neuropathies, but little is known about their pathogenic effects. Research into the roles of these antibodies is limited in humans because the production of human monoclonal antibodies is fraught with difficulties. Those which have been produced have been shown to have poor binding qualities in tissue, which is problematic in establishing their roles and whether they are in fact responsible for causing the disease (O'Hanlon et al., 1998).

The use of animal research has partially addressed this problem by utilising hybridoma technology. This technique involves immunising animals with an antigen conjugated to an adjuvant, which produces an immune response. After several immunisations, the spleen of the animal is removed and fused with a myeloma cell line to create an immortal antibody producing cell called a hybridoma. This is considerably cheaper and easier to produce than human monoclonal antibodies and allows for the production of high numbers of diverse antibodies.

In recent years these hybridoma cell lines have been used to generate monoclonal antibodies targeting gangliosides and other lipid components. Various lower mammals including rabbits, rats and mice have been used to produce these antibodies, which have then been investigated *in vivo* to establish their pathogenic roles (O'Hanlon et al., 2001; Rupp et al., 2012). This research has subsequently led to the identification of new therapeutic targets and treatments, which demonstrates the importance that these antibodies have in investigating neuropathies (Halstead, Humphreys, et al., 2005).

The Willison laboratory employs the use of mice in research as they are the lowest mammals that synthesise gangliosides and are cheap to maintain and breed. Various different genotypes of these mice have been used to produce

approximately 100 monoclonal antibodies through active immunisations with ganglioside liposomes or lipopolysaccharides derived from C. *jejuni*.

These antibodies are diverse in their targets, isotypes and specificity. Some are cross reactive and are capable of binding multiple gangliosides, whereas others are only capable of binding one. They also differ widely in their abilities to bind to tissue, with some antibodies requiring very specific environments for binding to take place (Greenshields et al., 2009).

There has been recent interest in the importance of anti-ganglioside complex antibodies in patients in which antibodies bind specifically to a complex epitope composed of two different glycolipids, without binding to the single components. These antibodies could be key to identifying why certain tissues are targeted by antibodies whilst others remain unaffected, which could explain the mechanism of disease of certain autoimmune neuropathies. This chapter explores the attempts to produce these antibodies in mice through immunisations with liposomes composed of various ganglioside complexes.

6.2 Results

6.2.1 Technique Development

Antibodies were produced by immunising mice with ganglioside liposomes over a period of four weeks (Table 6.1). The spleens were then collected and fused with a myeloma cell line to create a hybridoma that produced the antibodies indefinitely. During the immunisation process, blood was collected at regular intervals and was screened using the combinatorial glycoarray to establish the development of the immune response.

6.2.1.1 Mouse Control Sera

The variability between print runs on the glycoarray could occasionally be high depending upon a number of factors related to the normal operation of the equipment. To ensure that this variation was not skewing the sample results, a control sera was created, which was applied during each experiment and used to normalise the data.

Table 6-1 – Immunisation Protocols for the Production of various Complex-Dependent Antibodies

Desired Antigen	Liposome Components	Liposome Ratio	Mouse Genotype	Immunisation Protocol	Section
GM1:GalC	GM1:Chol:SM:DCP:GalC	1:5:4:1:1	GalNAc T B6CGTGN x DBA	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.2.1
GM1:GalC	GM1:Chol:DCP:GalC	1:5:1:1	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.2.2
GM1:GalC	GM1:Chol:DCP:GalC	1:5:1:2	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.2.3
GM1:GalC	GM1:Chol:DCP:GalC	1:5:1:20	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.2.4
GM1:GD1a	GM1:Chol:DCP:GD1a	1:5:1:1	GalNAc T	IP: Day -112, -98, -84, 7, 14 IV: 19, 20	6.2.3.1
GM1:GD1a	GM1:Chol:DCP:GD1a	1:5:1:1	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.3.2
GM1:Sulf	GM1:Chol:SM:DCP:Sulf	1:5:4:1:1	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.4
GM1:Sulf	GM1:Chol:SM:DCP:Sulf	1:5:4:1:1	CST-/- x GalNAc T-/- x Thy 1	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.4.2
Gang Complexes	5mg of WLE in Chol:SM:DCP	5:4:1	GalNAc T	IP: Day 0, 7, 14, 21 IV: 25, 26, 27	6.2.5

Three B6CGTGNxDBA mice were immunised with GM1 liposomes created as per Section 2.4. These mice were selected as they had previously been shown to respond well to immunisation and were readily available. The mice were injected IP with liposomes three times a week. After six immunisations the mice were killed and their serum was collected and pooled.

The sera were screened against a standard grid of lipids and were found to have good IgM responses to GM1 and the other lipids which were included in the liposomes.

A serial dilution was created to establish the optimal concentration for use in screening (Figure 6.1). At an exposure time of 16ms the GM1 response was 20525 FI units at a 1 in 100 dilution. As a linear relationship existed between exposure time and GM1 response, this dilution was found to be acceptable for comparisons between experiments.

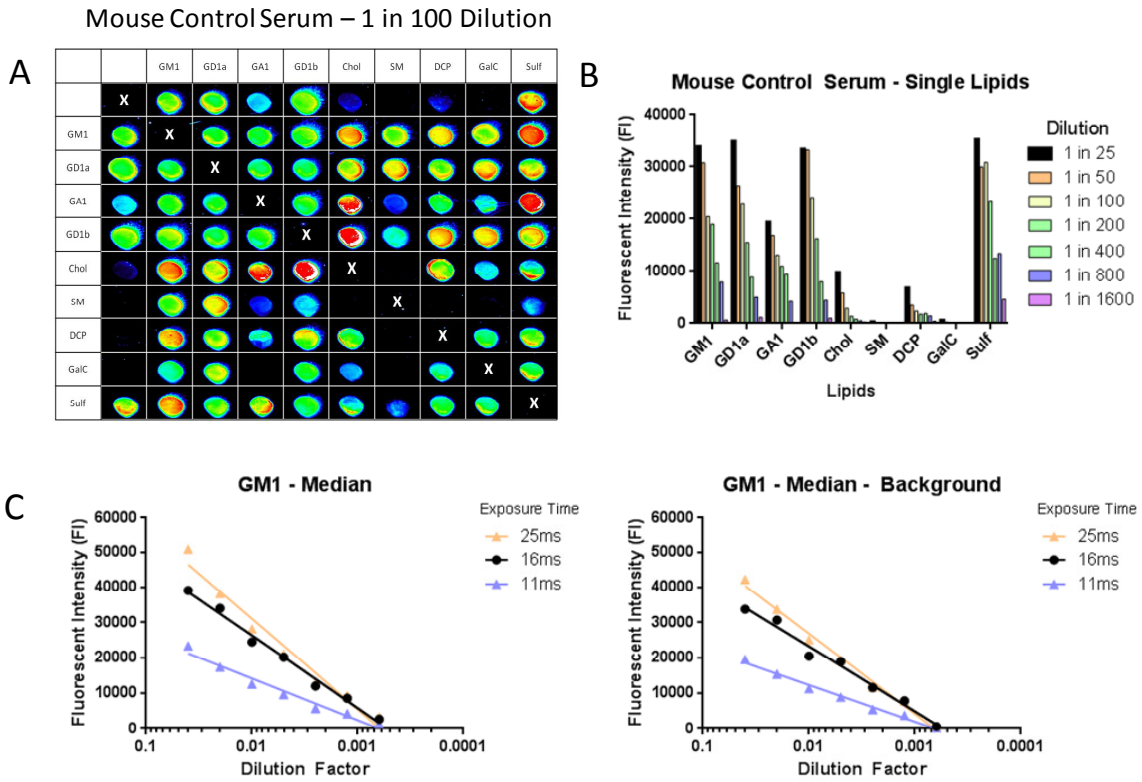


Figure 6.1 - Mouse Control Serum

IgM response in mice immunised with GM1 liposomes. A: Binding fingerprint at a 1 in 100 dilution and scanned at 16ms exposure. B: Binding signals to the single lipids at the different dilutions. C: Semi-log graphs of GM1 binding at different dilution factors at different exposure times with and without the subtraction of the background signal. A linear relationship existed between the dilution of serum and fluorescent intensity.

6.2.1.2 IgG Control Antibody

Unfortunately, for some unknown reason, the IgM antibodies in the mouse control sera did not class switch to IgG, so a different control was required for this isotype. An in-house IgG monoclonal antibody, DG2, was selected as this had previously been well characterised within the laboratory (Townson et al., 2007). An initial DG2 concentration of 400µg/ml was serially diluted 1:1 with 1% BSA to a final concentration of 49ng/ml and screened using the combinatorial glycoarray. The values for the median signal and the median signal minus background of GM1 binding were plotted to create a semi-log graph (Figure 6.2). The background signal was particularly high at increased concentrations of DG2 which is reflected in the graph where there is a decreased binding signal at

these concentrations. The steepest point on both graphs, which was not negatively affected by background binding, was therefore selected as the antibody concentration for control slides. This was 3µg/ml.

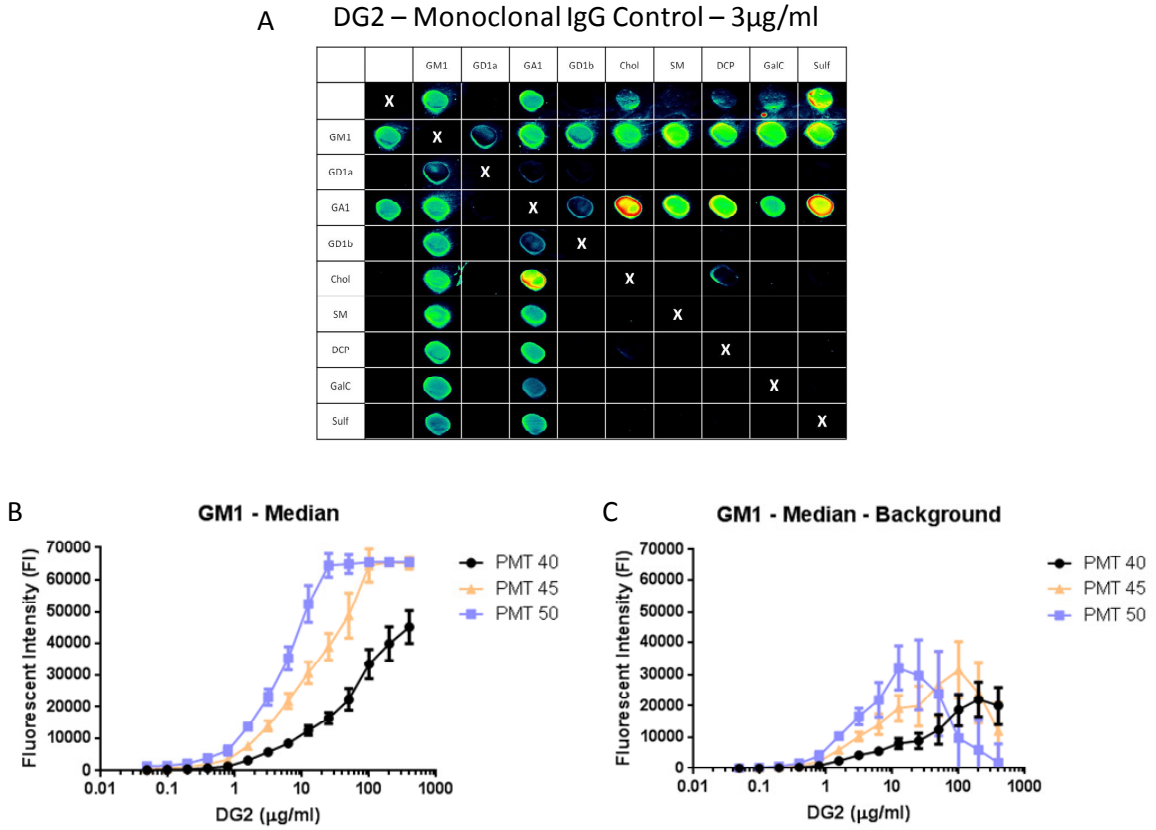


Figure 6.2 - IgG Control Antibody

The in-house antibody DG2 was characterised to create an IgG isotype control. A: A representative blot of DG2 at 3µg/ml. The antibody was serially diluted 1:1 from 400µg/ml to 49ng/ml and plotted with (B) and without the background subtracted (C). The optimal concentration where background was not influencing the signal was found to be 3µg/ml which was selected for use in the control slides of future experiments.

6.2.1.3 Control Slides

To accommodate these controls, the twelfth slide of each print was designated a control slide. These slides contained the same lipids as the other slides, but were instead probed with either the mouse control sera of DG2 at the predetermined concentrations. The results of these slides were then compared with the original slides and used to normalise the data.

6.2.1.4 Coefficient of Variation

The control slides were also used to calculate the coefficient of variation (CV) for each experiment, which could be used to determine the intra and inter assay variability. The data was first manipulated by truncating and setting values below 200 to 200 units as data at the lower end of the intensity scale was highly variable. The mean and SD of each spot was used to determine the overall CV of the experiment which was an indication of the intra-assay variability. This was found to be in the range of 17.8-23.4%. The inter-assay variability was calculated using the overall mean and SD of each assay across several experiments and was determined to be 15%. Although these values were high in comparison to different types of assay, they were deemed acceptable for a carbohydrate assay based upon the current literature (Oyelaran et al., 2010).

6.2.2 Immunisations with GM1:GalC Liposomes

As mentioned in Chapter 1, complexes composed of GM1 and GalC are potential targets for antibodies in MMN. In order to study the possible pathogenic effects of these antibodies, mice were immunised with GM1:GalC complexes to try and trigger a specific immune response. The sera was analysed using slides printed upon the combinatorial glycoarray to determine if complex specific antibodies had been generated.

6.2.2.1 GM1:GalC Liposomes with Sphingomyelin

Three different genotypes of mice (GalNAc T-/-, GalNAc T+/+ and B6CGTGNxDBA) were immunised with liposomes composed of cholesterol, DCP, SM, GM1 and GalC to establish which genotype was best suited for immunisations (Figure 6.3). It was hypothesised that the GalNAc T-/- mice would produce the highest responses as they do not express complex gangliosides and are therefore antigen naïve. In contrast, the GalNAc T+/+ mice are exposed endogenously and would therefore be expected to have a reduced immune response (Bowes et al., 2002). In-house studies also indicated that B6CGTGNxDBA mice responded well to gangliosides immunisations so these mice were included to establish if they were suitable for these experiments (unpublished data).

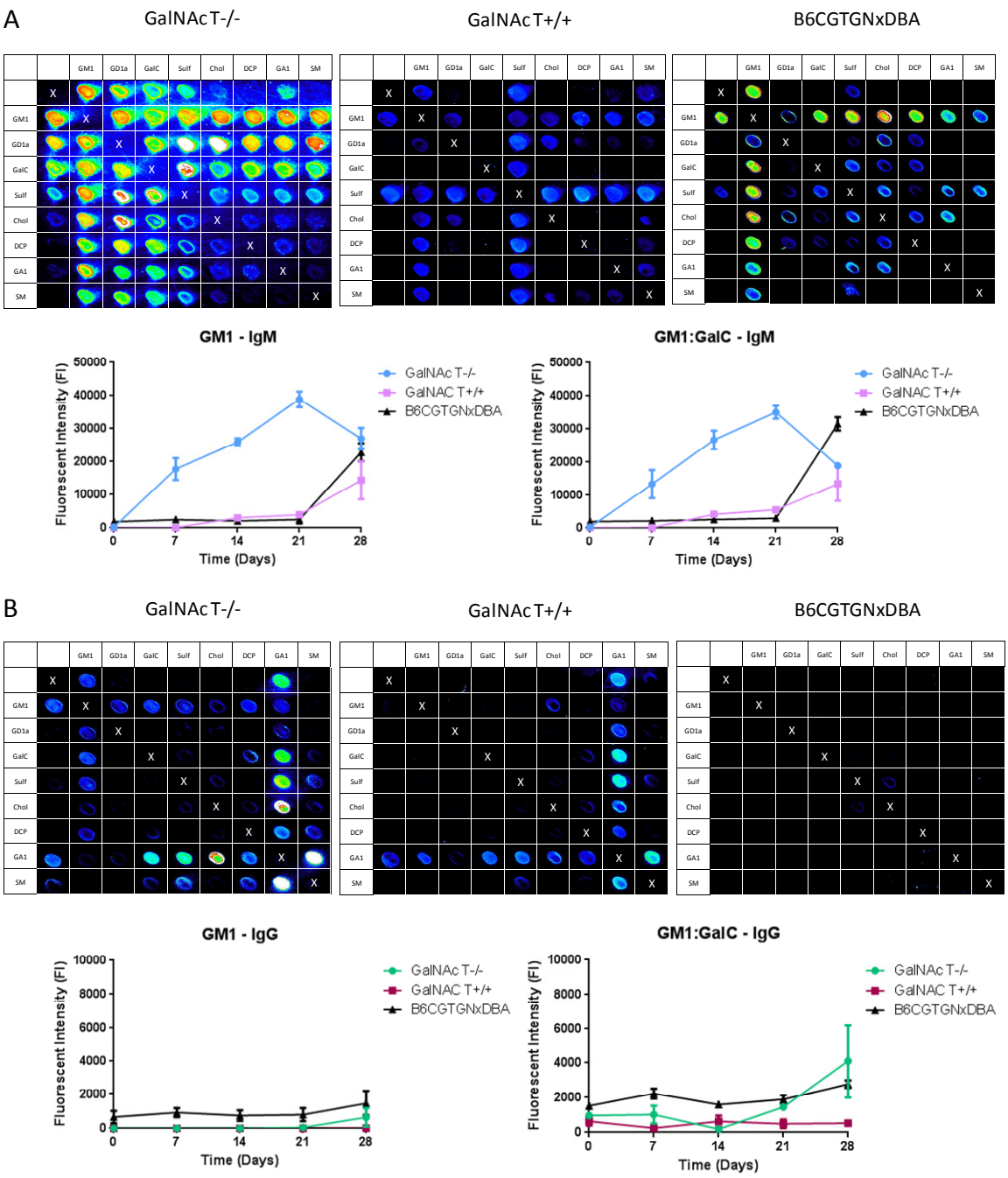


Figure 6.3 - GM1:GalC:SM Liposome Immunisations

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype and the binding signal to GM1 and GM1:GalC were plotted over time. A: IgM responses. All genotypes of mice responded to immunisations, with the GalNAc T-/- mice producing the earliest and largest intensity signals over time. B:IgG responses. Only the GalNAc T-/- mouse responded to GM1 and GM1 complexes. Both the GalNAc T-/- and GalNAc T+/+ mice showed high responses against GA1 and GA1 complexes. The B6CGTGNxDBA mice showed no IgG response to any gangliosides under these conditions. GalNAc T-/- (n=2), GalNAc T+/+ (n=3), B6CGTGNxDBA (n=3)

As predicted, the GalNAc T-/- mice were found to have the highest levels of anti-ganglioside IgM antibodies over the immunisation period. This immune response rose steadily to a peak at day 21, where it was assumed that the antibodies began class switching, as the signals decreased again at day 28. Neither the GalNAc T+/+ or the B6CGTGNxDBA mice produced a response until day 28, with the latter producing a much higher signal. There was little difference between the signals to GM1 and GM1:GalC except in the B6CGTGNxDBA mice, which showed a slightly higher response to GM1:GalC.

The IgG responses remained relatively weak for all the mice throughout the immunisation period. The B6CGTGNxDBA had higher background binding with IgG antibodies, which resulted in higher baseline levels at all timepoints. The mice therefore did not show any net change in IgG intensity over the immunisation period. The GalNAc T+/+ mice displayed no response to GM1 or GM1 complexes at any timepoint, whilst the GalNAc T-/- mice produced a peak response against GM1 and GM1:GalC at day 28. This correlated with the IgM results that showed a drop between day 21 and day 28, which can be attributed to antibody class switching.

Unexpectedly, both the GalNAc T-/- and GalNAc T+/+ mice had high IgG responses to GA1 and GA1 complexes. This suggested that the immune system of the mice preferred the oligosaccharide portion common to GA1 and GM1 over the sialic acid residue unique to GM1 alone. This, alongside the lack of a specifc anti-GM1:GalC complex antibody, led to a reconfiguration of the liposomes.

6.2.2.2 GM1:GalC 1:1 mol:mol Liposomes without SM

Examination of the literature suggested that phospholipids with long fatty acid chains such as SM may inhibit antibody binding to smaller lipids due to steric hindrance (Alving & Richards, 1977). Sphingomyelin was therefore eliminated from the liposome composition to establish if a specific GM1:GalC response could be generated. Only the GalNAc T-/- and GalNAc T+/+ mice were used for these immunisations as the delayed immune response of the B6CGTGNxDBA mice made them an undesirable candidate for monoclonal antibody production.

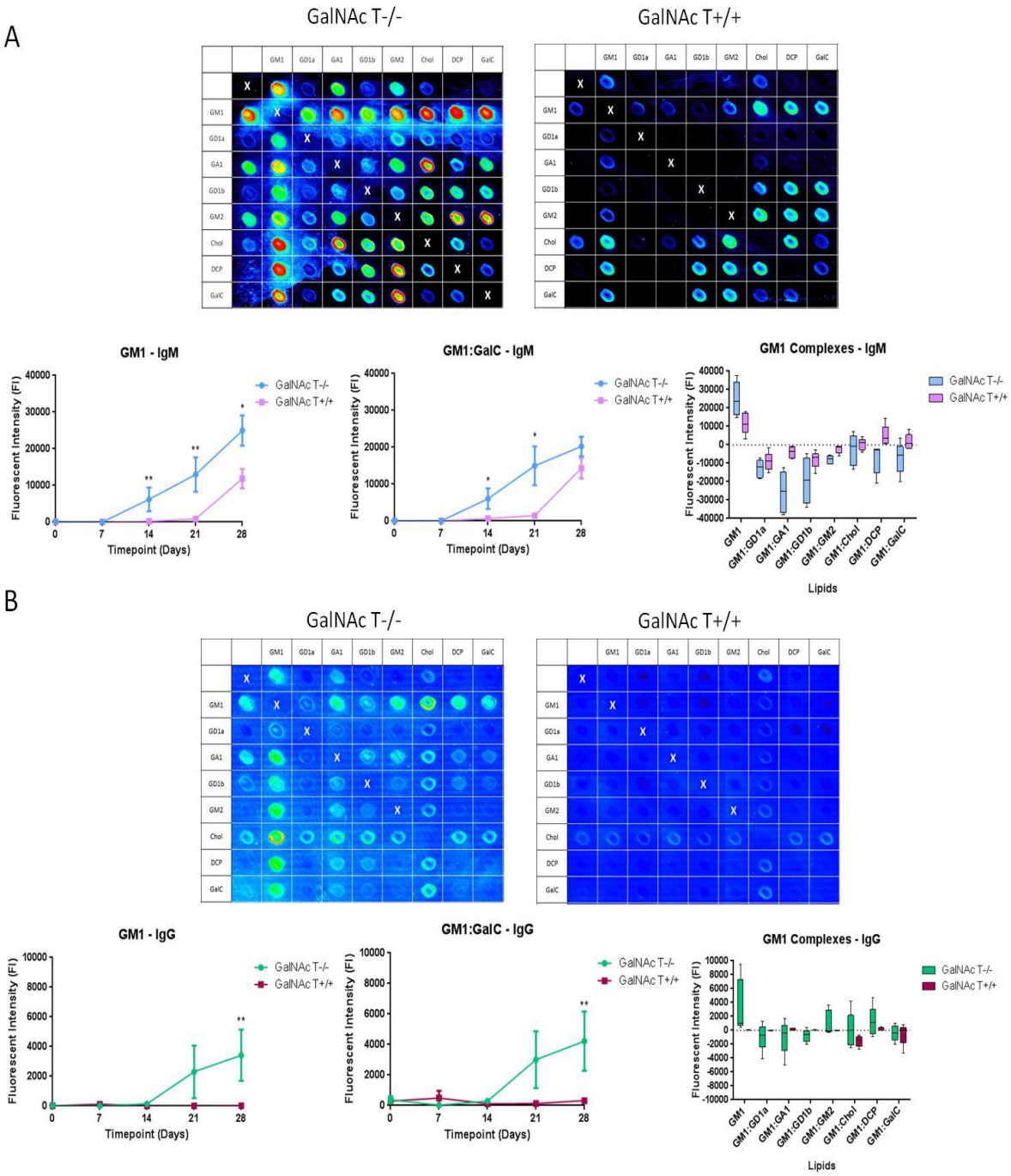


Figure 6.4 –GM1:GalC Liposome Immunisation

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype. The antibody binding signals to GM1 and GM1:GalC were plotted over time for each genotype and a comparison of the GM1 complexes with the single components subtracted is plotted. A: GalNAc T -/- mice had significantly higher IgM antibody intensity to GM1 at day 14, 21 and 28 and to GM1:GalC at day 14 and 21 compared to GalNAc T+/+ mice. GM1 complexes were compared to one another and showed no enhanced binding. B: GalNAc T-/- mice had significantly higher binding to GM1 and GM1:GalC at day 28 compared to GalNAc T+/+ mice. An analysis of the complexes showed enhancement with DCP and Chol but not GalC. (Mann Whitney, $P<0.05$) (n=5)

The sera were analysed from the mice following the same protocol as before (Figure 6.4). The GalNAc T-/- mice had significantly higher IgM intensity values for GM1 and GM1:GalC at day 14 and day 21 compared to the GalNAc T+/+ mice (Mann Whitney Test, $P<0.05$). The GM1 values were also significantly higher at day 28 but this was not reflected by the GM1:GalC complex. A comparison of the complexes with the single components subtracted showed no enhancements.

The IgG intensity values for GM1 and GM1:GalC were only significantly higher at day 28 in the GalNAc T -/- mice compared to the GalNAc T+/+ mice. This was consistent with the reduction in signal in the IgM intensity values, which suggested that class switching had taken place. The removal of SM from the liposomes appeared to allow for the production of a GM1 and GM1 complex specific IgG response, but the only enhanced signals were showed with complexes containing Chol and DCP, not GalC.

6.2.2.3 GM1:GalC 1:2 mol:mol Liposomes

A GM1:GalC specific response was still not generated *in-vivo* using the new liposome configuration, so the proportion of GalC was doubled to try and induce a specific antibody. This resulted in liposomes composed of GM1:GalC in a 1:2 mol:mol ratio instead of the previously used 1:1 mol:mol ratio.

The sera were screened against the same array of lipids as the GM1:GalC 1:1 mol:mol immunised mice except that an additional row and column of GalC was added to the grid at double the proportion previously used. The corresponding complexes consisted of ganglioside:GalC in a 1:2 mol:mol ratio instead of the previously used 1:1 ratio (Figure 6.5).

The IgM antibody response was higher in the GalNAc T-/- mice than the GalNAc T+/+ mice as previously shown. This was only significantly higher in the GM1:GalC 1:2 complex at day 21 (Mann Whitney Test, $P<0.05$). There was no apparent difference between GM1 and either configuration of the GM1:GalC complex and no GM1 complexes appeared to enhance antibody binding.

The IgG antibody binding intensity was significantly higher in the GalNAc T-/- mice with GM1, GM1:GalC 1:1, and GM1:GalC 1:2 at day 28 compared to the GalNAc T+/+ mice. It was also significantly higher in the GM1:GalC 1:2

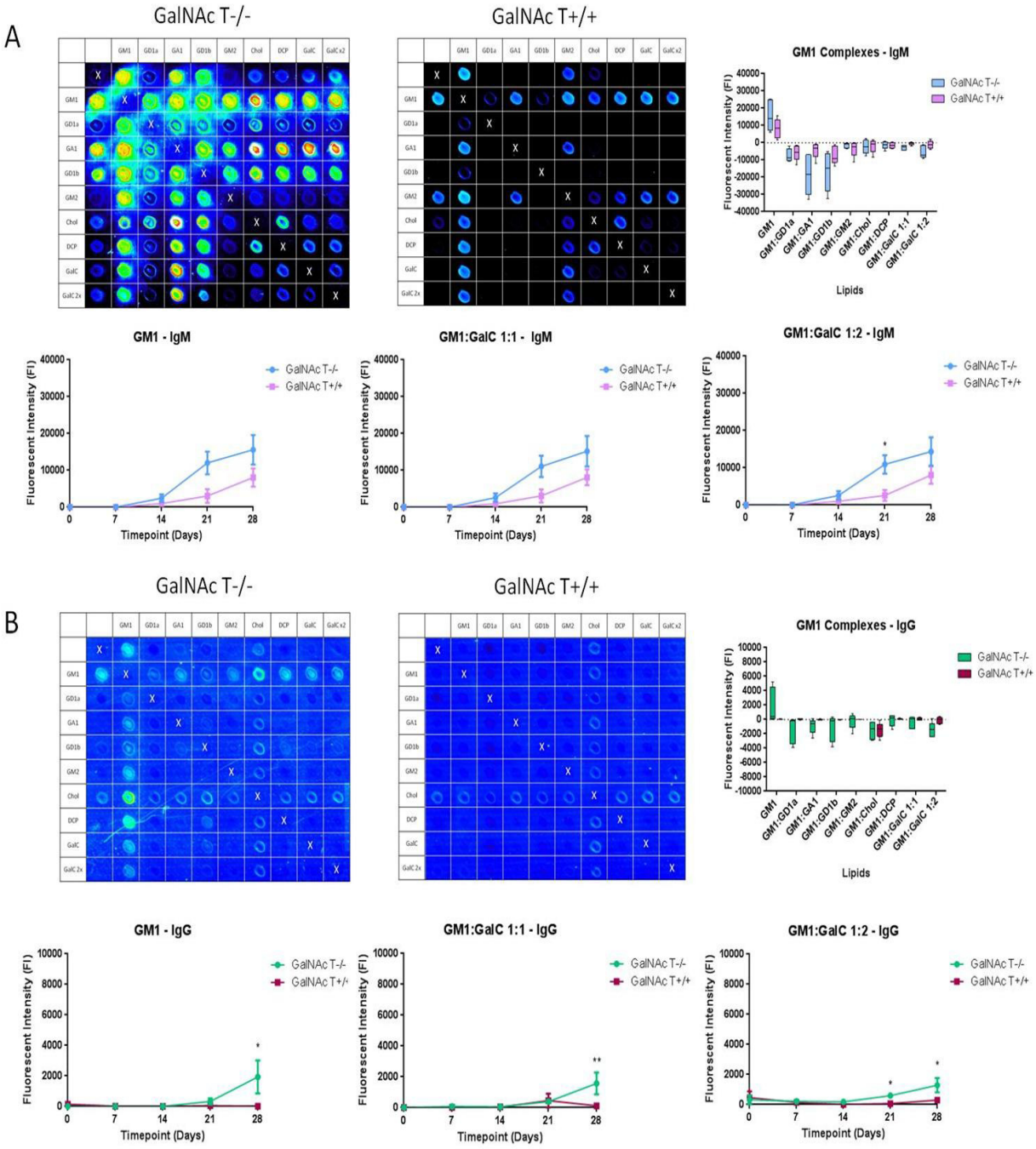


Figure 6.5 – GM1:GalC 1:2 Liposome Immunisation

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype. The antibody binding signals to GM1, GM1:GalC 1:1 and GM1:GalC 1:2 were plotted over time for each genotype and a comparison of the complexes with the single components subtracted is plotted. A: GalNAc T-/- mice showed higher IgM antibody binding signals than the GalNAc T+/+ but this was only significant on day 21 against the GM1:GalC 1:2 complex. There were no enhancing complexes. B: IgG binding intensity in GalNAc T-/- mice was significantly higher compared to the GalNAc T+/+ mice at day 28 when screened against GM1, GM1:GalC 1:1 and GM1:GalC 1:2. No complexes showed enhancement of IgG antibody binding. (Mann Whitney, $P<0.05$) (n=5)

immunised mice at day 21 (Mann Whitney Test, $P<0.05$). As with the IgM results there was no enhancement of antibody binding with GM1 complexes.

6.2.2.4 GM1:GalC 1:20 Liposomes

A distinguishable GM1:GalC specific response was still not detected in the sera using the 1:2 liposome configuration. It was plausible that the antibody was present but was being masked by the polyclonality of the sera; however, this would remain unclear until a hybridoma fusion was performed. This was an undesirable option as the fusion was labour intensive and required expensive reagents. To overcome this problem, the liposome composition was reconfigured again but for this experiment the number of moles of GalC was increased 20 fold.

As per the previous protocol, an additional row and column of GalC was added to the lipid array used for screening. This was 20x the original GalC proportion, with the corresponding complexes configured to give a molar ratio of ganglioside to GalC of 1:20.

The GalNAc T-/- mice responded better to immunisation than the GalNAc T+/+ mice as shown with the other immunisations (Figure 6.6). The IgM response to GM1:GalC 1:20 was much higher than the response to either GM1 or GM1:GalC 1:1 throughout the immunisation period. This was most apparent at day 28, where the GM1:GalC 1:20 signal was found to be approximately 12000 FI units higher than both the GM1 and GM1:GalC 1:1 signals. The IgG response reflected these results with the GM1:GalC 1:20 complex producing a binding signal that was approximately 6000 FI units higher than either GM1 or GM1:GalC 1:1 at day 28.

This suggested that a distinguishable GM1:GalC response had been generated *in-vivo,* which led to a number of these mice being used for hybridoma fusions. After two weeks of incubation, the supernatant was screened for specific GM1:GalC antibodies and, whilst many wells were found to produce GM1 antibodies, none were found to produce antibodies that were capable of binding specifically to GM1:GalC in a 1:1 or 1:20 ratio.

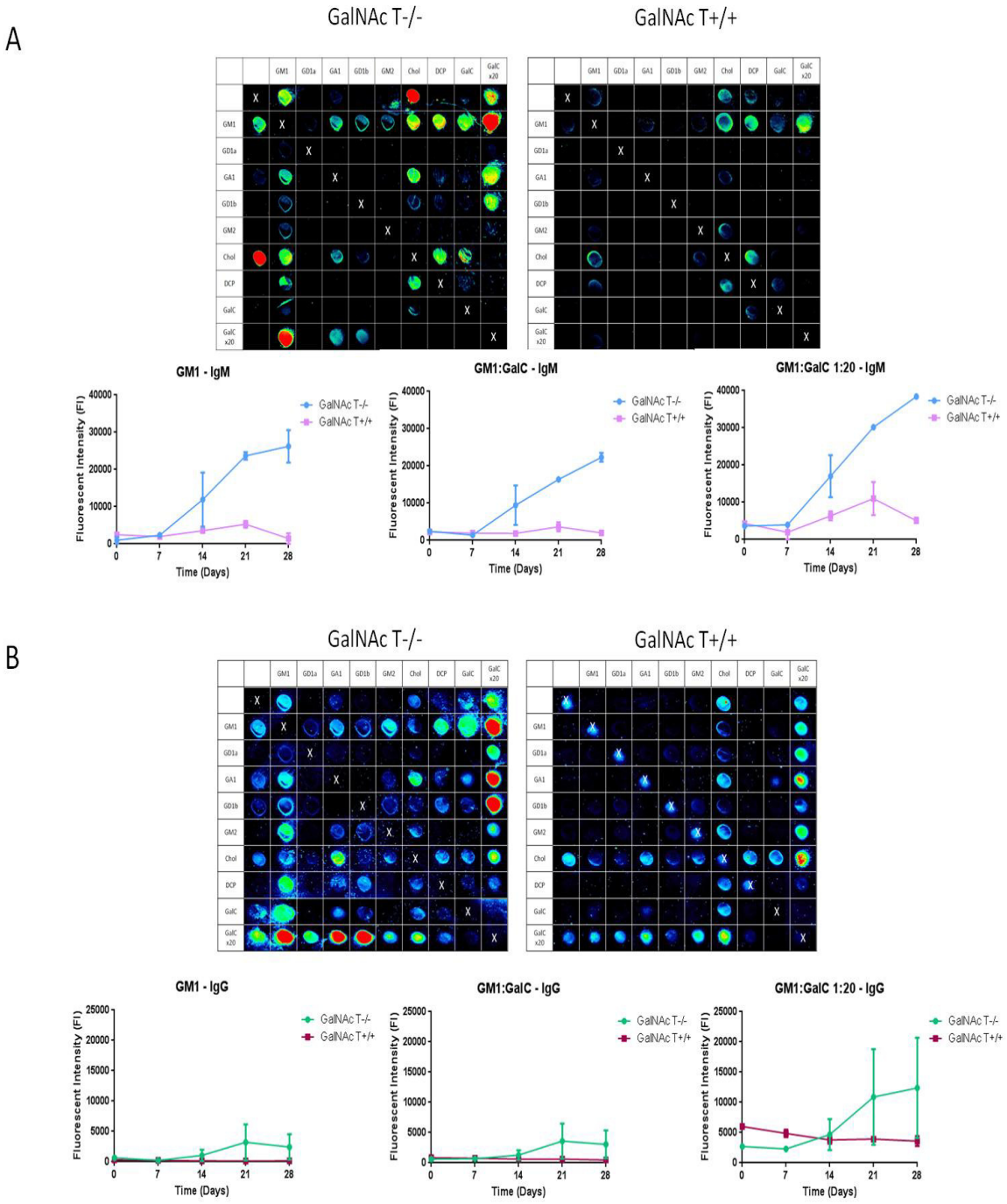


Figure 6.6 - GM1:GalC 1:20 Liposome Immunisation

The sera was screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype. The antibody binding signals to GM1, GM1:GalC 1:1 and GM1:GalC 1:20 were plotted over time for each genotype. A: GalNAc T-/- mice showed higher IgM antibody binding signals than the GalNAc T+/+. GM1:GalC 1:20 appeared to have a much higher binding signal than GM1 or GM1:GalC 1:1 B: IgG binding intensity in GalNAc T-/- mice were higher compared to the GalNAc T+/+ mice at day 21 and day 28 when screened against GM1, GM1:GalC 1:1 and GM1:GalC 1:20. The binding signal against GM1:GalC 1:20 was higher than both GM1 and GM1:GalC 1:1 however this signal was highly variable. There was a high baseline IgG antibodyto GalC but this remained steady throughout the immunisation period. (n=2)

6.2.2.5 Screening of Sera Against Complexes containing different Ratios of GalC

The lack of a GM1:GalC specific antibody was unexpected but it was hypothesised that the presence of increased GalC in the array may have resulted in the formation of a complex that enhanced anti-GM1 antibody binding. This enhanced binding may have given the illusion that a separate GM1:GalC antibody was present. It would also explain why no GM1:GalC specific antibodies were detected from the hybridoma fusion as the arrays were only detecting GM1 antibodies.

To test this hypothesis, the terminal sera from all the immunised mice were screened against a panel of different ratios of GalC. This array followed the same configuration as that used in Section 4.2.1 except that GM1 was the only ganglioside screened.

The best IgM response was found in the GM1:GalC:SM 1:1:1 immunised mice, which all showed a clear trend of enhancement as the ratio of GM1:GalC increased (Figure 6.7). This was found to be strongest in the GalNAc T+/+ mice but was also found in both the GalNAc T-/- and B6CGTGNxDBA mice. The signal to the GalNAc T-/- sera was lower than the other genotypes as there was binding to the single GalC epitope, which increased as the ratio increased. Due to the polyclonal nature of sera it was not possible to determine if this was the result of the GM1 antibody cross reacting or a separate GalC antibody.

In contrast to these results, the GM1:GalC 1:1 immunised mice showed a neutral response overall and were not enhanced by increasing concentrations of GM1:GalC complexes. Examination of the individual mouse serum showed that two mice did have weakly enhancing signals; however, three other mice did not produce an enhancement, as they contained antibodies that also bound to the individual GalC epitope. This attenuated the binding signal to the GM1:GalC complexes, which resulted in a neutral signal.

The GM1:GalC 1:2 immunised mice had similar issues; however, they produced weakly positive enhancements at high ratios. The binding signals were found to be higher in the GalNAc T+/+ mice as the sera did not tend to cross react to the single GalC epitopes as it did in the GalNAc T-/- mice.

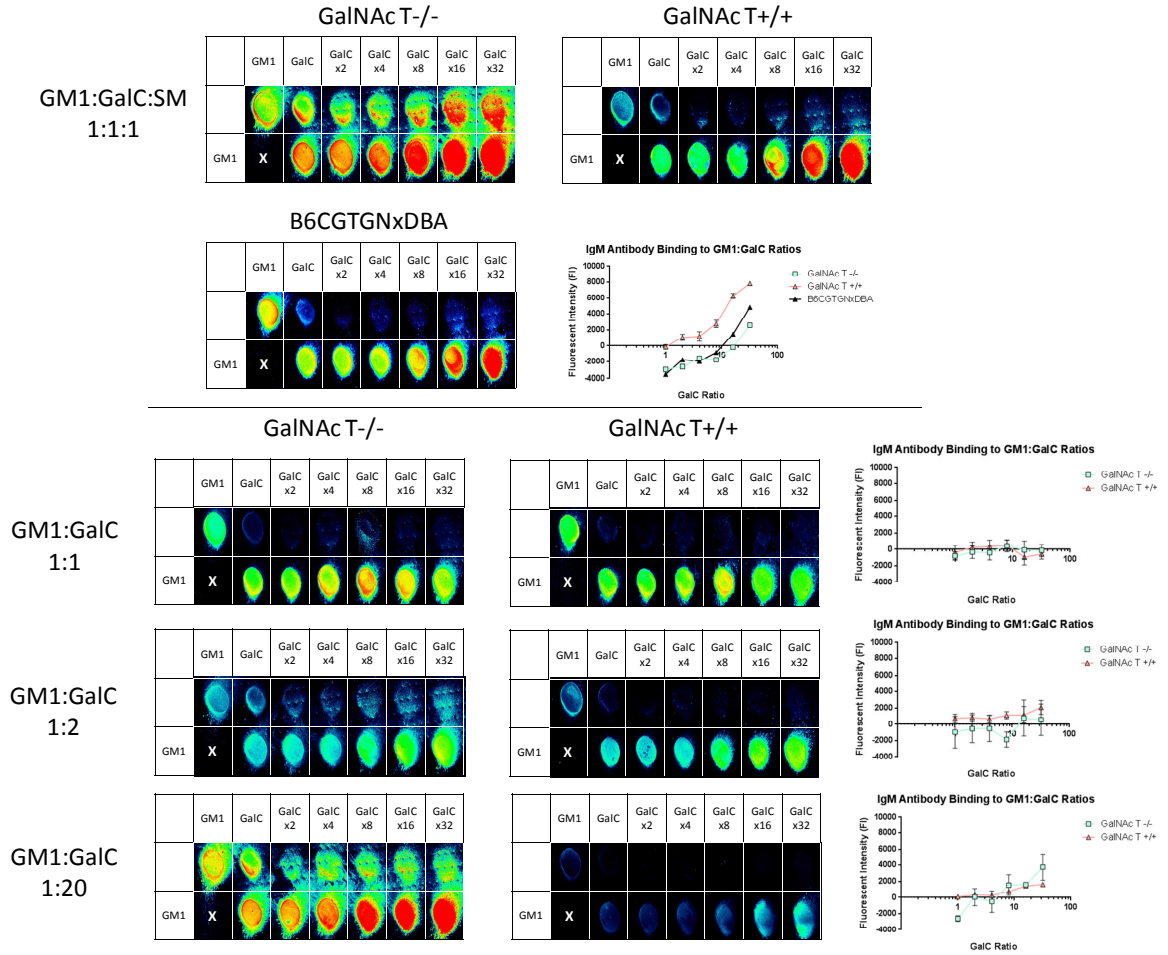


Figure 6.7 - IgM responses to different ratios of GM1:GalC

The sera from the different immunisations were screened against GM1:GalC complexes that contained differing proportions of GalC. Representative blots indicated the antibody reactivities against complexes following subtraction of single components and were plotted on semi log graphs for each genotype. GM1:GalC:SM 1:1:1 immunised mice responded well to different ratios of GM1:GalC complexes. The highest peak in all three genotypes was found with a ratio of 1:32. GM1:GalC 1:1 immunised mice shown a poor IgM response at any ratio. GM1:GalC 1:2 immunised mice showed an enhanced but low response in both genotypes at higher ratios. This was also shown with GM1:GalC 1:20 immunised mice. Some antibodies in the sera also bound to the single GalC epitopes.

Mice immunised with GM1:GalC 1:20 showed a higher level of enhancement as the proportion of GalC increased in the complexes. This was found with both the GalNAc T-/- and the GalNAc T+/+ mice particularly between the ratios of 1:16 and 1:32. As before, the GalNAc T-/- sera tended to bind to the single GalC epitope; however, this appeared to have less of an impact on the overall binding signal compared to the other immunised mice.

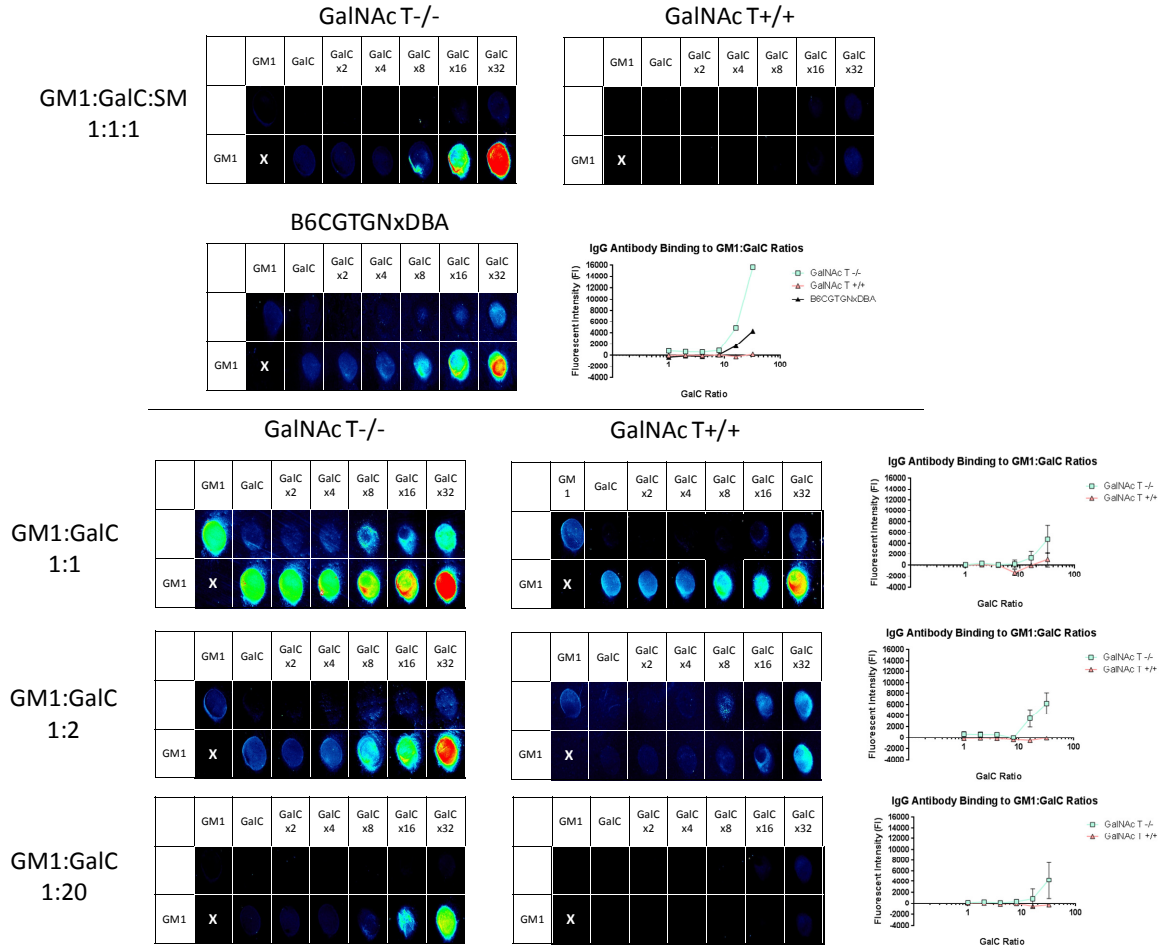


Figure 6.8 - IgG responses to different ratios of GM1:GalC

The sera from the different immunisations were screened against GM1:GalC complexes that contained differing proportions of GalC. Representative blots indicated the antibody reactivities against complexes following subtraction of single components and were plotted on semi log graphs for each genotype. All the GalNAc T-/- mice showed similar levels of enhancement against complexes containing higher ratios of GalC except for the GM1:GalC:SM immunised mice which had a much higher enhancement. The B6CGTGNxDBA mice had a similar binding pattern as these mice. The GalNAc T+/+ mice tended to have low responses even at higher GM1:GalC ratios except in the GM1:GalC 1:1 immunised mice which showed an enhancement at the higher ratios. Some antibodies in the sera also bound to the single GalC epitopes but this had little effect on the binding signals.

The IgG responses to increasing ratios of GM1:GalC complexes were more consistent than the results seen with IgM antibodies (Figure 6.8). Generally, the GalNAc T-/- mice, which had the highest levels of GM1 antibodies, appeared to show the most enhancement with increasing concentrations of GalC. This enhancement was highest in the GM1:GalC:SM immunised mice, which displayed a binding signal of 15932 FI units at a ratio of 1:32. This was substantially higher

than the signals achieved by the GM1:GalC 1:1; 1:2, and 1:20 immunised mice, which all displayed similar signals between 4000 and 6000 FI units at the same ratio.

Very little binding was observed in the GalNAc T+/+ mice regardless of their antigen. Signals were only detected at high ratios of GM1:GalC, where binding was also detected to the single GalC epitope. This resulted in neutral levels overall. The only exception was the GM1:GalC 1:1 immunised mice, which showed an improvement in binding as the ratio of GalC increased in the complexes. This resulted in an enhanced signal; however, it was still lower than the signals detected in the GalNAc T-/- mice.

The GM1:GalC:SM immunised B6CGTGNxDBA mice also showed an increase in binding signal as the ratio of GM1:GalC increased, although this was lower than the signal seen with the GalNAc T-/- mice. This binding was unexpected as the original characterisation suggested that no GM1 IgG antibodies were present (Figure 6.3).

6.2.2.6 Summary

There was no clear trend found with the IgM sera results in regards to increasing GalC ratios, except in the GM1:GalC:SM immunised mice. The initial screening indicated that these mice produced a larger response to GM1, so it is possible that the increased number of antibodies led to higher levels of enhancement. The low enhancement in the other immunised mice was impacted by the presence of antibodies that bound to GalC, which resulted in the attenuation of the GM1:GalC complex signal relative to the signals of the single component lipids. The polyclonality of the serum made it difficult to determine if anti-GalC antibody reactivity was caused by the anti-GM1 antibody cross reacting with this lipid, or a separate GalC antibody that had arisen during the immunisation period.

Another issue was the effect that class switching had upon the IgM antibody repetoire. The number of anti-GM1 IgM antibodies was reduced during class switching, whilst the number of antibodies targetting other antigens appeared to be unaffected. This resulted in a lower raw signals against GM1 complexes, whilst the signals against GalC remained high. This would have impacted the

complex binding signals following subtraction of the single components, which may have given a false impression that the complexes were not enhancing anti-GM1 antibody binding. Screening the sera at an earlier timepoint would have addressed this issue and may have indicated a more clear trend.

The IgG data was much more supportive of the hypothesis that increased concentrations of GalC in GM1:GalC complexes enhanced GM1 binding. The GalNAc T-/- mice from all the immunisations showed a similar trend with the enhancement increasing as the ratio of GalC in the complex increased. This was also found in the B6CGTGNxDBA mice, which had shown no GM1 binding in the original characterisation.

This data added credence to the hypothesis that a GM1:GalC complex dependent antibody was not responsible for the enhanced signals and that they were in fact produced by a GM1 antibody that bound better to different configurations of the complex .

This enhancing behaviour was not a phenomenon unique to sera alone and was also found with monoclonal antibodies. Two in-house anti-GM1 antibodies, DG1 and DG2, were also screened using the same grid and demonstrated the same binding behaviour (data not shown). This data alongside that in Chapters 3 and 4 suggested that a GM1:GalC response in serum was unlikely to be a GM1:GalC complex-dependent antibody.

These antibodies may still exist in patients; however, the major drawback of producing them in mice is the uncertainty in the configuration and presentation of the liposome antigen. The interaction between GM1 and GalC in liposomes is unclear as it is not known if the molecules are even in close proximity to one another, let alone forming a neo-epitope complex. To produce a specific antibody the lipids would have to be fused into a true dimer with a known structure as shown in other studies (Mauri et al., 2012). This technique is relatively new however, and was not attempted within these studies. It was for these reasons that attempts to produce GM1:GalC dependent antibodies were abandoned and instead the focus shifted to other ganglioside complexes.

6.2.3 Immunisation with GM1:GD1a Liposomes

The inhibitory effects of GM1:GD1a complexes on antibody binding have been well described in both humans and mice (Greenshields et al., 2009; Kusunoki & Kaida, 2011). An antibody which could specifically target this complex would prove beneficial in establishing its location in tissue and could explain why some tissues are targeted in disease whilst others are not. GalNAc T-/- and GalNAc T+/+ mice were immunised against liposomes that contained GM1, GD1a, Chol and DCP to try and produce one of these antibodies. They were bled at regular intervals and their sera was analysed using the combinatorial glycoarray.

6.2.3.1 Immunisation of GM1:GD1a Pre-Immunised Mice

A colleague immunised mice with GM1:GD1a liposomes three times over a 6 week period and then discontinued the experiment for three months (Galban Horcajo, Unpublished data). The mice then received two IP and two IV immunisations over a three week period followed by a hybridoma fusion. Only the sera collected from the second immunisation cycle were analysed. For clarity, the timepoints for serum collection were reset to reflect the second immunisation cycle only.

As shown with the GM1:GalC immunisations, the GalNAc T-/- mice produced a higher level of anti-ganglioisde antibody than the GalNAc T+/+ mice (Figure 6.9). The day 0 results indicated that the mice still had low levels of circulating anti-ganglioside IgM antibodies prior to the commencement of the new immunisation cycle. Anti-GM1 IgM antibodies increased modestly throughout the immunisation period, whilst anti-GD1a and anti-GD1a complex antibodies showed a more substantial increase. There were no enhancements or inhibitions by GD1a complexes.

An analysis of the IgG data demonstrated that there were no circulating anti-ganglioside antibodies in the sera of the mice prior to the second round of immunisations. There was also no detection of an IgG response in the GalNAc T+/+ mice throughout the immunisation cycle. In the GalNAc T-/- mice, the anti-GM1 antibodies increased slightly at day 14, whilst anti-GD1a and anti-GD1a complex antibodies increased substantially at both day 7 and day 14.

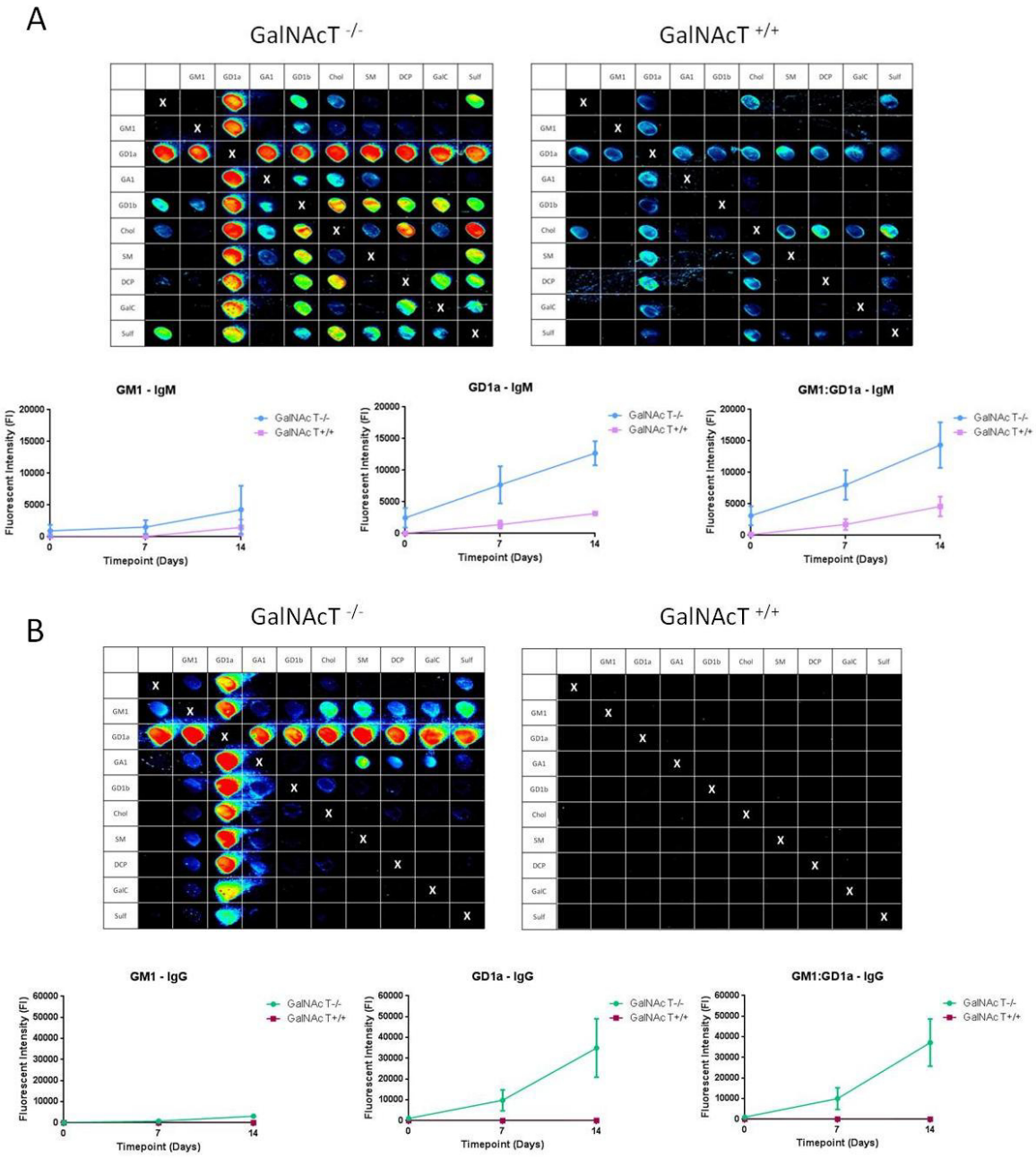


Figure 6.9 – GM1:GD1a Pre-Immunised Mice

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype. As shown previously the GalNAc T-/- mice responded better to immunisation than the GalNAc T+/+ mice. A: The mice had residual IgM antibody reactivity from the initial immunisation cycle. GM1 antibody increased modestly over the immunisation period whilst GD1a and GM1:GD1a increased to a substantially higher level. There appeared to be no GD1a complex enhancements. B: There was no IgG response in the GalNAc T+/+ mice. The GalNAc T-/- mice had no IgG antibody reactivity at day 0 but this increased substantially at each subsequent immunisation. At day 14 the GD1a and GD1a complex response was approximately 40000 FI units. There was no GD1a complex enhancement or inhibition. (n=2)

6.2.3.2 Hybridoma Screening by ELISA

A specific GM1:GD1a antibody was not identified in the serum using the defined protocol; however, a hybridoma fusion was performed to determine if B cells were present that had been raised against this target. The spleens from both GalNAc T-/- mice were harvested and fused with a myeloma cell line and the supernatant was screened after two weeks of incubation. Due to the limited volumes of supernatant available, only one lipid and one antibody isotype were able to be screened using the standard ELISA protocol. As a result, the supernatant was only analysed for IgM reactivity against GM1:GD1a complexes. Several wells had ODs above the threshold of 0.1 but only the well with the highest OD of 2.1 was expanded and rescreened against the single lipids, GM1 and GD1a alone. Further analysis of supernatant on the combinatorial glycoarray indicated that it was an antibody that bound GD1a in its single form and when in heteromeric complexes. This was cloned further and antibody stocks were produced for characterisation as shown in Chapter 7. This antibody was dubbed GAME-M1

6.2.3.3 Immunisation of Naive Mice with GM1:GD1a Liposomes

Following on from this experiment, another immunisation was performed with GM1:GD1a liposomes using naive mice. These were immunised following the standard protocol as per Section 2.7.

An analysis of the sera indicated that the IgM response targeted GM1 and its structural analogues GA1 and GD1b (Figure 6.10). In the GalNAc T-/- mice, the signle GM1 response developed at day 14 and became significantly higher than the GalNAc T+/+ mice at day 21 and day 28 (Mann Whintey, $P<0.05$). This was also found with the GM1:GD1a complex except that the overall binding signal was much lower than the GM1 signal. A response to GD1a also developed; however, it was much lower than that found with GM1 and was similar between genotypes.

An IgG response did not develop in the GalNAc T+/+ mice but in the GalNAc T-/- mice a response to GM1 and GM1 complexes developed at day 21. This was

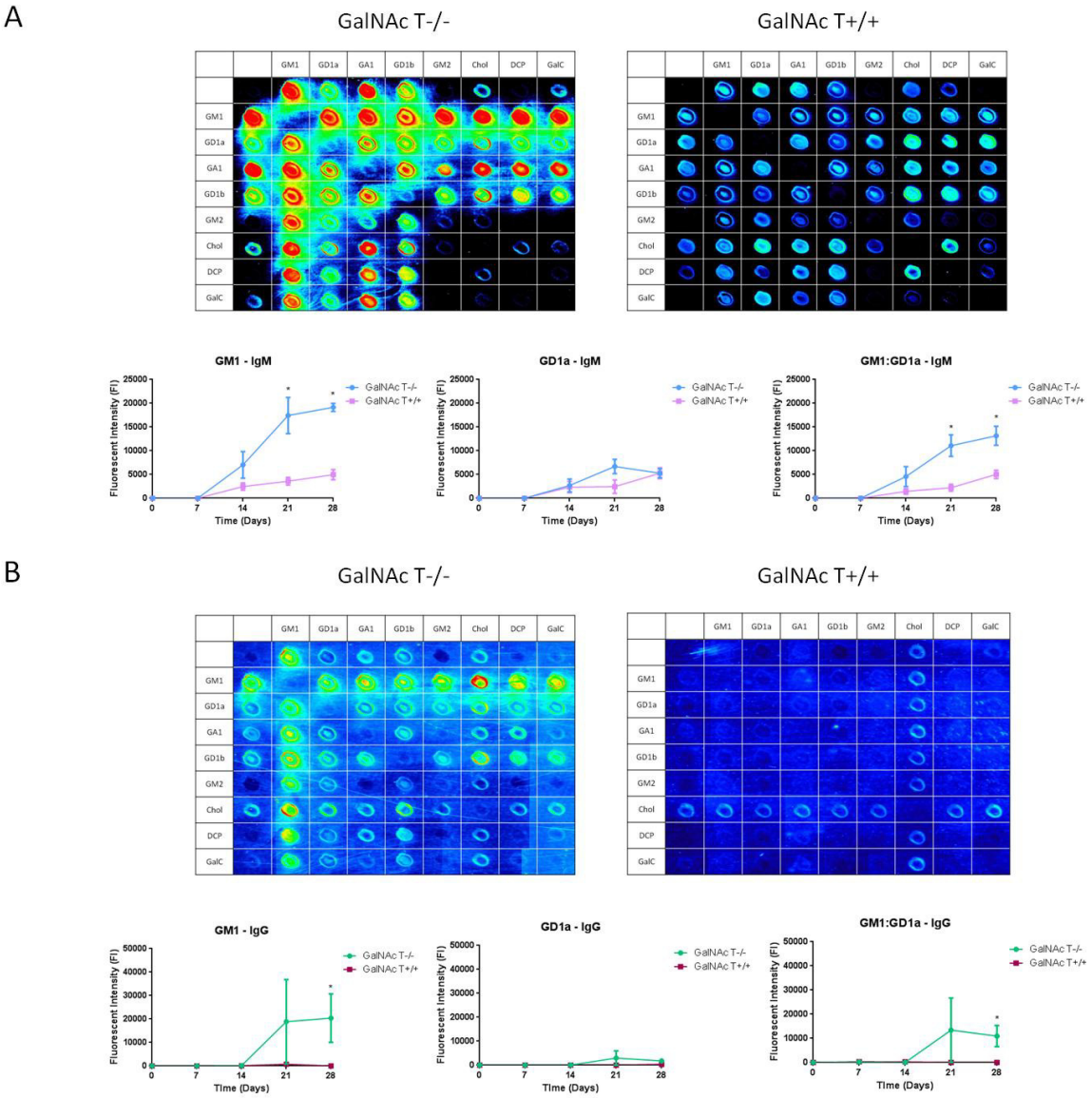


Figure 6.10 - GM1:GD1a Immunised Mice

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype. As shown previously the GalNAc T-/- mice responded better to immunisation than the GalNAc T+/+ mice. A: The IgM results indicated a larger response to GM1 and GA1 than GD1a. The GM1 and GM1 complex responses rose steadily during the immunisation period. They became significantly higher in the GalNAc T-/- mice in at day 21 and 28 but showed a higher response to GM1 than to GM1:GD1a. There was little GM1 complex enhancement. B: The GalNAc T+/+ mice did not have any IgG antibodies in the sera except to cholesterol. The GalNAc T-/- mice had a response develop at day 21 that targeted GM1 and GM1 complexes. This was significantly higher than the GalNAc T+/+ mice at day 28. The antibody response to single GM1 was again higher than the response to GM1:GD1a. (Mann Whitney, $P<0.05$) GalNAc T-/- (n=3) GalNAc T+/+ (n=5)

significantly higher at day 28 (Mann Whitney, $P<0.05$). A very low response developed to GD1a but this was again much lower than the binding signals found with GM1.

6.2.3.4 Hybridoma Screening by Lipid Microarray

Although a response specifically targeting the GM1:GD1a complex was not established in the serum, the mice were still fused to determine if any anti-complex antibodies had developed. One GalNAc T+/+ mouse and three of the GalNAc T-/- mice were culled and their spleens harvested for the hybridoma fusion. The original PCR indicated that the GalNAc T+/+ mouse was a knock out; however, analysis of the serum and resampling of the DNA proved that it was actually a GalNAc T+/+ mouse. This was discovered after the hybridoma fusion had been performed.

The previous experiment showed the limitations of using an ELISA as the screening method for anti-ganglioside complex antibodies. The volume of supernatant available for sampling was only 100µl and half of this had to be used as a negative control.

In addition to these issues, only one lipid could realistically be probed at one time and only one isotype of antibody could be analysed. The number of assays that had to be performed was also detrimental to the screening process, so a new method was developed using the lipid microarray.

The microarray was capable of printing grids that resembled those used for screening the sera. Different single lipids and combinations of various complexes could be analysed in the one assay, which would permit quick identification of complex dependent antibodies. The major advantage of the microarray was that it could print 20 slides in one cycle which consisted of 16 arrays each. The slides could then be sampled using just 50µl of undiluted supernatant and probed for both IgM and IgG reactivity simultaneously (Figure 6.11).

The initial screening of the supernatant from the GM1:GD1a immunised mice indicated several different targets that were both IgM and IgG (Figure 6.11). The

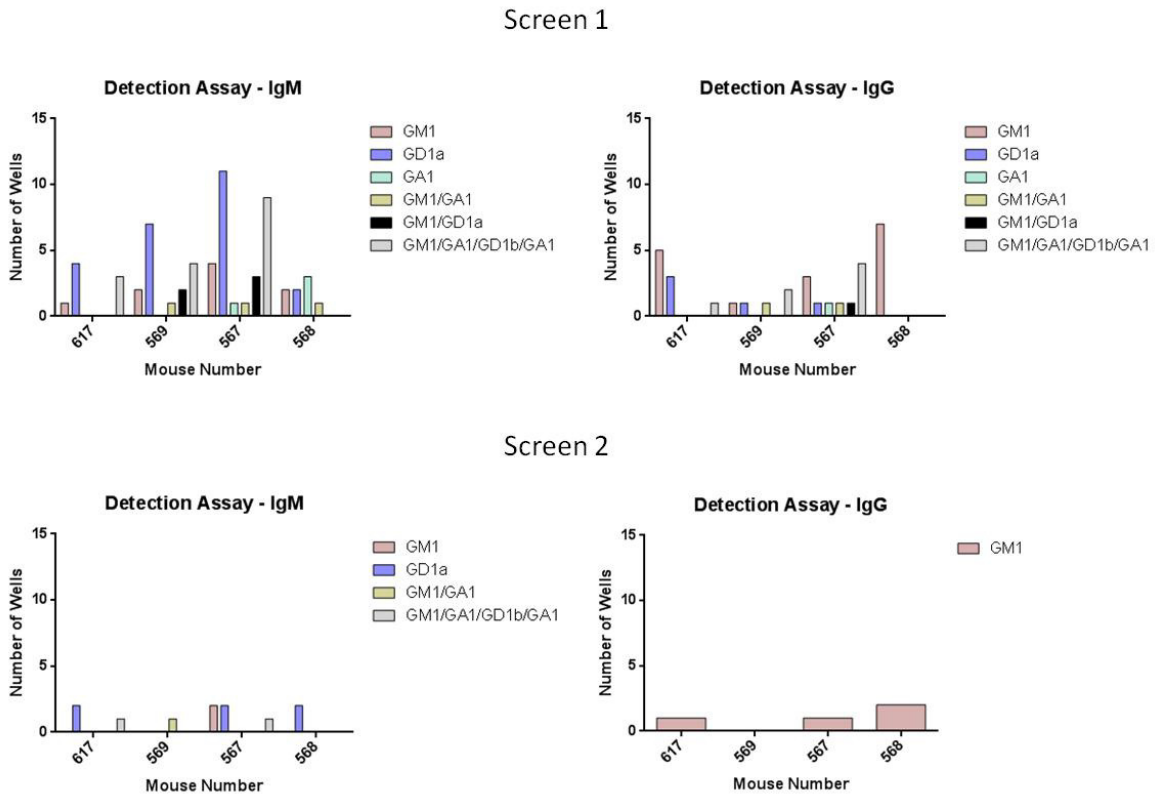


Figure 6.11 – Initial Supernatant Screen of GM1:GD1a Immunised Mice Hybridoma Cells

Hybridoma supernatant was screened to determine antibody positive wells. Screen 1 was performed two weeks after the initial hybridoma fusion. Wells were found containing IgM and IgG antibodies that targeted GM1, GD1b, and GA1. Wells were also found containing some antibodies that cross reacted to various different gangliosides. Screen 2 was performed on the expanded cells. Overall, fewer antibody positive wells were detected. Wells were found containing IgM antibodies that bound to GM1, GD1a and cross reacted to different gangliosides. The only IgG positive wells found were those that contained antibodies targeting GM1.

most frequent IgM target was GD1a followed by GM1, which was surprising considering that anti-GM1 antibodies were the predominant response in the sera. There were a higher number of anti-GM1 IgG antibodies, which is an indication that the GM1 antibodies were undergoing class switching. This was an example of the discrepancy between B2 cells in the spleen and what antibodies were being produced by the long lived plasma cells in the serum. As the B2 cells had recently undergone class switching, they were producing IgG antibodies but the plasma cells in the serum were still producing IgM antibodies. This led to a difference between the antibody repertoires in the hybridoma supernatant versus those in the sera.

Asides from GM1 and GD1a, IgM and IgG antibodies also targeted GA1 and several were present that cross reacted to several different gangliosides. No antibodies were detected that were complex dependent.

The positive wells were expanded into 24 well plates and after three days, all the media was removed and replaced. This addressed the possibility that the positive results detected during the first screen were due to unfused splenocytes or unstable hybridoma cells. The process of fusing two nuclei together often results in unstable cells, which experience chromosome loss. This leads to short lived hybridoma cells that produce antibody for a few days and then die. The supernatant was therefore tested again after a further three days of growth.

The number of positive wells dropped substantially. Several IgM antibodies were detected that still bound GM1, GD1a and cross reacted to several different gangliosides. IgG antibodies were detected in several wells but these only bound to GM1. A selection of cells were taken forward and expanded further for cloning.

Some cell lines were lost during the cloning process but a GM1 IgG antibody dubbed GAME-G2 was developed. This was characterised in Chapter 7.

6.2.4 Immunisation with GM1:Sulfatide Liposomes

Although there was little success in producing complex antibodies against GM1:GalC and GM1:GD1a, an attempt was made to produce GM1:sulfatide dependent antibodies. These have been associated with AMAN, with a recent study finding that patients who had no detectable anti-GM1 antibodies, commonly produced antibodies targeting the GM1:sulfatide complex (Rinaldi et al., 2013). There was also an association between MMN and GM1:sulfatide antibodies, which raised the possibility that they were targeting a specific structure common to both conditions (Galban-Horcajo et al., 2013).

To produce these antibodies, mice were immunised against liposomes containing GM1, sulfatide, SM, DCP and Chol following the standard immunisation protocol. Blood was collected regularly and the serum was screened using slides printed on the combinatorial glycoarray (Figure 6.12).

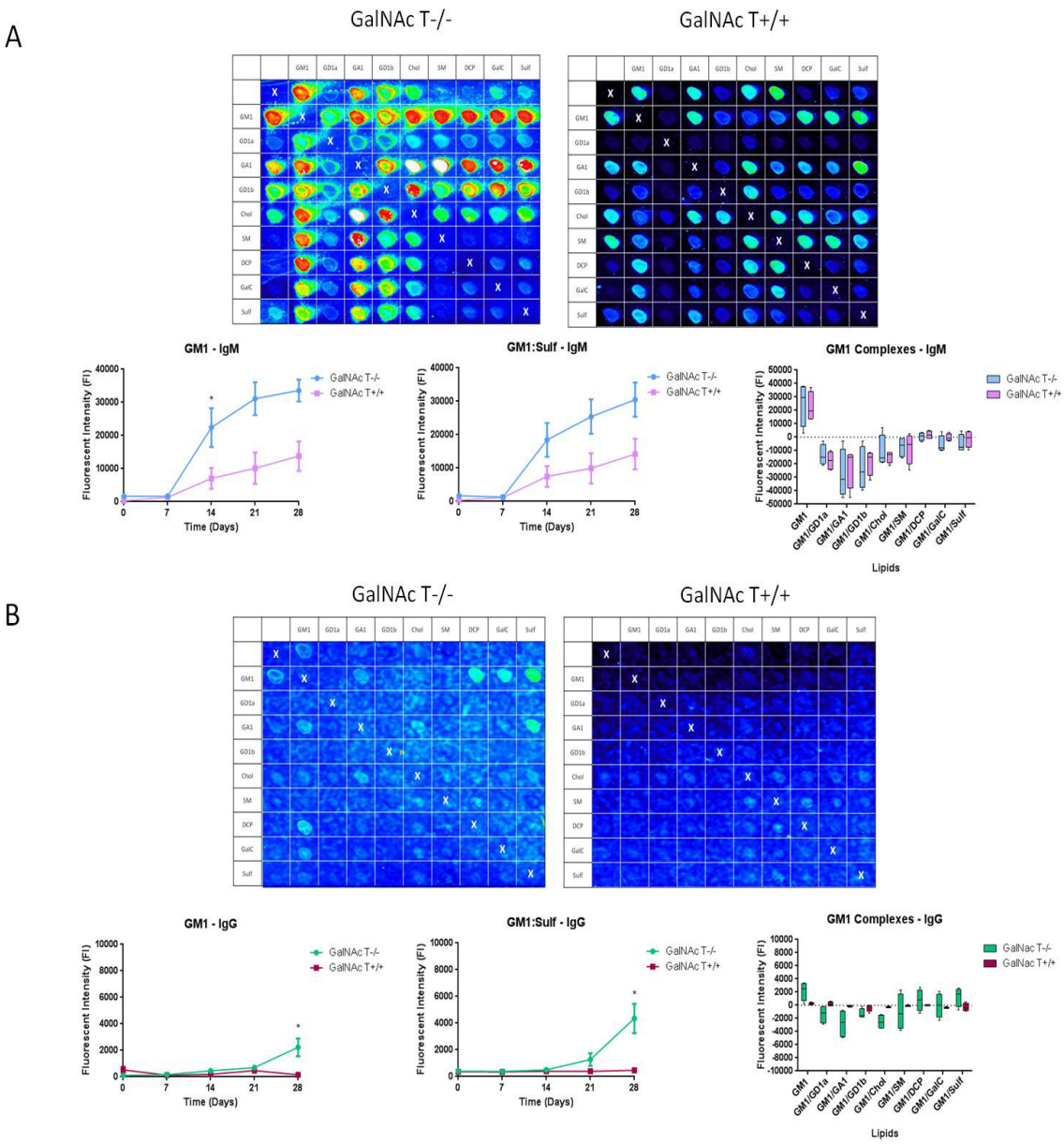


Figure 6.12 - GM1:Sulfatide Immunised Mice

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding behaviour is displayed for each genotype and a comparison of the GM1 complexes with the single components subtracted is plotted. As shown previously the GalNAc T-/- mice responded better to immunisation than the GalNAc T+/+ mice. A: The IgM results indicated a similar response to GM1 and GM1:sulfatide. This was only significantly higher with GM1 in the GalNAc T-/- on day 14. There were no enhancements with GM1 complexes at day 28. B: The GalNAc T+/+ mice had a minimal IgG response to any target. The GalNAc T-/- mice had a response that was significantly higher on day 28 that targeted GM1. There was also an enhanced signal to GM1:sulfatide that was also significantly higher than the GalNAc T+/+ mice on day 28. There was enhancement with GM1:DCP and GM1:sulfatide complexes. (Mann Whitney, $P<0.05$) (n=4)

The GalNAc T-/- mice had the largest response showing a steady increase in IgM antibodies targeting both GM1 and GM1:sulfatide. This was consistently higher than the response of the GalNAc T+/+ mice but was only significantly higher against GM1 on day 14 (Mann Whitney, $P<0.05$). There was no enhancement with any GM1 complexes.

The IgG response was mostly absent at all timepoints in the GalNAc T+/+ mice. In the GalNAc T-/- mice the GM1 response developed at day 21 and became significantly higher than the GalNAcT +/+ mice at day 28. This was also found with the GM1:sulfatide responses except that the signal was much higher than that found against the single GM1. Enhancements were found against both GM1:DCP and GM1:sulfatide at day 28.

6.2.4.1 Hybridoma Screening by Lipid Microarray

The spleens from all the GalNAc T-/- mice were harvested and fused with the myeloma cells to create hybridomas. After two weeks of incubation, the supernatant was screened for antibodies following the method established with the lipid microarray. The data from the first screen indicated that a large number of IgM antibodies were found that cross reacted to GM1 and GA1 (Figure 6.13). There were also antibodies that bound to the single GM1, sulfatide and SM. IgG antibodies were detected that bound to the single GM1 and other antibodies were found that cross reacted to GM1 and GA1. No complex dependent antibodies were detected in either isotype. The positive wells were expanded into 24 well plates and grown for a further 3 days before the supernatant was removed and replaced. The cells were incubated for a further 3 days and rescreened.

Screen 2 showed a large drop in the number of GM1/GA1 cross reacting antibodies as well as the loss of those targeting sulfatide. There was also a drop in the number of both IgG and IgM GM1 antibodies. This was again associated with unfused splenocytes and unstable hybridoma cells as described with the GM1:GD1a supernatant screening.

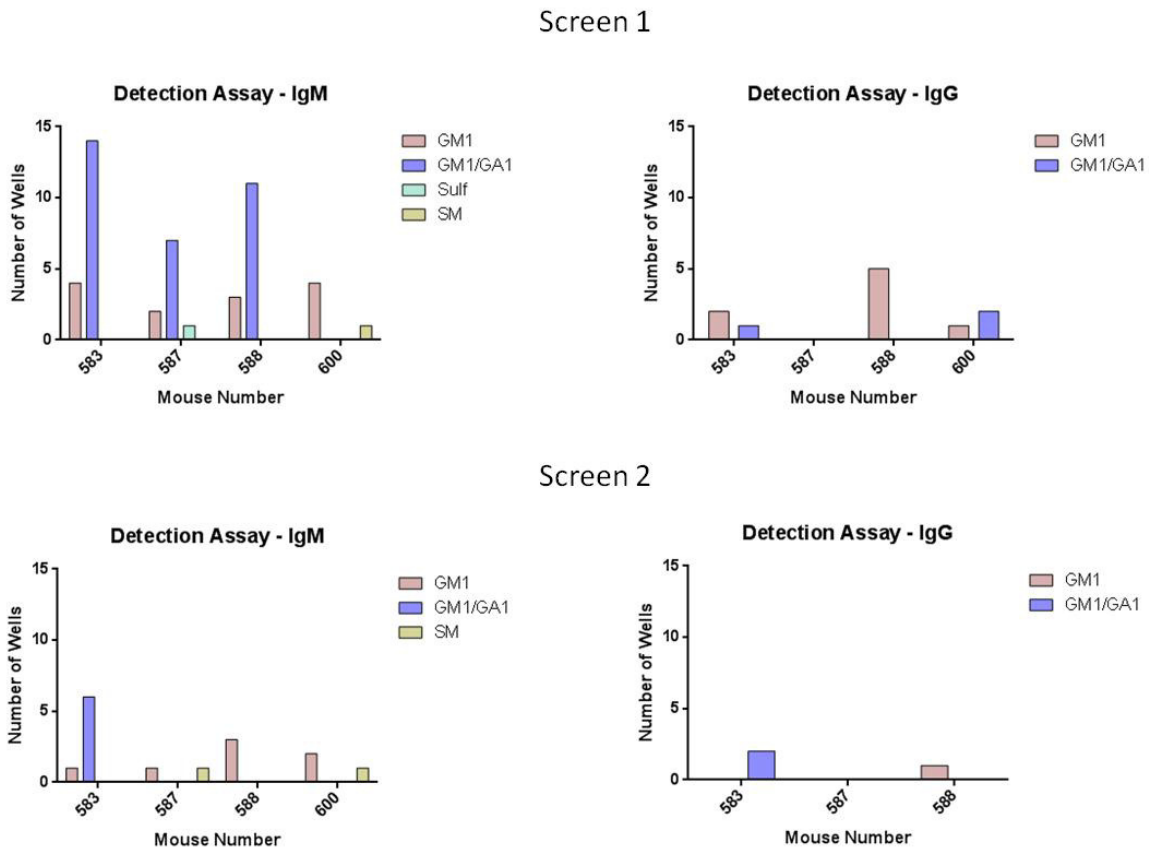


Figure 6.13 - Initial Supernatant Screen of GM1:Sulfatide Immunised Mice Hybridoma Cells

Hybridoma supernatant was screened to determine antibody positive wells. Screen 1 was performed two weeks after the initial hybridoma fusion. Wells were detected containing IgM and IgG antibodies that cross reacted to GM1 and GA1 and antibodies were also found that bound to the single GM1. Other wells contained IgM antibodies that bound to sulfatide and SM. Screen 2 was performed on the expanded cells. Overall, fewer antibody positive wells were detected particularly those containing antibodies that bound GM1/GA1. The anti-sulfatide antibody positive wells were lost completely.

A selection of the antibody producing cells were expanded further and tested routinely to determine if they were still producing antibody. As with the GM1:GD1a hybridomas, some cells were lost during expansion but an anti-GM1 IgM and an anti-SM IgM were successfully cloned. These antibodies were dubbed GAME-M3 and GAME-M4 respectively and were characterised in Chapter 7.

6.2.4.2 GM1:Sulfatide Immunisation with Transgenic Rescue Mouse

A transgenic mouse was created to try and address the potential issues with tolerance that may have been hindering the ability to produce a complex specific antibody. A mouse has recently been created in the Willison laboratory

that lacks all complex gangliosides except in places where the Thy 1 promoter is active. This restricts ganglioside expression to the axons in these mice, which makes them useful for studying autoimmune neuropathies.

An immunisation study, performed by a former colleague, indicated that these mice lacked the tolerance to gangliosides seen in normal WT mice but not to the same degree as GalNAc T-/- mice (Rupp, Unpublished Observations). The suitability of the mice for immunisation studies led to the desire to try and produce a mouse that lacked tolerance to both GM1 and sulfatide.

This was achieved by crossing the Thy1 mice with the CST-/- mice to create a new mouse – CST-/- x GalNAc T-/- Thy 1 - dubbed the double KO axonal rescue. These mice lacked sulfatide entirely and had only restricted complex ganglioside expression. They suffered from a reduced muscle mass and developed a tremor and ataxia as they aged which resembled the normal CST-/- mice. As a result of these features, the mice were immunised at 4 weeks to reduce any excess suffering.

A pilot study was performed using one mouse, which was immunised with GM1:Sulfatide liposomes that were created as per Section 2.6. The mouse was bled regularly but smaller volumes than normal were collected due to its reduced size. The serology indicated that no anti-GM1 antibodies were created throughout the immunisation cycle (Figure 6.14). Antibody responses were detected to other components of the liposomes particularly sulfatide and sulfatide complexes such as sulfatide:SM and sulfatide:Chol. There were also antibodies that targeted Chol complexes, which were enhanced beyond the signal detected to the single epitope.

A hybridoma fusion was performed using the spleen from the mouse, with the first screening indicating that several cells were producing antibodies. As expected most cells were producing sulfatide antibodies but there were also several cells producing anti-GM1 IgM and IgG antibodies. The blots indicated that these had high intensity values which supported the theory that the signals were not artefacts.

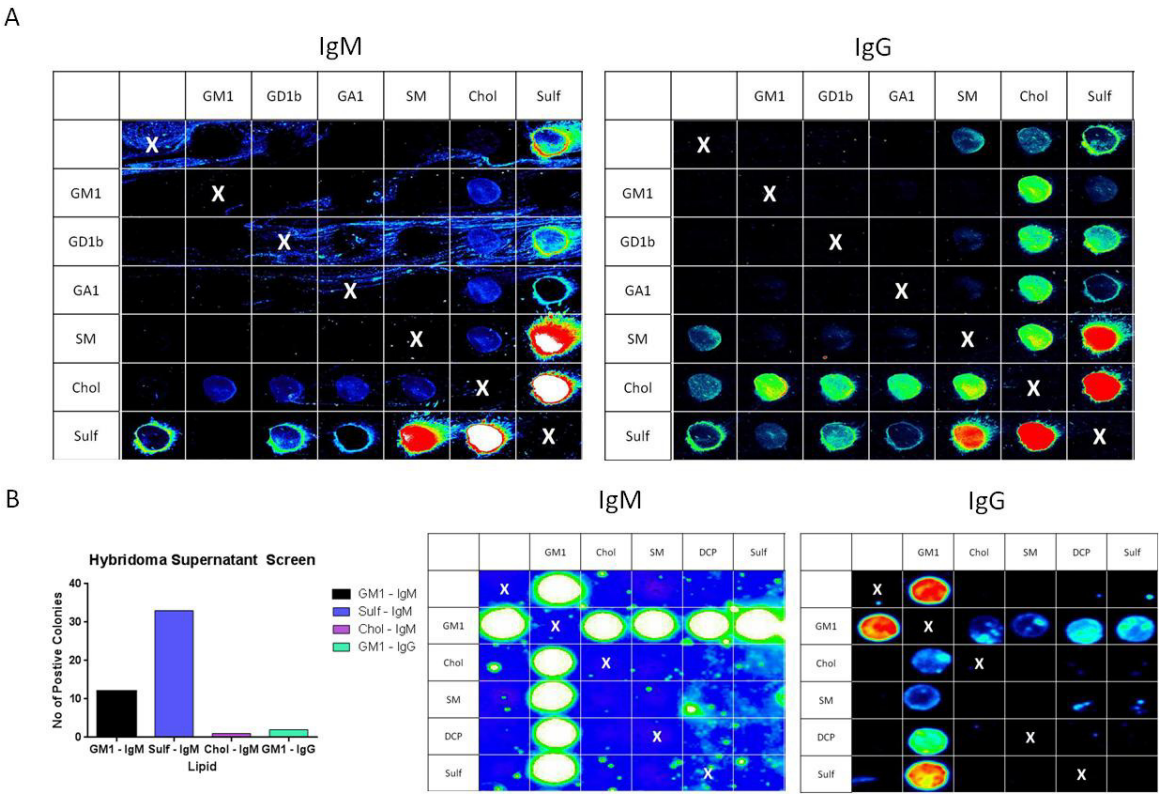


Figure 6.14 – Double KO Axonal Rescue Immunisation with GM1:Sulfatide Liposomes

Double KO axonal rescue mice were immunised with GM1:sulfatide liposomes and their sera were screened by combinatorial glycoarray. A: The IgM and IgG blots from the terminal bleed indicate a high sulfatide antibody response that is enhanced with SM and Chol. There is also minor responses to Chol complexes which are enhanced compared to the single Chol. No anti-ganglioside antibodies were detected. B: The spleens from these mice were used to create hybridoma cell lines. A high number of sulfatide antibodies were detected in the supernatant but there were also a high number of GM1 antibodies which were both IgM and IgG. The spot colour indicated that these were strong signals.

A selection of cells, which produced the highest signals, were expanded and attempts were made to clone them further but unfortunately the cells were outgrown and ceased antibody production.

6.2.5 Immunisations with WLE Liposomes

The lack of success in producing complex-dependent antibodies raised questions about the effectiveness of the liposome configurations. The simplistic composition, whilst useful for producing antibodies against single antigens, is not representative of the endogenous membrane and, as such, may be unable to

induce the same complex-dependent antibody responses that are observed in certain patient's sera.

In an attempt to address this issue, an immunogen was sought that more closely resembled the lipid composition of a nerve membrane. This resulted in the selection of whole lipid extract (WLE), which is derived from homogenised cauda equina. As this nerve is enriched in a variety of gangliosides (Ogawa-Goto et al., 1992), it was a particularly good candidate for inducing the generation of complex-dependent antibodies.

Initially, attempts were made to immunise mice with WLE suspended in CFA/IFA; however, this was found to be ineffective in inducing an immune response (data not shown). Instead, an alternative approach was taken, in which WLE was incorporated into liposomes in place of the glycolipids. Both GalNAc T-/- and

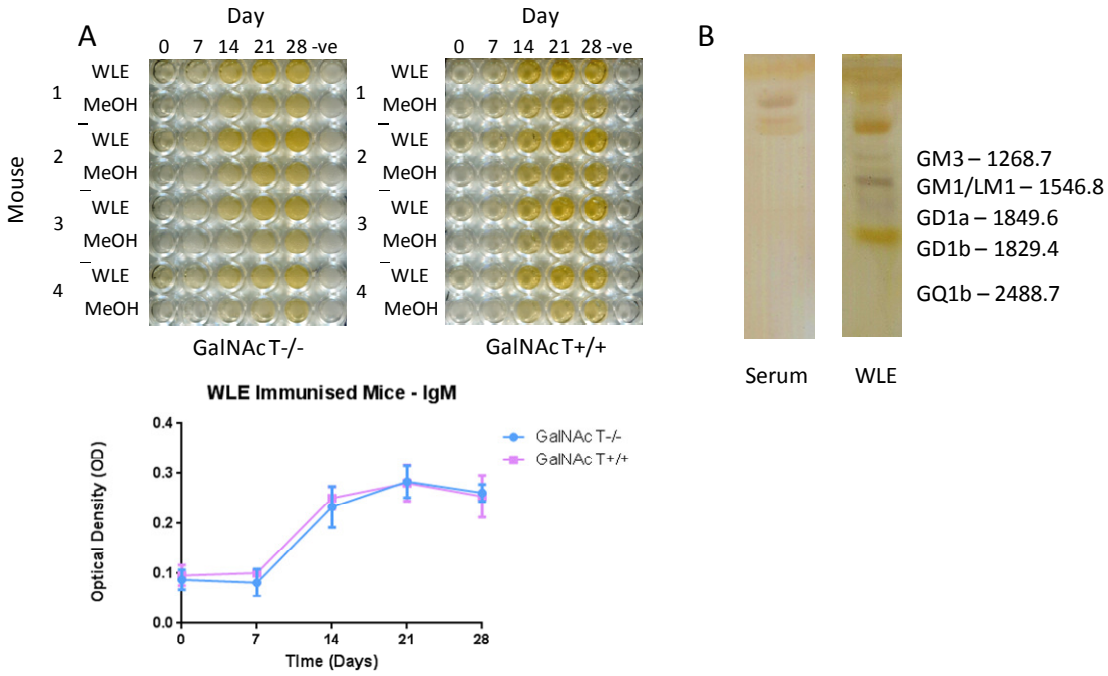


Figure 6.15 – WLE Immunised Mice

GalNAc T-/- and GalNAc T+/+ mice were immunised with liposomes containing WLE over a 4 week peiod. A: An ELISA was performed to analyse the development of the immune response over the immunisation cycle. The response rose steadily in both genotypes to a peak OD of approximately 0.3 at day 21. There was no difference between genotypes. B: TLC was performed on the WLE alongside ganglioside markers to determine its composition. An immuno-overlay was performed with the serum of the immunised mice, which determined that the antibody was not binding to one of the gangliosides screened.

GalNAc T+/+ mice were immunised with these liposomes over a 4 week period as per Section 2.7.1.

During the immunisation cycle, thin layer chromatography (TLC) was performed on the WLE and a series of ganglioside markers, to establish its composition and determine which antigens to include in the serum screening (Figure 6.15). As predicted from the literature, WLE contained GM3, GD3, GD1a, GD1b, and GM1/LM1 (Ogawa-Goto et al., 1992). Unfortunately, due to their identical molecular weights, it was not possible to distinguish between GM1 and LM1 using this method, so both lipids were included in the serum screening.

After 4 immunisations, the serum from the mice was screened against the panel of aforementioned gangliosides, Chol, DCP, SM, WLE and associated complexes. Unusually, neither genotype had a detectable antibody response against the ganglioside antigens; however, IgM antibodies were detected to WLE, suggesting that the mice were producing an antibody that was targeting a lipid not included in the initial screening.

Analysis of the serum responses over the immunisation period appeared to correlate well with this theory, as there was no distinguishable difference between the mouse genotypes suggesting that the mice were not generating

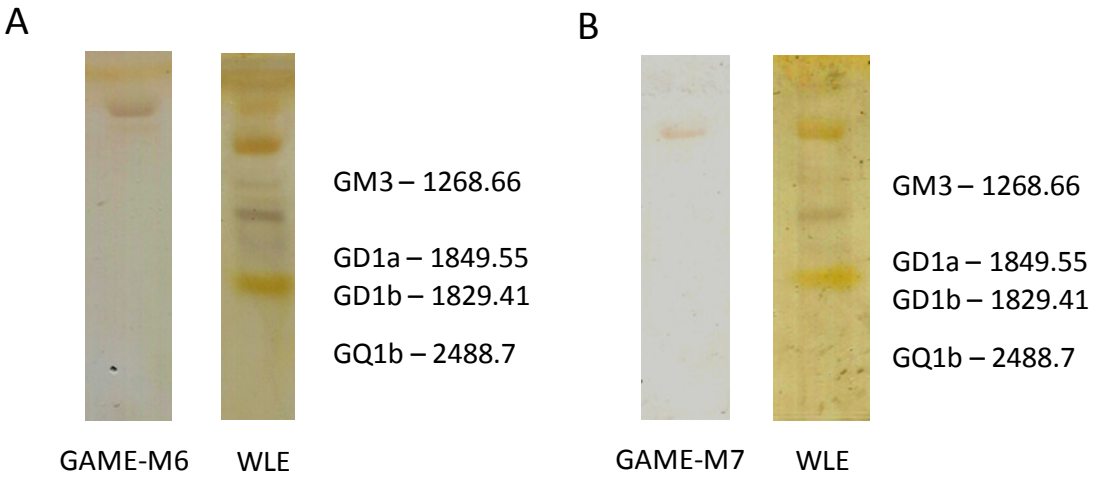


Figure 6.16 – GAME-M6 and GAME-M7 TLC immuo-overlay

A TLC immuno-overlay was performed with both GAME-M6 and GAME-M7. The antibodies appeared to bind to a lipid that had a smaller molecular weight that all of the ganglioside markers.

antibodies against a complex ganglioside (Figure 6.16). Further analysis by TLC immuno-overlay proved that the serum antibodies were in fact binding to another lipid that had a smaller molecular weight than the ganglioside markers.

As the investigations into the antigens continued, the four mice with the highest antibody responses were fused with a myeloma cell line to produce a hybridoma as per the standard protocol. Following two weeks of incubation, the cell supernatant was screened by ELISA against WLE instead of a specific antigen. A series of wells with ODs above 0.5 were expanded and rescreened after a further two weeks, resulting in the production of two stable hybridoma cell lines, dubbed GAME-M6 and GAME-M7.

In an attempt to establish which antigen the monoclonal antibodies were binding, a further TLC immuno-overlay experiment was performed (Figure 6.16). The results from this experiment correlated with those from the serum TLC immuno-overlay, confirming that the antibodies were not binding to a ganglioside. Rather than screen the antibodies against a series of non-gangliosides lipids, the decision was taken to perform mass spectrometry on the lipid band to determine its composition. A colleague carried out this experiment and found that the antibodies were binding to sulfatide, which was confirmed through ELISA and combinatorial glycoarray. Further characterisation of these antibodies was carried out in both Chapters 8 and 9.

6.3 Discussion

Antibodies raised against neo-epitopes composed of ganglioside complexes are a relatively new concept in autoimmune neuropathies. They were first speculated to exist when antibodies from GBS patients were found to preferentially bind to dimers composed of different gangliosides (Kaida et al., 2004). Since this initial discovery, a vast amount of research has been performed to try and identify potential targets of these antibodies, their pathogenic roles and their tissue binding capabilities (Créange et al., 2014; Kaida et al., 2007; Notturno et al., 2009).

Little research has been performed, however, to clone these antibodies, which is essential to establish what roles, if any, they have in disease. This chapter

explored the attempts to produce one of these antibodies in mice though immunisations with ganglioside and WLE liposomes.

This method is well established and has been used in the past to induce antibody production *in vivo* (Bowes et al., 2002). In this experiment it was possible to produce a variety of anti-ganglioside antibodies in serum, but no antibodies were detected, under these conditions, which were purely complex dependent.

The data from the GM1:GalC immunisations suggested that an anti-complex antibody had been created but further analysis, using an array of GM1:GalC complexes with differing ratios of GalC, implied that this response was most likely due to preferential presentation of the epitope. This was confirmed by the use of mouse monoclonal antibodies, which displayed the same behaviour, and was further supported by the data in Chapter 4.

It is still possible that a GM1:GalC specific antibody exists; however, induction of such an antibody would require a precisely designed epitope. Other researchers have shown that dimers can be produced by fusing the oligosaccharide tails of two different gangliosides (Mauri et al., 2012). These dimers represent a true complex; however, by artificially changing the way they interact the researchers may have inadvertently modified their natural behaviour. The combination of the oligosaccharide tails may have added a level of rigidity to the complex, which would prevent the normal flexibility of the headgroups and thus modify the ability of the gangliosides to interact with other lipids.

Aside from the potential issues with the structure of these complexes, they did consist of a guaranteed 1:1 dimer, which is a feature that cannot be attributed with those created through liposomes. The method of creating the liposomes involved mixing the components together simultaneously, which may result in any combination of lipids. There is no evidence to support that the desired complex has formed or that the constituent lipids are even in proximity to one another. This may explain why no complex dependent antibodies were identified.

The immunisations themselves yielded new information in regards to the development of the antibody response. The differences between the GalNAc T+/+ and GalNAc T-/- mice have often been attributed to tolerance (Lunn et al., 2000). The GalNAc T-/- mice are antigen naive as they have never been exposed

to gangliosides whereas the GalNAc T+/+ are exposed endogenously. This is often purported as the reason that the GalNAc T-/- mice have such a high antibody response as they do not recognise the antigen.

The double KO axonal rescue raised questions about this assumption as the hybridoma fusion indicated that anti-GM1 antibodies were being generated. There was no response in the serum, however, which may suggest that the antibodies were being degraded or absorbed. There could be a similar response in the GalNAc T+/+ mice where antibodies were being produced at similar levels to the GalNAc T-/- mice but were being removed from the serum. This would result in lower levels of circulating antibodies giving the impression that there was a lower immune response.

This hypothesis has been strongly supported by the work of a colleague who has shown that antibody internalisation is one of the main mechanisms for the removal of antibodies from serum (M Cunningham, Unpublished Data). The consequences of this work suggest that the antibody repertoire in the serum may not be representative of the immune response, which may have impacts on further immunisation studies.

Although the desired outcome of anti-complex antibodies did not come to fruition, the development of the hybridoma supernatant screening technique proved beneficial for future studies. The lipid microarray was an optimal tool in establishing a high throughput screening method that could quickly identify a large variety of different reactivities. It was also essential for determining the presence of complex dependent antibodies and was vastly superior to ELISA in achieving this goal.

The benefits of this technique included: the identification of antibodies with different targets; the ability to screen for different antibody isotypes simultaneously; reduced false positives as the background signal was subtracted; and the characterisation of an antibody and its potential targets.

Although no anti-complex antibodies were identified, the technique did prove useful in identifying complex independent antibodies. In the GM1:sulfatide immunised mice, it allowed for the selection of two monoclonal antibodies that had different lipid targets. This would not have been possible using ELISA as the growth rate of the cells would not have permitted the supernatant to be

screened twice. The resultant monoclonal antibodies produced from these experiments are characterised in Chapter 7.

7 CHARACTERISATION OF ANTI-GANGLIOISDE ANTIBODIES

7.1 Introduction

The liposome immunisations performed in Chapter 6 yielded several monoclonal antibodies. These were dubbed GAME-M1, GAME-M3, GAME-M4, and GAME-G2 and were found to predominantly bind to GD1a, GM1, SM, and GM1 respectively. Although an initial screening was performed by ELISA and microarray, the antibodies required a more robust characterisation to determine their pathogenicity and whether they would be useful in future studies. This chapter explores this characterisation through analysis of the antibodies binding behaviours to solid phase assays and live tissue preparations.

7.2 Results

7.2.1 Combinatorial Glycoarray

Although the main targets of the antibodies had been ascertained through the initial screening process, a more thorough characterisation was required to identify their fine specificities. This was carried out by probing the antibodies against various ganglioside and glycolipid targets printed using the combinatorial glycoarray.

7.2.1.1 GAME-M1

GAME-M1 was derived from a GalNAc T-/- mouse that was immunised with GM1:GD1a liposomes. On ELISA, the antibody had been shown to bind to GD1a and GD1a:GM1 complexes but on the combinatorial glycoarray it was found to bind to other glycolipid targets (Figure 7.1).

These included GT1b, sulfatide and associated complexes. As per the original screening, the antibody bound primarily to GD1a and produced equivalent signals with all complexes containing the ganglioside. GT1b binding remained stable

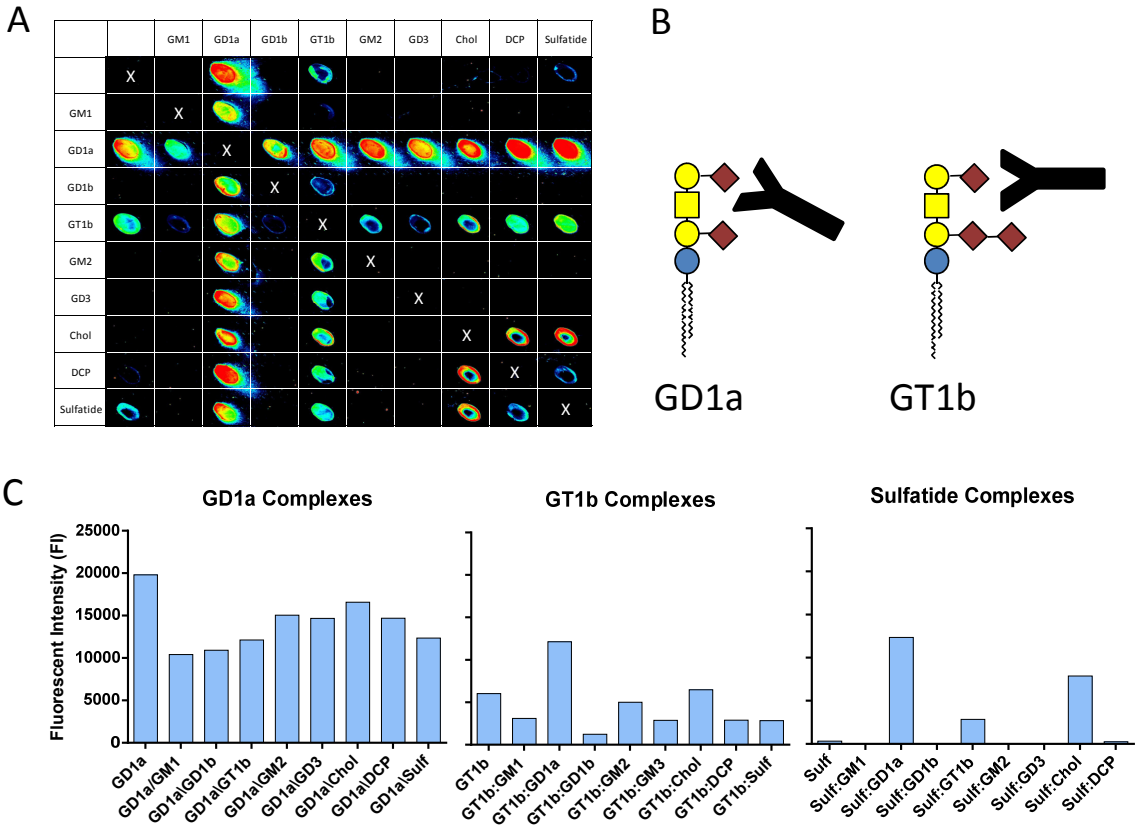


Figure 7.1 - GAME-M1 Characterisation on Combinatorial Glycoarray

A: A representative blot indicates the binding pattern of the antibody to various lipids and complexes. GAME-M1 was found to bind predominantly to GD1a, GT1b, sulfatide and associated complexes. B: Hypothetical binding of the antibody to the different gangliosides based upon its relative binding intensities. The antibody is likely to bind to the terminal sialic acid, common to GD1a and GT1b. C: Graphs of the raw data indicated the antibody's binding signals. GAME-M1 binds predominantly to GD1a with equivalent signals found against GD1a complexes. It also weakly binds to GT1b and most GT1b complexes, although it is slightly inhibited by GM1 and GD1b. Very weak binding was detected against sulfatide. All sulfatide complex binding was inhibited by gangliosides except those to which the antibody bound in their own right (GD1a and GT1b).

with most complexes except GM1 and GD1b, which slightly inhibited the signals. Sulfatide binding was inhibited by all gangliosides except those to which the antibody bound in their own right, e.g. GD1a and GT1b.

From this data it was concluded that the antibody preferentially bound to the terminal sialic acid, common to both GD1a and GT1b, that is absent from the other gangliosides screened. The weak sulfatide binding was attributed to the sulfatide specific binding domain that is common to many anti-carbohydrate antibodies (Alving, 1986; Townson, Greenshields, et al., 2007).

7.2.1.2 GAME-M3

GAME-M3 was derived from a GalNAc T-/- mouse that was immunised with GM1:sulfatide liposomes. It was partially characterised during the hybridoma supernatant screening but a more thorough characterisation was performed on the combinatorial glycoarray, to further establish its binding pattern (Figure 7.2).

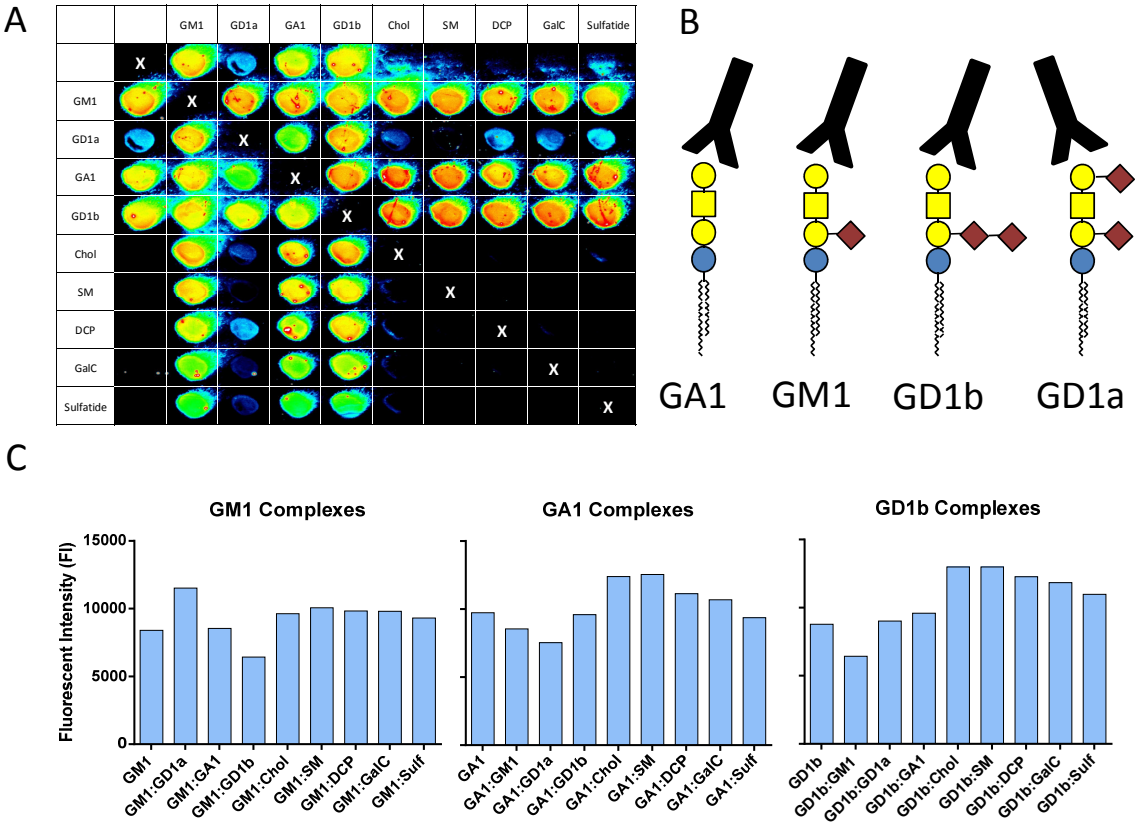


Figure 7.2 - GAME-M3 Characterisation on Combinatorial Glycoarray

A: A representative blot indicates the binding pattern of the antibody to various lipids and complexes. Strong binding was detected to both single targets and complexes composed of GM1, GA1 and GD1b with smaller signals detected against GD1a and sulfatide. B: Hypothetical binding of the antibody to the different gangliosides based upon its relative binding intensities. The antibody is likely to bind to the terminal galactose of the ganglioside headgroup. C: Graphs of the raw data indicating the antibody's binding signals. GAME-M3 binds to the single GM1, GA1, and GD1b epitopes and weakly to GD1a and sulfatide. GM1, GA1 and GD1b display similar binding signals and are all slightly enhanced with complexes containing accessory lipids. GD1a binding is weak and is inhibited in the presence of SM. All sulfatide complex binding was inhibited by gangliosides except those to which the antibody bound in their own right.

The data from the array indicated that the antibody bound to GM1, GA1 and GD1b with similar values. The only complexes that appeared to modify these binding signals were those containing accessory lipids, which were found to have a slightly enhancing effect. In addition to these three gangliosides, weaker binding signals were also detected to GD1a and sulfatide. All GD1a complexes showed similar values as the single target antigen except GD1a:SM, which inhibited antibody binding. In contrast, sulfatide binding signals were completely abolished by all lipids except those to which the antibody bound in their own right.

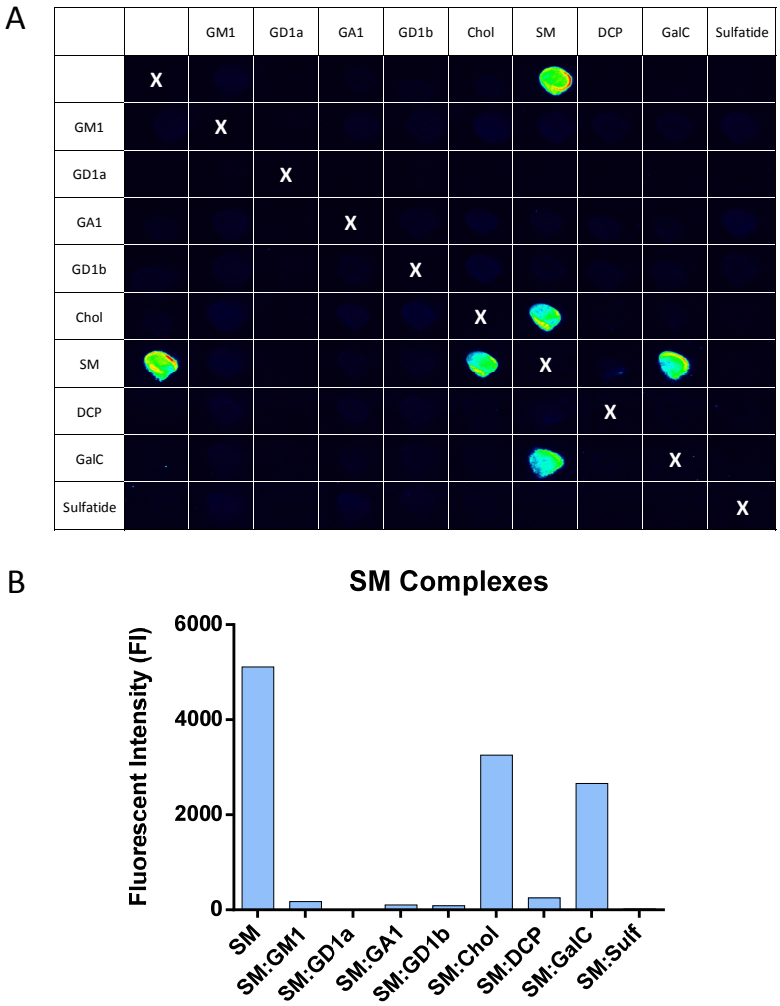


Figure 7.3 - GAME-M4 Characterisation on Combinatorial Glycoarray

A: A representative blot indicates the binding pattern of GAME-M4 to various lipids and complexes. Binding was only detected to SM, SM:Chol and SM:GalC. Graphs of the raw data indicated that high binding signals were detected against SM with lower signals detected against SM:Chol and SM:GalC complexes. No binding was detected against any other lipids or SM complexes.

From this data it was concluded that GAME-M3 was likely binding to the terminal galactose that was common to all four gangliosides; however, it was also possible that it was binding to another non-sialyated component of the ganglioside headgroup. The binding to the single sulfatide antigen was, again, likely to be due to the sulfatide binding domain common to many anti-carbohydrate antibodies (Alving, 1986; Townson, Greenshields, et al., 2007).

7.2.1.3 GAME-M4

GAME-M4 was derived from a GalNAc T-/- mouse that was immunised with GM1-sulfatide liposomes. As with the previous antibodies, it was partially characterised during the hybridoma supernatant screening but a more thorough characterisation was performed to further establish its binding pattern (Figure 7.3).

The results indicated that the antibody had a very restricted binding fingerprint. It only bound to the single SM epitope and SM complexes containing either Chol or GalC. Binding was completely abolished in complexes composed of any gangliosides, DCP or sulfatide.

7.2.1.4 GAME-G2

GAME-G2 was derived from a GalNAc T-/- mouse that was immunised with GM1:GD1a liposomes. The lipid microarray screening determined that the antibody primarily targeted GM1 but the combinatorial glycoarray screening established that the antibody also bound to other antigens (Figure 7.4).

These included GD1b and sulfatide. GM1 binding was found to be enhanced with complexes containing Chol, SM, DCP, and sulfatide. A similar pattern was found with GD1b although the binding signals were much lower than those found with GM1. Both GM1:GD1a and GD1b:GD1a complexes inhibited the binding of GAME-G2.

The antibody also produced a minor binding signal against sulfatide, which was abolished when sulfatide was in complex with most other lipids. The only exceptions were complexes containing gangliosides that were known targets for the antibody (GM1 and GD1b) and unusually GA1:sulfatide.

As no binding was detected to single GA1, it was concluded that the antibody preferentially bound to the internal sialic acid (Neu5Acα3) that is common to both GM1 and GD1b. The binding signal that was detected against sulfatide was, again, assumed to be the sulfatide binding pocket that was found with the other antibodies (Alving, 1986; Townson, Greenshields, et al., 2007).

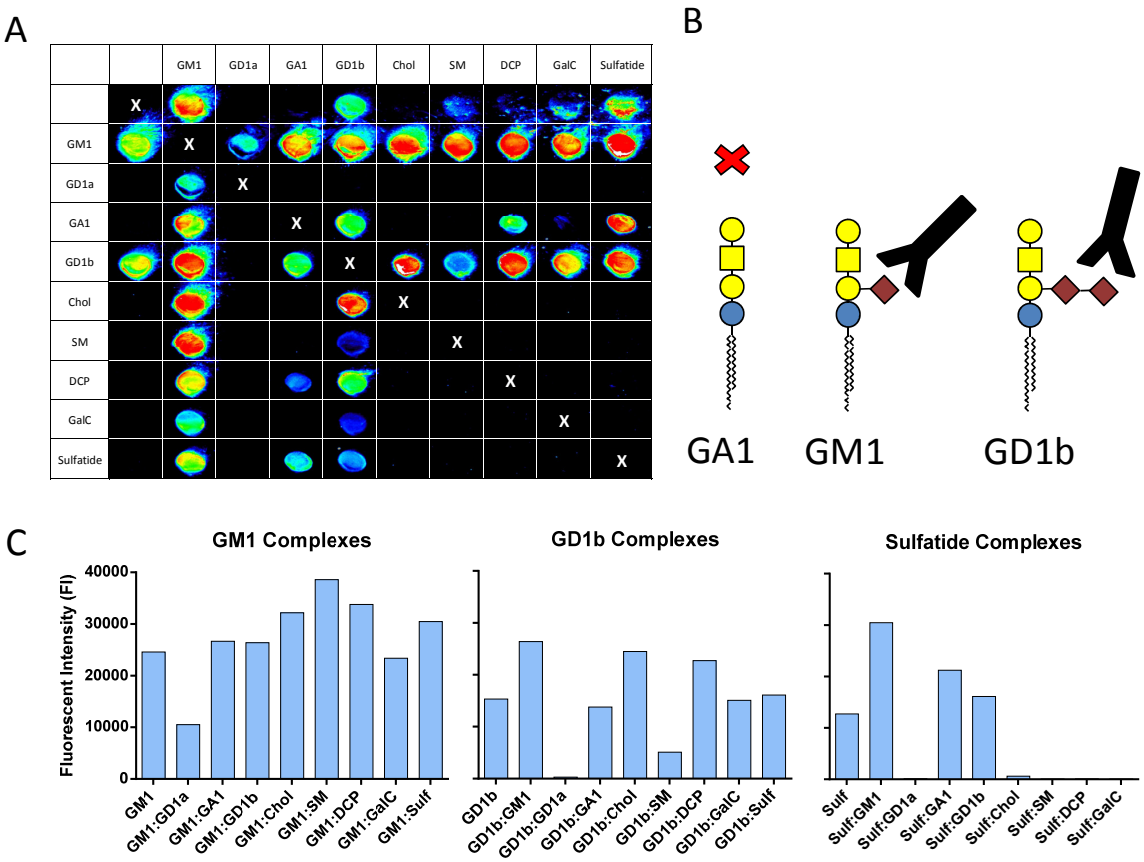


Figure 7.4 - GAME-G2 Characterisation on Combinatorial Glycoarray

A: A representative blot indicates the binding pattern of the antibody to various lipids and complexes. GAME-G2 binds to GM1, GD1b and sulfatide. B: Hypothetical binding of the antibodies to the different gangliosides based upon their relative intensities. The antibody likely binds to the internal sialic acid (Neu5Acα3). C: Graphs of the raw data indicating the antibody's binding signals. GAME-G2 binds to the single GM1 and GD1b epitopes and weakly to sulfatide. There is enhancement with GM1 and GD1b complexes that contain Chol, SM, DCP and sulfatide. GM1:GD1a and GD1b:GD1a complexes inhibit antibody binding. Sulfatide binding is weak and is only present to the single epitope or complexes containing the positive binding gangliosides.

7.2.2 Tissue Characterisation

Following on from the array characterisation, the antibodies were probed against tissue to determine their binding capabilities to biological environments.To best achieve this aim, the decision was made to use *ex vivo* preparations of mouse TS. This allowed the antibody to be viewed under physiological conditions, unlike sections, which would involve staining pre-fixed tissue. The TS was selected as it contains an abundance of easily visualised NMJs, which are known to be a rich source of gangliosides (McArdle et al., 1981).

7.2.2.1 GAME-M1

In order to ascertain the specific targets of GAME-M1 in tissue, a variety of different mouse genotypes were probed to determine whether binding was taking place. Initial tests were performed with tissue from a WT mouse, which was incubated at two different temperatures (4°C or 32°C) to establish the optimal conditions for antibody detection. Once the experiment was complete, the tissues were imaged and the resultant binding signals overlying the NMJ were quantitated. The same tissues were then permeabilised and reimaged to determine if any antibody had been internalised (Figure 7.5).

The binding signals in the unpermeabilised WT tissue were similarly low between the 4°C and 32°C experiments after 160 mins. However, following permeabilisation with 0.5% Triton X-100, the binding signals increased substantially for both temperatures. The median signal in the 4°C experiment rose to 162 AU, whilst in the 32°C experiment it rose to 25 AU. This suggested that the antibody was being readily internalised in the WT tissue at both temperatures, although it appeared that 4°C was optimal for antibody detection.

In addition to NMJ binding, it was also noted that the antibody bound well to the Schwann cell plasmalemma. This binding was found to overly the CFP positive axon and extended from the nerve trunk to the terminal heminode (Figure 7.6).Unfortunately, due to the nature of this binding, it was not possible to quantitate these results; however, it was observed that the signals appeared to be more intense following permeabilisation (data not shown)

Having now established that GAME-M1 was capable of binding to live tissue, an

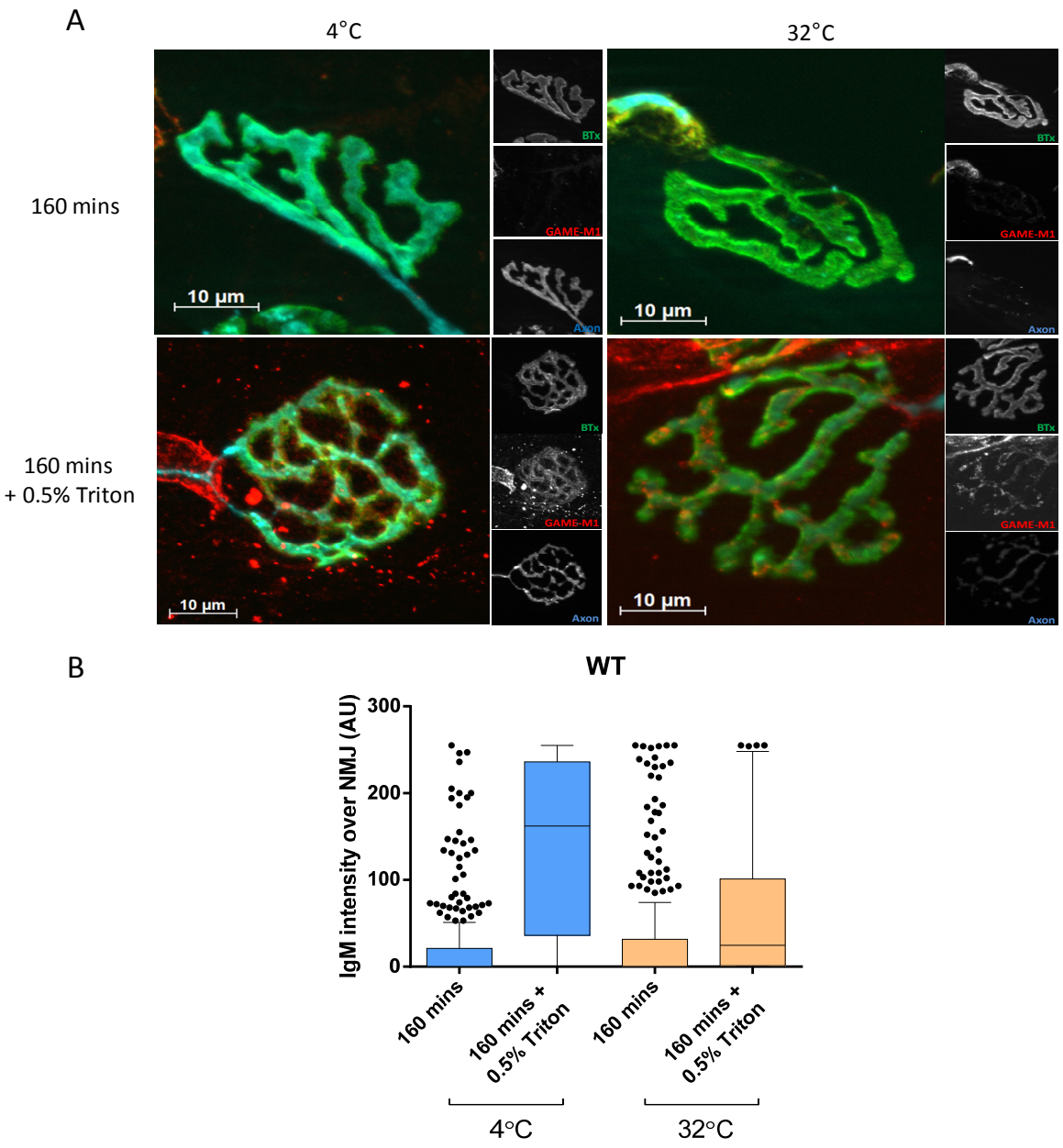


Figure 7.5 - WT Tissue Characterisation of GAME-M1

Ex vivo WT TS preparations were probed with GAME-M1. The tissue was incubated at two different temperatures (4˚C and 32˚C) to determine the optimal binding conditions. It was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. The tissue was then permeabilised and reimaged to determine whether antibody was being internalised (n=2). A: Illustrative examples of GAME-M1 binding over the NMJ indicated higher binding in the permeabilised tissues at both temperatures. B: Box and whisker plots of fluorescent intensity indicated weak binding at both temperatures in the unpermeabilised tissue. These signals increased substantially following permeabilisation and were found to be particularly high in the 4°C treated tissue. In addition to binding at the NMJ, it was noted that GAME-M1 bound to the Schwann cell plasmalemma under all conditions.

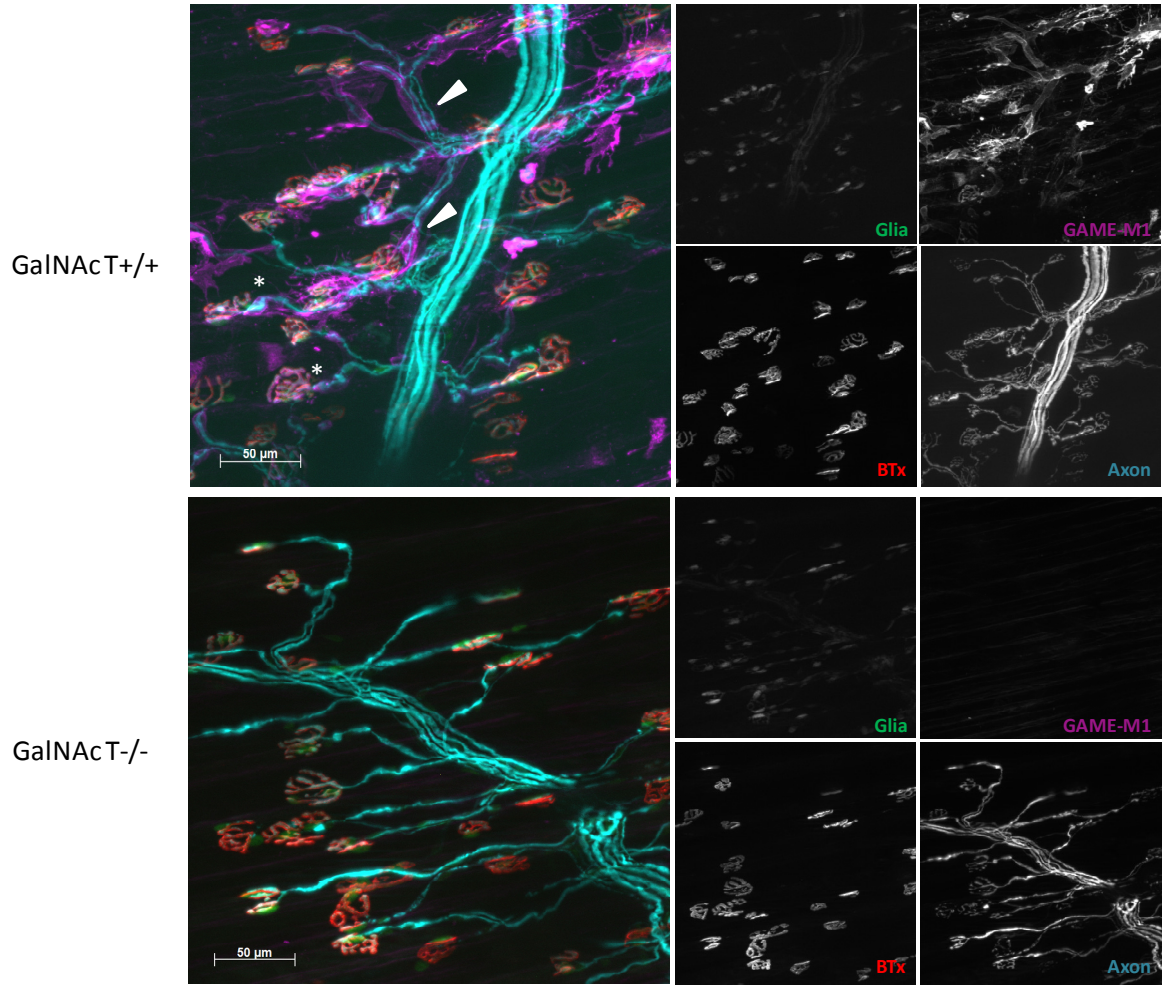


Figure 7.6- GalNAc T-/- Tissue Characterisation of GAME-M1

Ex vivo TS preparations from GalNAc T+/+ and GalNAc T-/- mice were probed with GAME-M1 and then permeabilised. In the GalNAc T+/+ tissue, the antibody bound strongly to the Schwann cell plasmalemma (arrows), which overlies the CFP positive axon. Binding was also detected over the NMJs (asterix). This was not replicated in the GalNAc T-/- tissue, which suggests that GAME-M1 is binding to complex gangliosides in these structures and not sulfatide.

additional experiment was performed to determine if the antibody was targeting complex gangliosides or sulfatide. This was achieved by probing GAME-M1 against tissue from a GalNAc T+/+ and a GalNAc T-/- mouse for 160 mins at 32°C, followed by permeabilisation (Figure 7.5). The results of this experiment indicated that no binding was present in the GalNAc T-/- tissue, whereas strong binding signals were detected on the Schwann cell plasmalemma and overlying the NMJs in the GalNAc T+/+ tissue (Figure 1.6). This suggested that GAME-M1 was binding to complex gangliosides on these structures and that no sulfatide

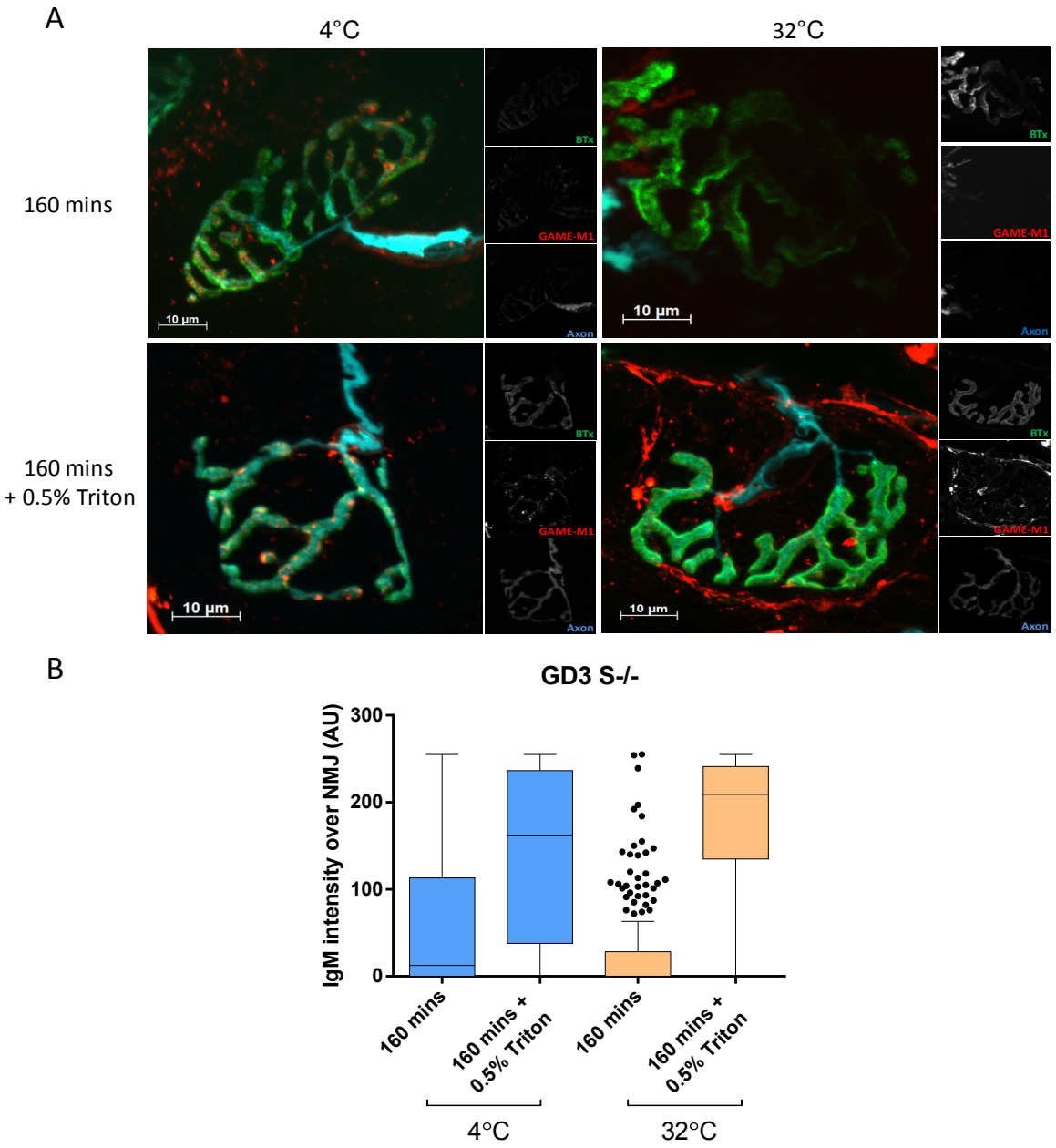


Figure 7.7 – GD3 -/- Tissue Characterisation of GAME-M1

Ex vivo GD3 S -/- TS preparations were probed with GAME-M1. The tissue was incubated at two different temperatures (4˚C and 32˚C) to determine the optimal binding conditions. It was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. The tissue was then permeabilised and reimaged to determine whether antibody was being internalised (n=2). A: Illustrative examples of GAME-M1 binding over the NMJ indicated higher binding in the permeabilised tissues. B: The quantitation results were graphed and indicated weak binding at both temperatures in the unpermeabilised tissue, although the 4˚C treated tissue was higher than that treated at 32˚C. Following permeabilisation, the binding signals substantially increased for both temperatures. This was higher in the 32˚C treated tissue. In addition to binding at the NMJ, it was also noted that GAME-M1 bound in differing degrees to the Schwann cell plasmalemma under all conditions.

binding was taking place.

Despite these findings, it was still unclear whether the antibody was targeting GD1a, GT1b or both in tissue. To investigate this further, another transgenic mouse (GD3 S-/-) was used, which lacks the ability to synthesise b-series gangliosides such as GT1b. As a consequence of this modification, the mouse overexpresses the a-series gangliosides such as GD1a, which made this tissue well suited for investigating this antibody's targets.

As with the WT tissue, the experiment was performed at two temperatures to determine the optimal conditions for antibody detection (Figure 7.7). All other settings remained the same, with the secondary antibody signal overlying the NMJ being quantitated, to determine the binding intensities in the different tissues. In the unpermeabilised tissue, incubation at 32°C produced similar binding to that found in the WT mice, whereas incubation at 4°C produced a higher binding signal of 13AU. The permeabilised tissue produced median signals of 209 AU and 162 AU in the 32°C and 4°C experiments respectively. The elevated binding signal in the 32°C treated tissue suggested that the higher temperature was increasing the biological activity of the membrane, which resulted in higher levels of antibody internalisation.

As the binding signals were higher in the GD3 S-/- tissue, it could be proposed that GAME-M1 primarily targets GD1a; however, it cannot be definitively stated that binding to GT1b does not also occur to a certain degree in the WT tissue.

7.2.2.2 Removal of Cryptic Epitope

It has previously been established that the in-house antibody – DG1 – is inhibited from binding GM1 when it is in complex with GD1a (Greenshields et al., 2009). This complex is so ubiquitously expressed in living membranes that the only way to achieve DG1 binding is to first treat tissue with neuraminidase, which removes the terminal sialic acid of GD1a and converts it to GM1. As GAME-M1 binds to GD1a, it was hypothesised that it may be able to disrupt the GD1a:GM1 complex

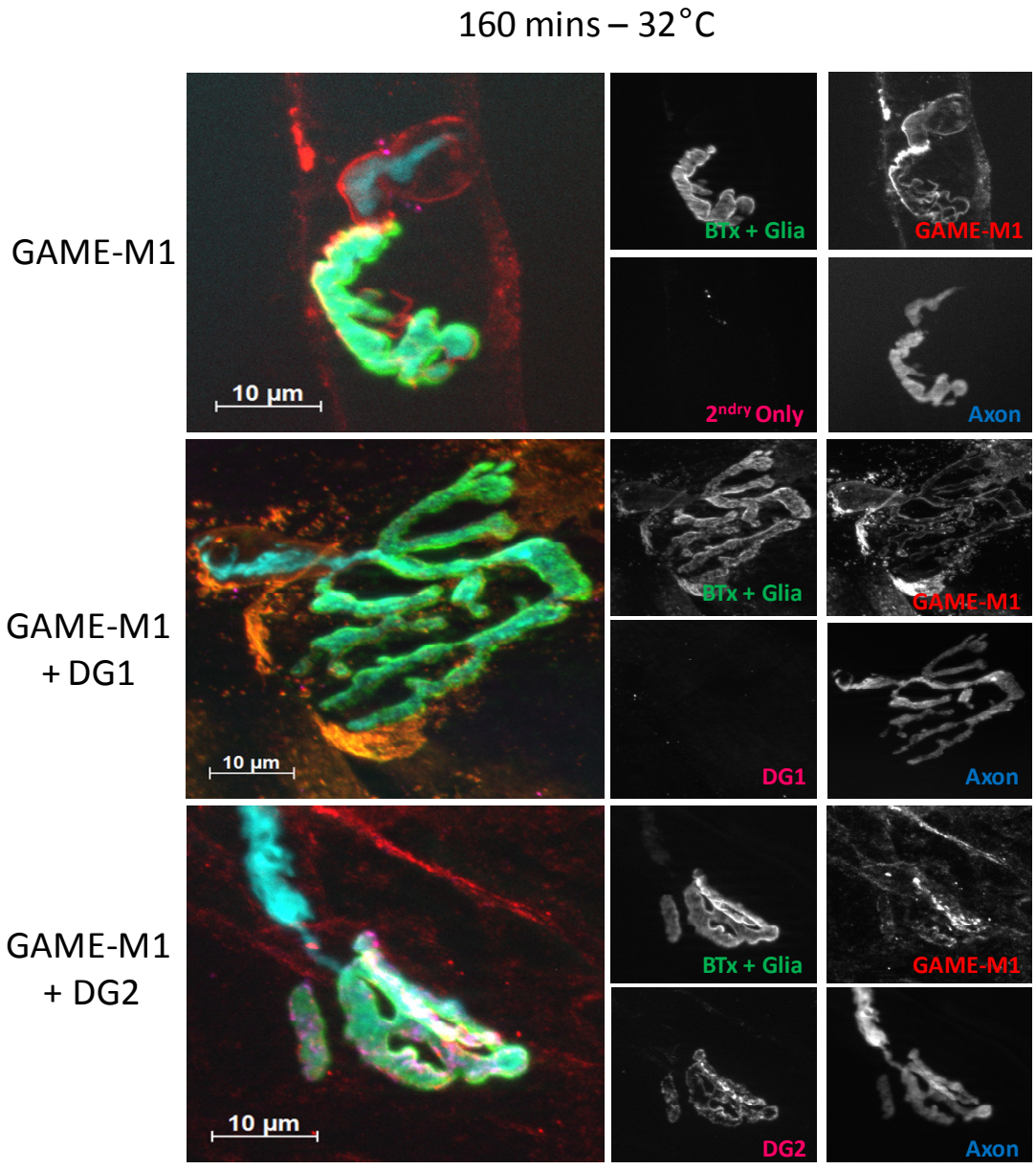


Figure 7.8 – DG1 does not bind nerve terminals incubated with GAME-M1

Ex vivo WT TS preparations were probed with GAME-M1. One preparation was also probed with DG1 to establish if the GM1 binding epitope had been revealed by GAME-M1. Another was probed with DG2 to prove that GM1 was present over the NMJ. No DG1 binding was detected but DG2 verified that GM1 was present at the NMJ.

interaction and free the GM1 epitope, which would become available for DG1 binding.

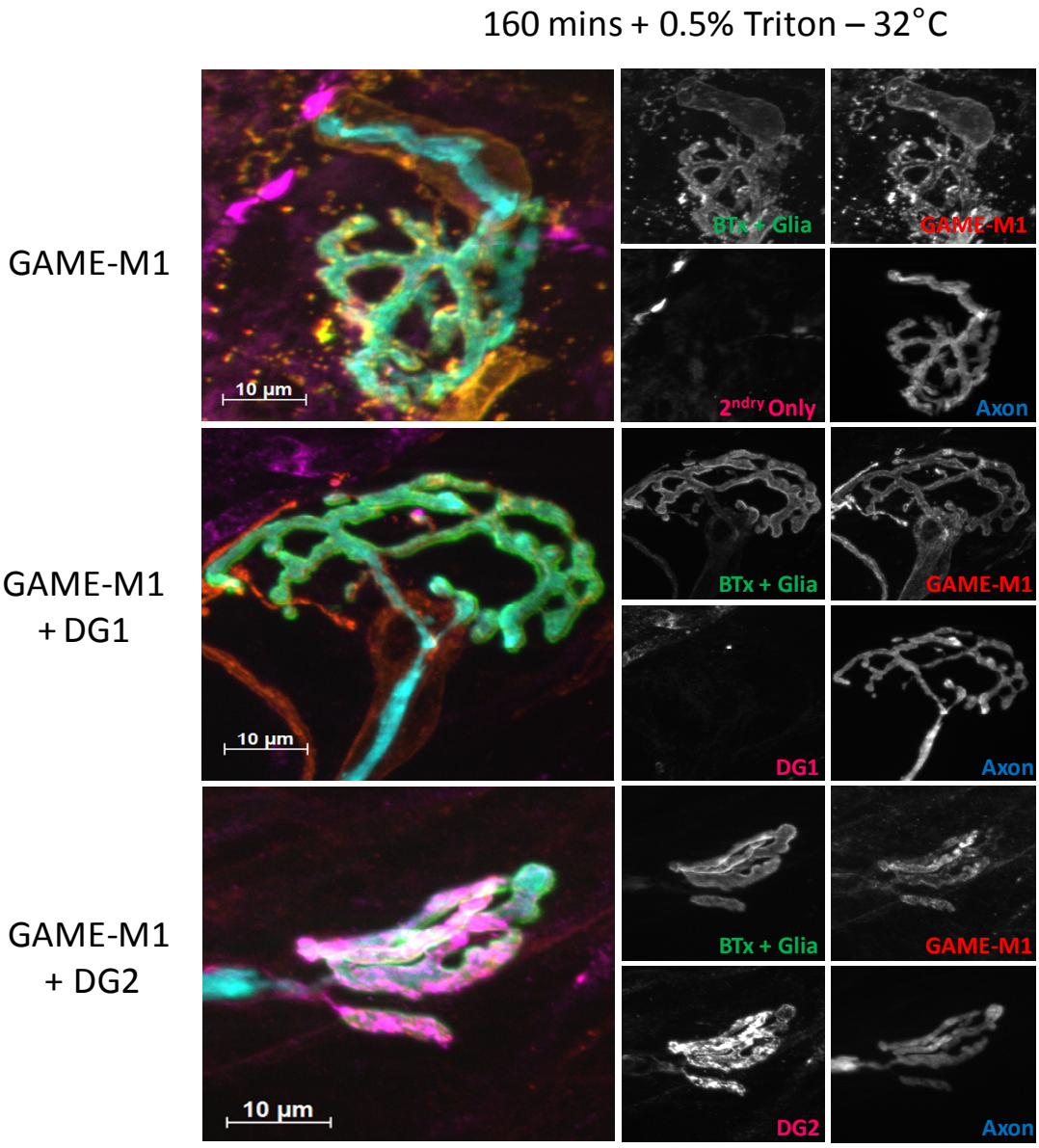


Figure 7.9 – DG1 does not bind nerve terminals incubated with GAME-M1 - Permeabilised

Ex vivo WT TS preparations were probed with GAME-M1 and then permeabilised. One preparation was also probed with DG1 to establish if the GM1 binding epitope had been revealed by GAME-M1. Another was probed with DG2 to prove that GM1 was present over the NMJ. No DG1 binding was detected but DG2 verified that GM1 was present at the NMJ.

To test this theory, tissue from WT mice were probed with GAME-M1 in the presence of DG1 (Figure 7.8). Other tissue was probed with the complex independent antibody,DG2, to confirm that GM1 was present at the terminal and available for binding. Under these conditions, the GM1:GD1a complex was not disrupted by GAME-M1 and DG1 binding was not detected.

For certainty, the tissue was permeabilised to ensure that DG1 had not bound and been internalised (Figure 7.9). As expected, the tissue was still negative for DG1 binding, which suggests that GAME-M1 does not modify the configuration of GD1a in the GM1:GD1a complex.

7.2.2.3 GAME-M3

In an effort to establish the specific targets of GAME-M3 in tissue, it was first screened against TS collected from GalNAc T-/- and GalNAc T+/+ mice. This would determine if the antibody was binding to complex gangliosides or to sulfatide.

Ex vivo preparations of TS were probed with 100µg/ml of antibody for 60 mins at 4°C, which pilot studies had indicated were the optimal conditions for antibody detection (data not shown). Following incubation, the tissues were imaged and the secondary binding signals overlying the NMJs were quantitated. The tissue was then permeabilised with 0.5% Triton X-100 and reimaged (Figure 7.10).

The data confirmed that the antibody was preferentially binding to complex gangliosides, with the GalNAc T+/+ tissue producing high median binding signals of 79 and 108 AU in the pre and post permeabilised tissues respectively. The unpermeabilised tissue was found to be significantly higher than the unpermeabilised GalNAc T-/- tissue which had a median value of 0 AU (Mann Whitney, $P<0.05$). The permeabilised GalNAc T-/- tissue produced a small level of binding with a median signal of 26 AU; however, this was much lower than the signals detected in the TS from the GalNAc T+/+ mice. This suggested that a small amount of GAME-M3 was binding to sulfatide, a simple ganglioside or another unknown antigen.

To determine which complex gangliosides GAME-M3 was binding in tissue, an experiment was devised using GD3 S-/- and GD3 S+/+ TS with and without N'ase treatment. The rationale behind this experiment was to alter the ganglioside expression in tissues and use the differences between binding signals to deduce which antigen was likely being targeted by the antibody. The purpose of the GD3 S-/- tissue was to eliminate GD1b as a target, whilst the use of the N'ase treatment was to remove the terminal sialic acid of GD1a and convert it to GM1.

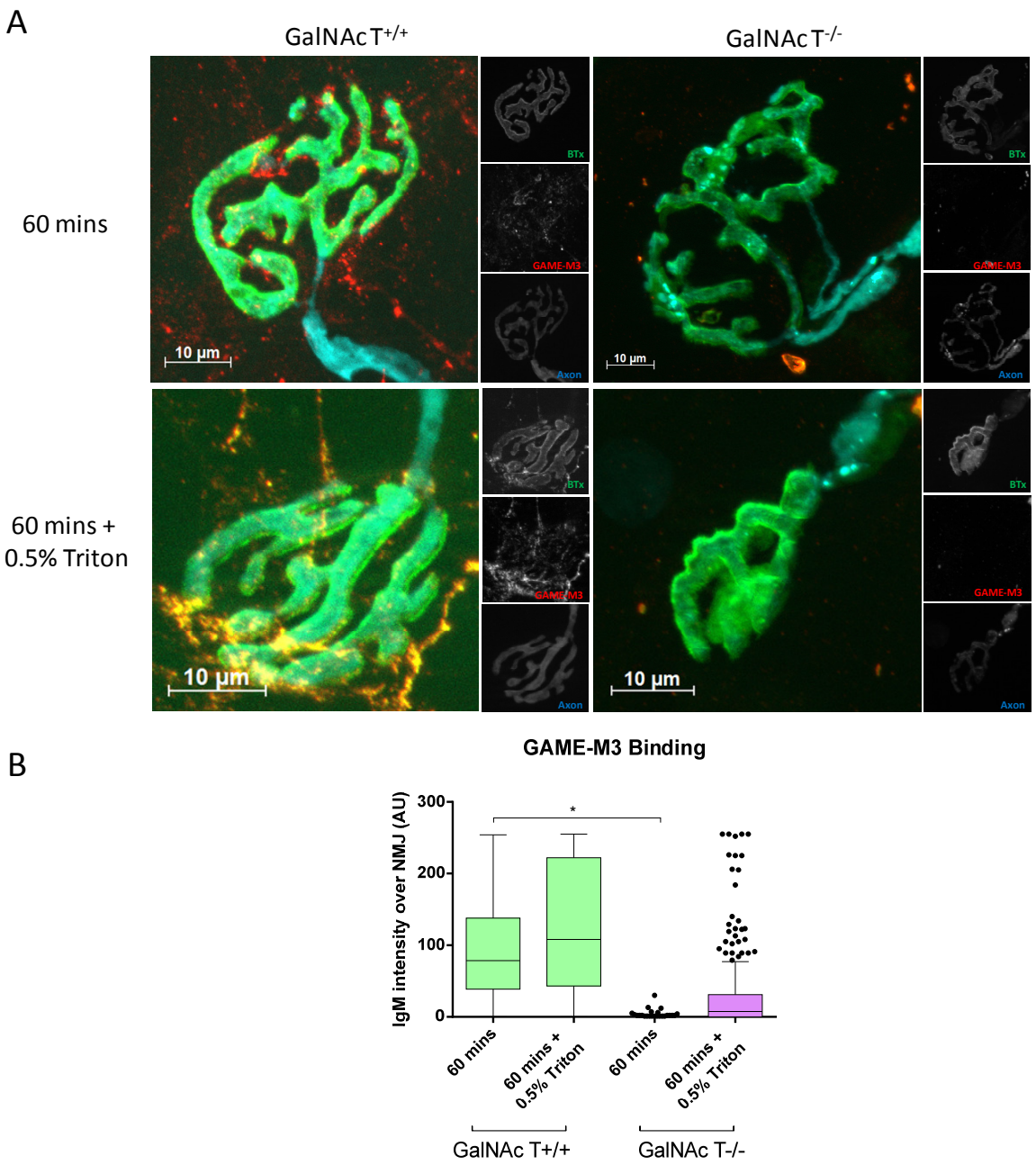


Figure 7.10 – GAME-M3 Binding at GalNAc T-/- and GalNAc T+/+ Mice Nerve Terminals

Ex vivo preparations of TS were probed with GAME-M3. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. The tissue was then permeabilised and reimaged to determine whether antibody was being internalised. A: Illustrative examples show high binding signals overlying the NMJ in the GalNAc T+/+ tissues. No binding is seen in the GalNAc T-/- tissue. B: High binding signals were found in the GalNAc T+/+ mice in both the pre and post permeabilised tissue. The unpermeabilised GalNAc T+/+ tissue was significantly higher than the unpermeabilised GalNAc T-/- tissue (Mann Whitney, $P<0.05$). Minor binding was detected in the permeabilised GalNAc T-/- tissue but this was much lower than the GalNAc T+/+ tissue (n=5).

The tissues were pre-treated with either N'ase or Ringer's solution and then probed with GAME-M3 for 60 mins at 4°C, as per the previous experiment. The results indicated that there was very little binding to the N'ase treated tissue in either genotype (Figure 7.10). Low binding signals were detected, however, in both the untreated GD3 S-/- and GD3 S+/+ tissue with the GD3 S-/- tissue showing the highest signal.

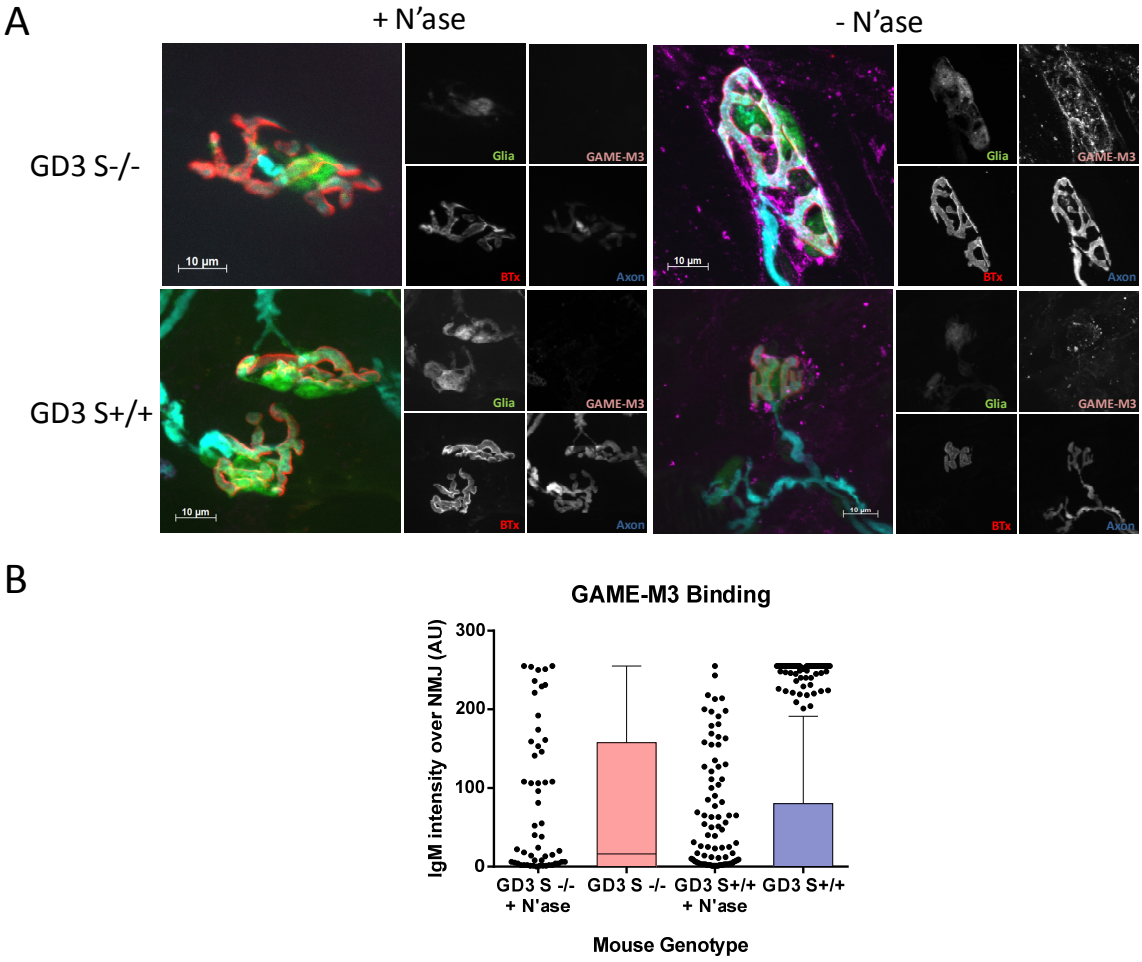


Figure 7.11 – GAME-M3 Binding in GD3 S-/- and GD3 S+/+ tissue with and without N'ase treatment.

Ex vivo preparations of TS from GD3 S-/- and GD3 S+/+ mice were probed with GAME-M3. Half the tissue had been pre-treated with N'ase whilst the other half only received Ringer's solution. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. A: Illustrative examples show binding overlying the NMJ in the untreated tissues whereas binding is absent in those treated with N'ase. B: The results indicated that low binding was detected in the GD3 S-/- and GD3 S+/+ tissues without N'ase, whilst binding was mostly absent from the N'ase treated tissue (n=5).

In the permeabilised tissue, binding increased in both the untreated GD3 S-/- and GD3 S+/+ tissue, which suggested that the antibody had been internalised (Figure 7.12). Binding was also detected in the N'ase treated GD3 S-/- tissue; however, no binding was detected in the treated GD3 S+/+ tissue.

Taken together, this data suggested that the antibody preferentially bound to GD1a in tissue, which was unexpected as it was not one of the main antigens on

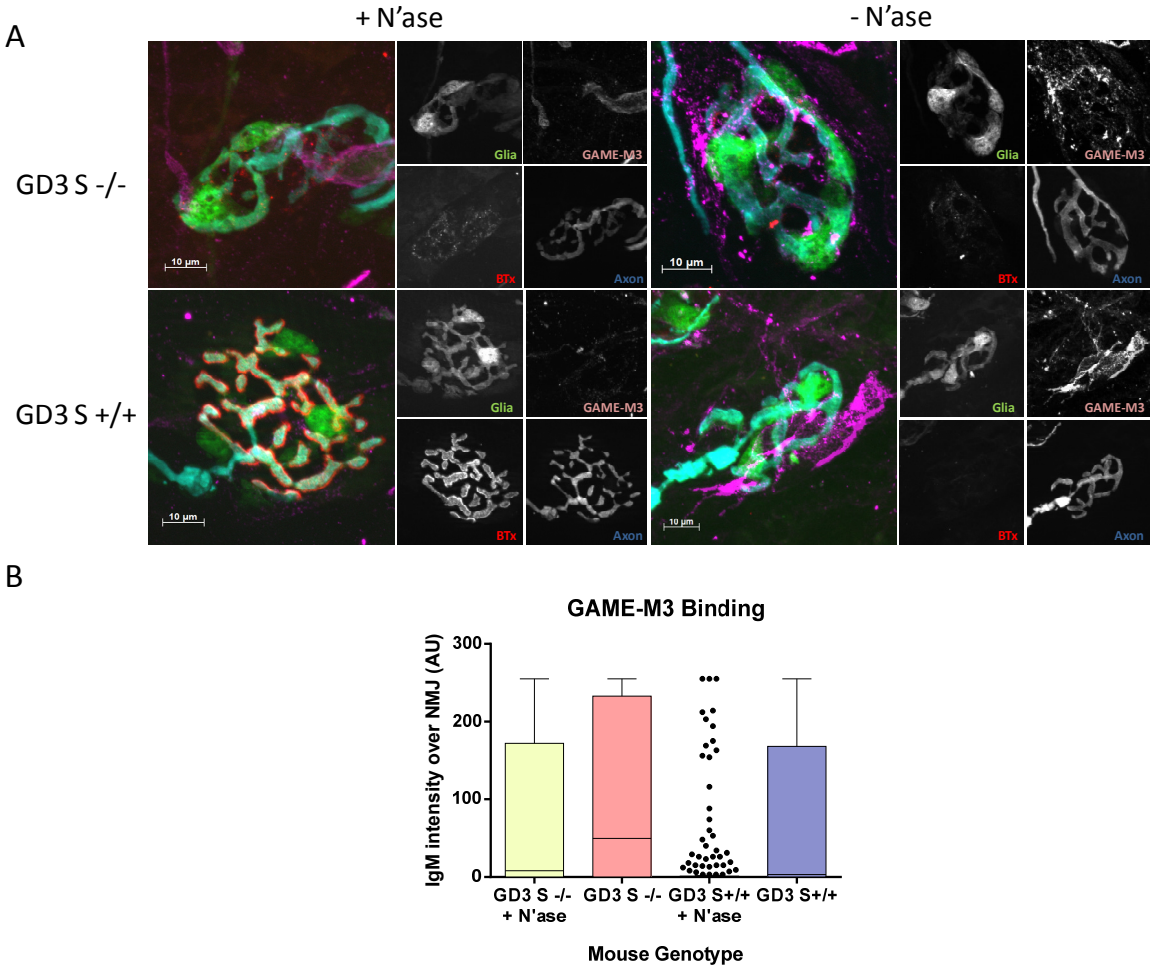


Figure 7.12 - GAME-M3 Binding in GD3 S-/- and GD3 S+/+ tissue with and without N'ase treatment - Permeabilised.

Ex vivo preparations of TS from GD3 S-/- and GD3 S+/+ mice were probed with GAME-M3 followed by permeabilisation. Half the tissue had been pre-treated with N'ase whilst the other half only received Ringer's solution. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. A: Illustrative examples indicate increased binding overlying the NMJ in untreated tissues. B: The results indicated that low binding was detected in the N'ase treated GD3 S-/- and untreated GD3 S+/+ tissues. A good signal was detected in the untreated GD3 S-/- tissue whilst very little binding was detected in the N'ase treated GD3 S+/+ (n=5).

array. There was still minor binding to GM1 as confirmed by the detection of antibody in the N'ase treated GD3 S-/- tissue, which lacked GD1a; however, this was surprisingly low considering the high expression of GM1 at the nerve terminal (Greenshields et al., 2009).

These unusual results could be explained if complex interactions were taken into account. It is well established that the presence of other lipids can modify the headgroup conformation of gangliosides and this can be influenced by the density of the molecules (Galban-Horcajo et al., 2014). On array it appears that GAME-M3 preferentially binds to the terminal galactose of gangliosides but it is not clear how this is presented in tissue. It is possible that in the membrane, the headgroup is kinked due to some unknown interaction, which makes it less accessible for antibody binding. GD1a contains a terminal sialic acid, which may counteract this effect and allow GAME-M3 to bind. Similarly, the signals detected in the N'ase treated GD3 S-/- tissue may be due to the overexpression of GM1, which is so high that it partially overcomes the complex attenuation of the ganglioside.

Further study would be needed to confirm this hypothesis but this acts as a good example of the differences between arrays and tissue and justifies why antibody characterisation must be carried out using both methods.

7.2.2.4 GAME-M4

The anti-SM antibody, GAME-M4, was probed against GalNAc T-/- and GalNAc T+/+ tissue to establish if it was capable of binding to a biological environment (Figure 7.13). As the array indicated that antibody binding was inhibited in the presence of gangliosides, it was thought that tissue from a GalNAc T-/- mouse would be optimal for detecting antibody staining.

Ex vivo preparations of TS were therefore probed with 100µg/ml of GAME-M4 for 60 mins at 4°C. Examination of the tissue suggested that no GAME-M4 binding took place at the nerve terminals or along the axon in either the GalNAc T+/+ or GalNAc T-/- TS. Background binding was seen, however, in all tissue but this did not appear to be localised to any particular structure and was therefore associated with non-specific binding. The tissue was permeabilised to clarify

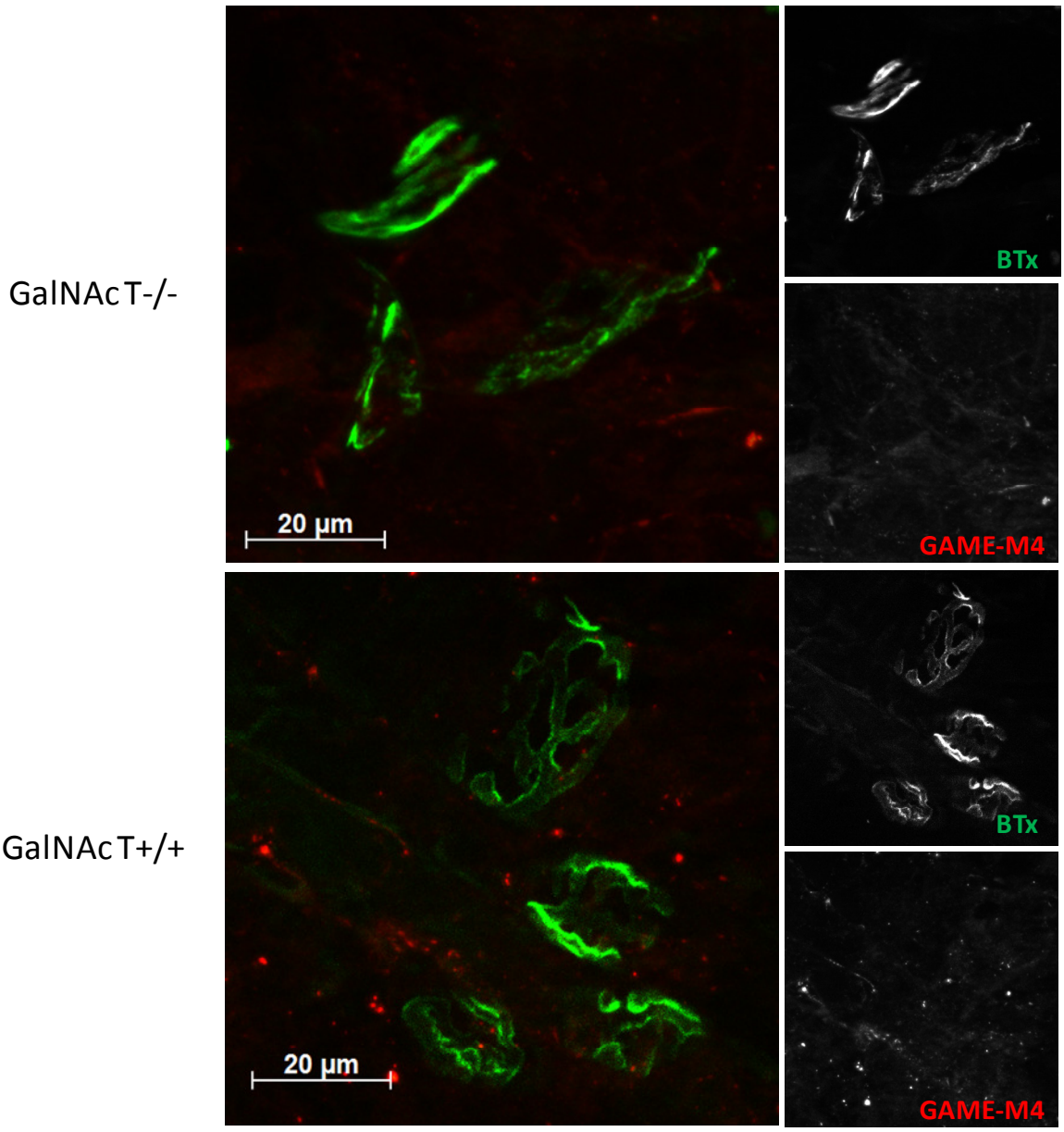


Figure 7.13 - GAME-M4 Binding in GalNAc T-/- and GalNAc T+/+ Mice

Ex vivo preparations of TS were probed with GAME-M4. Illustrative examples show that only background binding was detected in the GalNAc T-/- and the GalNAc T+/+ tissue. Quantitation was not performed due to the absence of a visual signal (n=5).

that the antibody had not been internalised but no binding signal was detected (Data Not Shown).

The lack of antibody detection was presumably due to the restrictive binding pattern of the antibody. Gangliosides and sulfatide are abundant in neural tissue and presumably form complexes with SM when they are co-localised. This severely reduces the availability of the epitope for GAME-M4 to bind, which may explain why no signal was detected.

It may be worthwhile in future studies, however, to incubate nerve sections with GAME-M4 to ensure that the lack of staining was not due to problems with antibody penetration. In addition, it may also be beneficial to co-stain for a myelin marker, to definitively prove that no staining was present along any myelinated structures in whole mount tissue.

7.2.2.5 GAME-G2

The anti-GM1/GD1b/sulfatide antibody, GAME-G2, was probed against TS from GalNAcT- /- and GalNAc T+/+ mice to determine if the antibody was binding to complex gangliosides or sulfatide (Figure 7.14). Analysis of the data, however, indicated that the antibody bound poorly to both permeabilised and unpermeabilised tissue from both genotypes. There was very low binding in the GalNAc T-/- TS; however, the background level of antibody was relatively high in some of these tissues, which may have skewed the results.

Due to the poor binding signals in the GalNAc T+/+ tissue, the antibody was probed against GD3 S+/+ and GD3 S-/- tissue with and without N'ase treatment to determine if any binding could be detected with enhanced ganglioside expression.

There was very little binding in the unpermeabilised tissue regardless of N'ase treatment (Figure 7.15). The only tissue which produced a visible signal was the N'ase treated GD3 S-/- TS but even this signal was relatively low. In the post-permeabilised experiment the binding signals increased for all the tissues, although their values were still low (Figure 7.16). The binding was again highest in the N'ase treated GD3 S-/- tissue which had a median signal of 37AU.

As this tissue lacked GD1a, it suggested that GD1a inhibition was preventing the binding of GAME-G2 in WT tissue. This was substantiated by the findings of the combinatorial glycoarray screening, which indicated that the presence of GD1a in complexes inhibited the binding signal of the antibody.

Even with N'ase treatment, the binding signals were still low, which suggested that the antibody may have had trouble accessing its epitope. This may be due to its location on the ganglioside, which is in a lower portion of the headgroup

and thus less freely available for binding. Further experiments would need to be performed, however, to test this theory.

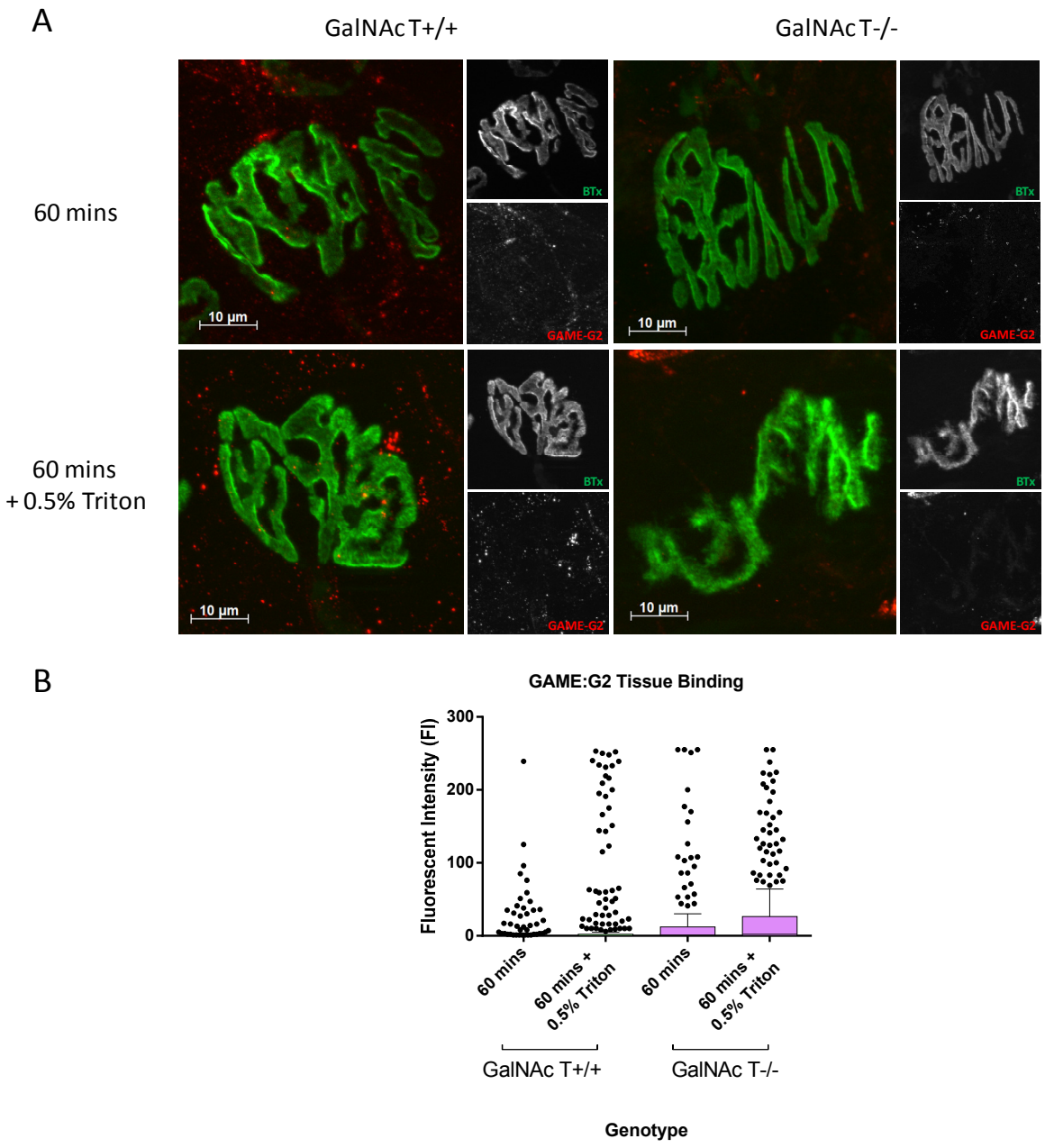


Figure 7.14 - GAME-G2 Binding in GalNAc T-/- and GalNAc T+/+ Mice

Ex vivo preparations of TS were probed with GAME-G2. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. The tissue was then permeabilised and reimaged to determine whether antibody was being internalised. A: Illustrative example of absent GAME-G2 binding over the NMJ in all tissues. B: Box and whisker plots were created of the fluorescent secondary binding signals overlying the NMJ. There were poor binding in both the GalNAc T-/- and GalNAc T+/+ tissue pre and post permeabilisation. Very minor binding was detected in the GalNAc T-/- tissue; however, this may have been caused by high background binding of the antibody in these tissues (n=5).

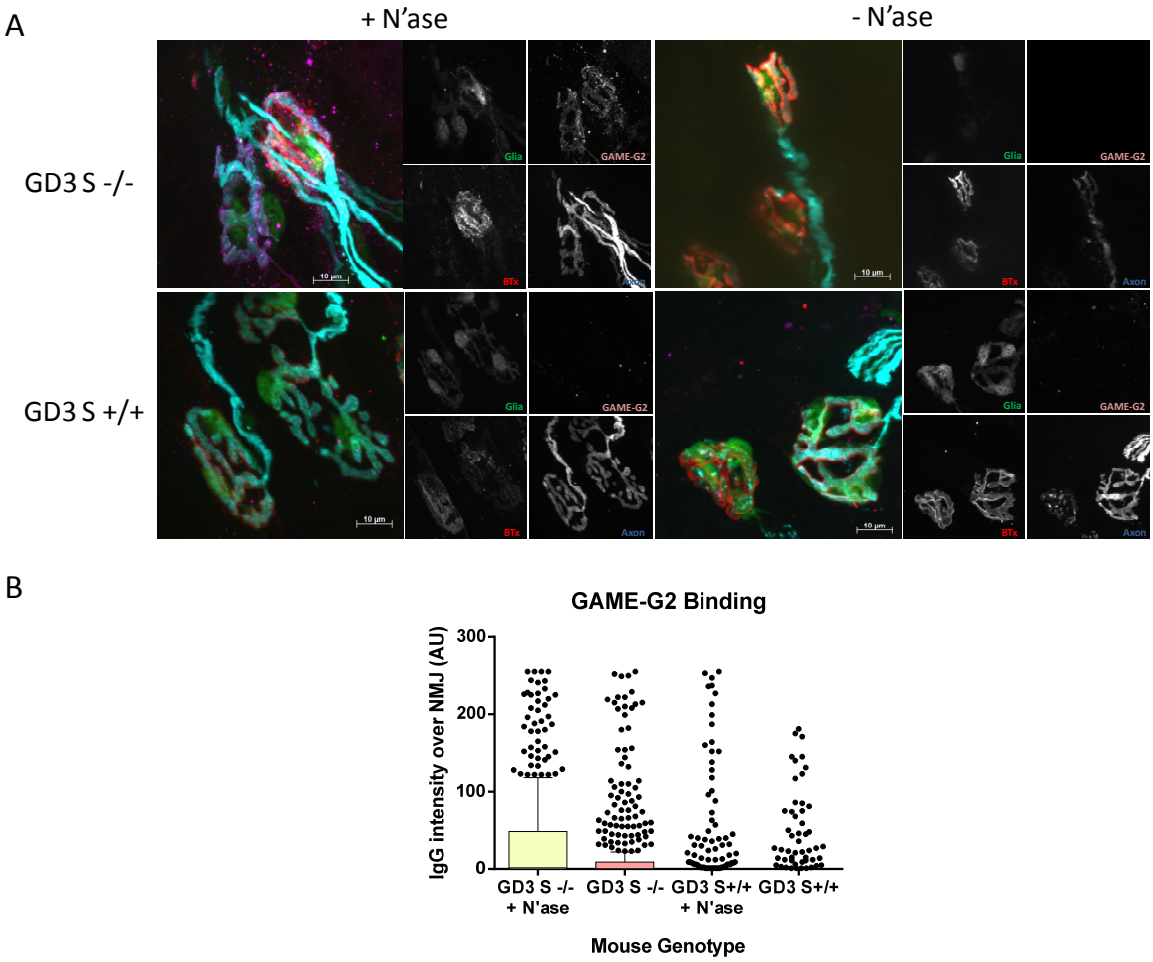


Figure 7.15 – GAME-G2 Binding in GD3 S-/- and GD3 S+/+ tissue with and without N'ase treatment.

Ex vivo preparations of TS from GD3 S-/- and GD3 S+/+ mice were probed with GAME-M3. Half the tissue had been pre-treated with N'ase whilst the other half only received Ringer's solution. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. A: Illustrative examples indicate GAME-G2 binding over the NMJ in the N'ase treated GD3 S-/- tissue. Binding was absent in the other tissues. B: The binding signals were generally low for all the tissues regardless of treatment although a small level of binding was detected in the N'ase treated GD3 S-/- tissue (n=5).

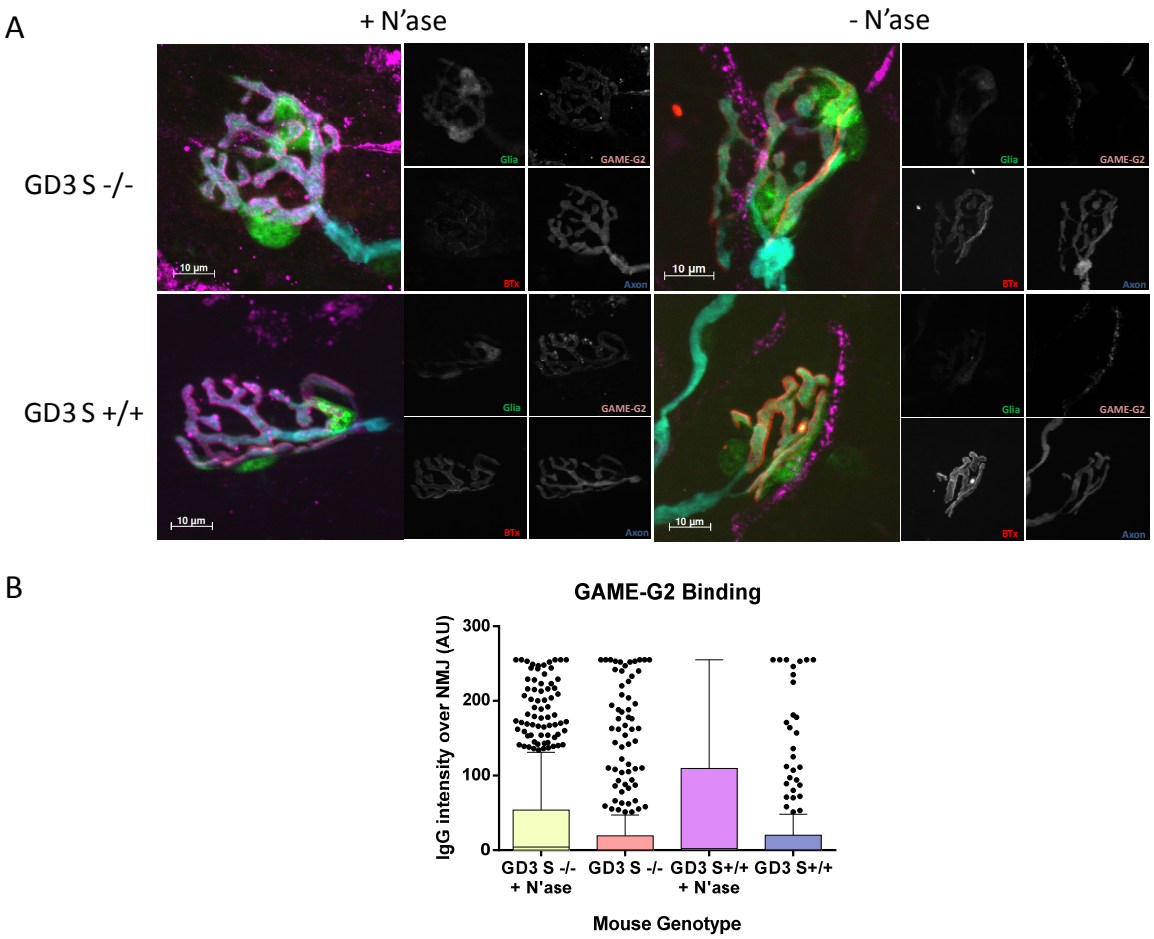


Figure 7.16 - GAME-G2 Binding in GD3 S-/- and GD3 S+/+ tissue with and without N'ase treatment - Permeabilised.

Ex vivo preparations of TS from GD3 S-/- and GD3 S+/+ mice were probed with GAME-G2 followed by permeabilisation. Half the tissue had been pre-treated with N'ase whilst the other half only received Ringer's solution. The tissue was imaged and the secondary antibody fluorescent intensity overlying the NMJ was quantitated. A: Illustrative examples indicate GAME-G2 binding over the NMJ in the N'ase treated tissues. Binding was absent in the other tissues. B: The binding signals were higher in the permeabilised tissue but the signals were still relatively low. The highest signals were detected in the N'ase treated tissues of both genotypes but there was no significant differences (n=5).

7.3 Discussion

Mouse anti-ganglioside antibodies are an ideal tool for investigating the pathogenesis of autoimmune neuropathies. They are easy to produce and have proven useful in identifying specific mechanisms of disease in several studies; ranging from the roles of complement in nerve injury, to the localisation of

gangliosides within the nervous system (Lunn et al., 2000; O'Hanlon et al., 2001).

To fully understand the behaviour of novel antibodies, it is vital that their binding properties are fully characterised prior to their use. The newly synthesised antibodies, reported in this thesis, underwent this characterisation through a robust screening program involving analysis of the antibodies both *in vitro* and *ex vivo*.

During the initial cell supernatant screening process, the primary antigen of each antibody was identified through tests with ELISA or microarray. Once the monoclonal antibodies were produced, however, they were rescreened against a panel of lipids to establish their cross reactivities. Traditionally, these initial binding specificity assays were carried out using ELISA (Goodyear et al., 1999); however, the combinatorial glycoarray has proved to be a superior tool for determining the various targets of novel antibodies. It allows for the simultaneous screening of a larger number of antigens using a much smaller amount of lipid and has the major advantage that it can be used to print complexes composed of different combinations of gangliosides and other lipids (Rinaldi et al., 2009).

The identification of complexes was particularly useful here as they established which lipids could enhance or inhibit antibody binding prior to tissue studies. This allowed for a methodical approach in analysing the behaviours of the antibodies and aided in explanations as to the observed binding patterns in tissue.

An example of this was shown with GAME-G2, which had a decreased binding signal on arrays when bound to complexes composed of GD1a. As the tissue studies indicated that the antibody bound relatively poorly to GD3 S-/- and GD3 S+/+ tissue, it implied that GD1a inhibition may be preventing antibody binding. To test this hypothesis, N'ase was applied to the tissue to remove the terminal sialic acid that is common to GD1a and GT1b. Binding signals were detected in these treated tissues, which substantiated the initial findings on array that GD1a was inhibiting antibody binding.

The inhibition of anti-GM1 antibodies by GD1a has been reported previously (Greenshields et al., 2009; Nobile-Orazio et al., 2010), which highlights how

antibody binding is impacted by the local environment of the ganglioside. The differences in this environment may explain why cytochemical analysis has been unable to adequately explain why similar amounts of GM1 and GD1a are present in both motor and sensory nerves, and yet only motor nerves are targeted in MMN (Ogawa-Goto et al., 1990; Svennerholm et al., 1992, 1994). It could be assumed that in MMN patients, who often have high titres of anti-GM1 antibody, injury does not occur in the sensory nerves because they have a high level of cis-interaction between GM1 and GD1a, which alters the presentation of GM1 to the immune system. By contrast the motor nerves may have GM1 and GD1a located in separate portions of the membrane, resulting in the normal presentation of GM1 to the immune system, which opens it up to attack by antibodies.

This had been partially shown by other researchers who found that a rabbit model of AMAN produced high titres of anti-GM1 antibodies. These antibodies were then responsible for high levels of complement deposition and node disruption in motor nerves, whilst the sensory nerves remained mostly unaffected (Susuki et al., 2012). This may be due to the cis-inhibition of anti-GM1 antibodies by the GM1:GD1a complex, although the exact mechanism was not established within this experiment.

This provides a potential use of GAME-G2 in future studies, where it could be applied, alongside a complex-independent antibody, to motor and sensory nerves. This would establish if the GD1a inhibition was responsible for the lack of injury in sensory nerves or if the lack of injury was related to another unknown factor.

Aside from GD1a, the identification of complex enhancement or inhibition was also useful in determining which antigens the antibodies were most likely binding. GAME-M1, GAME-M3 and GAME-G2 bound to the single sulfatide epitope on arrays, which may have led to the assumption that the antibodies were binding to sulfatide in tissue. Analysis of the sulfatide complexes on the array, however, indicated that binding was inhibited with all complexes except those that contained a ganglioside to which the antibody bound in its own right.

The inhibitory lipids included Chol and SM which are ubiquitously expressed on the plasma membrane (Cortes et al., 2013). This makes it highly unlikely that any of the antibodies would bind successfully to sulfatide as it would almost

always be in a complex with one of these lipids. Immunofluorescent studies confirmed this theory and proved that little or no binding was detected in tissues lacking complex gangliosides.

This same analysis was used to determine why GAME-M4 did not bind tissue. On array the antibody was inhibited from binding to SM when it was in complex with any ganglioside or sulfatide. As these lipids are enriched in neural tissue (Molander-Melin, 2003), it was unlikely that GAME-M4 would be capable of binding successfully to it. It may be able to bind to other non neural cells; however due to time constraints, these were not examined within these experiments.

Although the array tended to correlate well with the immunofluorescence results, it was not always an accurate measure of the antibodies targets on tissue. On array, GAME-M3 appeared to bind with similar levels to GM1, GA1 and GD1b but it also bound weakly to GD1a. As these gangliosides all contained a terminal galactose, it was assumed that this was the specific epitope of the antibody, although it may well have bound to other non-sialylated components of the headgroup.

When applied *ex vivo,* it appeared that GAME-M3 was only capable of binding to GD1a enriched tissues, which was surprising considering the array results, and the fact that the nerve terminal is known to be enriched with both GM1 and GD1b (Boffey et al., 2005; Greenshields et al., 2009). In contrast, GA1 is mostly absent in the mammalian nervous system and was therefore never considered as a potential target of the antibody in tissue (Seyfried et al., 1996).

The absence of binding to GM1 and GD1b suggested that an unknown cis-inhibition was occurring between the gangliosides and a component of the plasma membrane. It was hypothesised that this interaction was cryptically shielding the terminal galactose in such a manner as to prevent antibody binding. As GD1a contains a terminal sialic acid, it was postulated that it may be able to overcome this shielding and expose the terminal galactose to which GAME-M3 could bind.

This data was at odds, however, with molecular modelling studies, which have previously indicated that the terminal galactose lies perpendicular to the plasma membrane and is therefore accessible for antibody binding (Patel & Balaji,

2008). These models are relatively simple though, and only incorporate one or two other lipids, which downplays the complexity of the membrane (Lyubartsev & Rabinovich, 2011). It is possible that if all components of the membrane, including lipids and proteins, are taken into account that the terminal galactose may become somewhat shielded.

It is also possible that GAME-M3 does not actually bind to it but rather to another component of the headgroup (GalNAc, the internal galactose, or glucose). Originally it was assumed that the antibody bound to the terminal galactose due to the change in the binding signal that was associated with GD1a. As GD1a contains a terminal sialic acid, it was assumed that it was most likely influencing the presentation of the terminal galactose, but this assumption may be incorrect.

Molecular modelling has indicated that the glucose and internal sialic acid of the ganglioside headgroups are unavailable for interaction with macromolecules when they are in DPPC membrane bilayers (Humphrey et al., 1996). Further to this, they indicated that CTx was unable to bind to these components, due to access issues, whereas it could bind to both the terminal galactose and internal sialic acid (Neu5Acα3) of GM1 (Merritt et al., 1998; Neu et al., 2008).

The same effects could occur with GAME-M3 in tissue, where the specific epitope is inaccessible due to its location on the headgroup. This would prevent antibody binding to a membrane bound ganglioside but it would not necessarily prevent binding to a ganglioside printed by array. The reason for this is that the array would print lipids as dehydrated monolayers that do not necessarily behave in the same way as those in a biological membrane. Further research would need to be performed to establish the specific epitope of this antibody, which may aid in determining the reasons for the differences between the array and tissue results.

Aside from comparisons between the array and tissue, *ex-vivo* preparations also proved beneficial in establishing which neurological structures the antibodies were specifically targeting. This helped determine their pathogenic roles and, by extension, their possible uses in future experiments.

Whilst, GAME-M3 and GAME-G2 were found to bind primarily to the NMJ, GAME-M1 was found to bind avidly to both the NMJ and the Schwann cell

plasmalemma. This has been shown with anti-GD1a antibodies previously, with some researchers finding that certain antibodies were able to distinguish between motor and sensory nerves depending upon their specific epitope (Gong et al., 2002; P. H. H. Lopez et al., 2008). This is interesting from a disease perspective as several neuropathies are commonly associated with anti-GD1a antibodies (Carpo et al., 1996; Yuki & Hartung, 2012) The symptoms of these conditions can vary between patients, which may be due, in part, to the type of anti-GD1a antibodies that have arisen. Further research would need to be carried out to determine if GAME-M1 was capable of distinguishing between nerves, but the potential remains that the antibody could be useful for studying paralytic neuropathies.

Overall, the characterisation of the anti-ganglioside antibodies successfully determined their binding patterns both *in vitro* and *ex vivo*. This information will be essential to determining their use in future experiments.

8 PRODUCTION OF ANTI-SULFATIDE ANTIBODIES

8.1 Introduction

The immunisation experiments of the previous chapters tended to focus on the use of GalNAc T-/- mice as hosts for the generation of complex-dependent antibodies. These mice were selected for these studies as they lack the ability to synthesise complex gangliosides, such as GM1 and GD1a, and have been demonstrated to be more effective hosts in antibody generation than their WT counterparts (Lunn et al., 2000).

Although the exact reason for the responsiveness of these transgenic mice to immunisation has yet to be fully elucidated, it has been hypothesised that the lack of endogenous exposure to complex gangliosides during development prevents the formation of immunogenic tolerance (Bowes et al., 2002; Goodfellow et al., 2005; Willison & Plomp, 2008). The immune systems of these mice would therefore perceive complex gangliosides as foreign antigens, which, upon exposure, would result in the production of high titres of anti-ganglioside antibodies.

Whilst these factors made the mice ideal candidates for the production of antibodies targeting complexes composed solely of gangliosides, their usefulness in producing antibodies against complexes containing other glycolipids was questionable. In particular, it was unclear how effective they would be in the production of antibodies specifically targeting GM1:GalC or GM1:sulfatide complexes.

In these experiments, consideration was therefore given to transgenic mice that lacked the ability to synthesise other glycolipids, specifically GalC and sulfatide (Honke et al., 2002; K. Suzuki, 1998). Unlike the GalNAc T-/- mice, however, no previous studies had been performed on these mice to assess their receptiveness to immunisation against the single glycolipids.

This information was deemed to be necessary before proceeding with the ganglioside complex immunisations, as the mice would be of no use in these

studies if they maintained immunological tolerance to the single glycolipid species. Whilst it would have beneficial to perform these studies using both genotypes of mice, only those lacking sulfatide (CST-/-) were readily available within our laboratory, and were therefore the only mice that were examined within these experiments.

In addition to establishing the suitability of the CST-/- mice to immunisation, these experiments also sought to produce a series of anti-sulfatide antibodies for further study. These antibodies are implicated in a number of demyelinating diseases, including GBS and MS, but their roles in these conditions are still unclear (Brennan et al., 2011; Carpo et al., 2000; Ilyas, 2003; Petratos et al., 2000; Souayah et al., 2007). This chapter therefore had two mains aims: to establish the susceptibility of CST-/- mice to immunisation with sulfatide liposomes; and to produce anti-sulfatide antibodies to establish their roles in disease.

8.2 Results

8.2.1 Sulfatide Liposome Immunisations

Three different genotypes of mice were employed in the immunisation experiments to ascertain whether the absence of sulfatide influenced the generation of anti-sulfatide antibodies. These included CST-/-, CST+/+ and DBA mice. As already discussed the CST-/- mice were deficient in sulfatide and were therefore assumed to produce high titres of antibodies due to a lack of immunogenic tolerance. In contrast, the CST+/+ mice had a normal distribution of sulfatide throughout their systems and were assumed to be poor antibody producers due to endogenous exposure. A third genotype, that had a different genetic background than the CST+/+ mice (C57/B6), was also included in these experiments to ascertain whether background affected the ability of the mice to generate an immune response.

All the mice were immunised with sulfatide liposomes over a 4 week period as per Section 2.7.1. Serum was collected regularly throughout the immunisation cycle and was analysed using the combinatorial glycoarray (Figure 8.1).

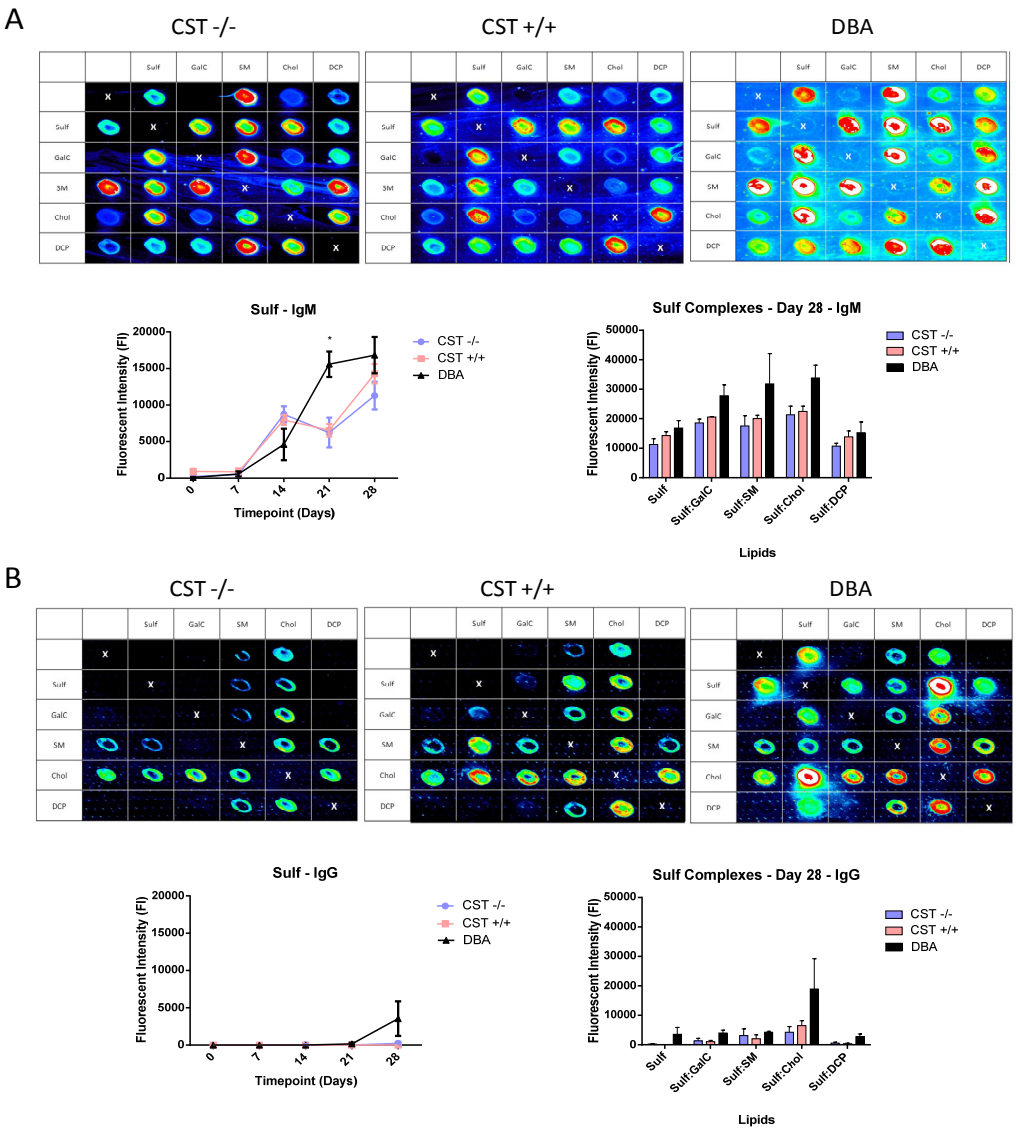


Figure 8.1 - Sulfatide Liposome Immunisations

The sera were screened against a panel of lipids and complexes using the combinatorial glycoarray. A representative blot of the terminal bleed antibody binding pattern is displayed for each genotype. The antibody binding signals to sulfatide were plotted over time for each genotype and a comparison of the terminal antibody binding to sulfatide complexes with the single components subtracted is also plotted. A: IgM Results. CST -/- and CST+/+ mice had virtually identical responses, displaying a sharp increase in anti-sulfatide antibodies on day 14, a small decrease on day 21, followed by a further increase on day 28. The antibody levels in the DBA mice increase steadily at each subsequent bleed and were found to be significantly higher than both the CST-/- and CST+/+ mice on day 21 (Mann Whitney, $P<0.05$). All genotypes had higher binding signals to sulfatide:GalC, sulfatide:Chol and sulfatide:SM complexes compared to the single sulfatide antigen. B: IgG Results. Neither the CST-/- or CST+/+ mice had detectable anti-sulfatide IgG antibodies at any timepoint. In contrast, the DBA mice developed anti-sulfatide antibodies on day 28. Sulfatide:Chol complexes appeared to greatly enhance this signal. CST+/+ (n=3), CST-/- (n=6), DBA (n=3).

The results from these experiments indicated that that there was very little difference between the CST+/+ and CST-/- mice. Both groups developed low levels of anti-sulfatide IgM antibodies on day 14, followed by a small decrease on day 21 and a sharp increase on day 28, reaching a high of approximately 12000 FI units. In contrast, the DBA mice developed a low immune response on day 14, followed by a substantial increase on day 21 that was significantly higher than both the CST+/+ and CST-/- mice (Mann Whitney, $P<0.05$). This eventually reached a plateau on day 28 of approximately 15000 FI units, where it was assumed that the antibodies began class switching. All three genotypes of mice were found to have higher binding signals to sulfatide:GalC, sulfatide:Chol and sulfatide:SM complexes compared to the single sulfatide antigen.

Analysis of the IgG responses indicated that neither the CST+/+ or CST-/- mice developed anti-sulfatide antibodies. In comparison, the DBA mice developed low levels on day 28, which correlated with the plateau observed in the IgM results. This signal was found to be substantially increased with sulfatide:Chol complexes compared to the single antigen.

Taken together, this data suggested that the CST-/- mice were not better suited to the production of anti-sulfatide antibodies than WT mice. This may be related to the C57/B6 background in which the mice were raised as the DBA mice were shown to be superior in producing both anti-sulfatide IgM and IgG antibodies; however, this could not be confirmed. Despite these results, the mice were still able to produce anti-sulfatide antibodies and, as such, their spleens were harvested and fused with a myeloma cell line to create a hybridoma.

8.2.2 Hybridoma Screening

Following two weeks of incubation, the supernatant of the hybridoma cells was screened for the presence of antibodies using the microarray method developed in Chapter 6 (Figure 8.2). This screening indicated that a large number of wells were producing both IgM and IgG antibodies against sulfatide and SM. Unlike the anti-ganglioside antibodies produced in the previous chapters though, none of these antibodies appeared to cross react with other glycolipids.

The positive wells with the highest binding signals were expanded into 24 well plates and incubated for a further three days, followed by removal and

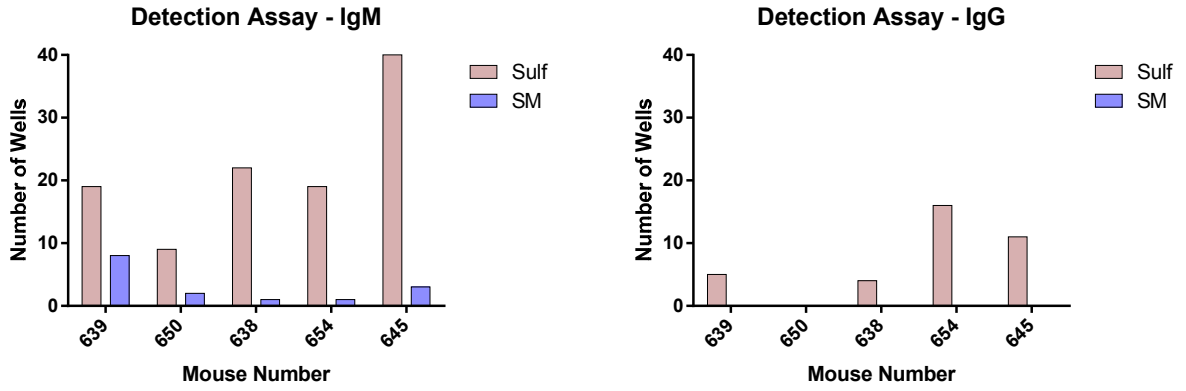


Figure 8.2 - Initial Supernatant Screen of sulfatide Immunised Mice Hybridoma Cells

Hybridoma supernatant was screened to determine antibody positive wells. The screening was performed two weeks after the initial hybridoma fusion. Several wells were found to contain IgM antibodies targeting both sulfatide and SM. IgG antibodies were also identified that targeted sulfatide.

replacement of the supernatant. As the aim of these studies was to produce anti-sulfatide antibodies, the supernatant was rescreened after a further three days of growth using a sulfatide ELISA. As expected, the number of positive wellsdropped substantially but the remaining hybridoma cells were expanded further and routinely rescreened for antibody activity. This led to the production of two IgM and two IgG anti-sulfatide monoclonal antibodies dubbed GAME-M2, GAME-M5, GAME-G1 and GAME-G3.

Following on from their production, the binding behaviours of the antibodies were characterised through the use of solid phase assays, cells and tissues. The results of these studies are shown in Chapter 9.

8.3 Discussion

The ability to successfully produce anti-glycolipid antibodies in mice is often purported to be related to their glycolipid repertoire. This is based upon the findings of several studies, which have shown that mice that lack the ability to synthesise particular gangliosides are better hosts for the production of anti-ganglioside antibodies than their WT counterparts (Bowes et al., 2002; Lunn et al., 2000).

The reason behind the superior antibody producing ability of these mice has been hypothesised to be due to a lack of endogenous ganglioside exposure, which prevents the formation of immunogenic tolerance (Bowes et al., 2002). As a result, the immune systems of these mice perceive complex gangliosides as foreign antigens and develop high titres of anti-ganglioside antibodies when challenged with the glycolipids.

Based upon this hypothesis, this chapter sought to determine whether sulfatide knockout mice behaved in a similar fashion and produced high titres of anti-sulfatide antibodies. Its findings, however, demonstrated that the knockout mice were no better in producing antibodies than their WT counterparts. This suggested that the mice had either developed tolerance through a different mechanism or that tolerance was not a factor in antibody production.

As the sulfate group is the only feature that distinguishes sulfatide from GalC, it is cogent to assume that it would be the only structure that the immune systems of CST-/- mice would perceive as foreign. However, this same sulfate group is expressed on a variety of sulfated proteins and lipids to which the mice would have developed tolerance. It is therefore possible that they would perceive the sulfate group on sulfatide as endogenous and would not develop anti-sulfatide antibodies in response to the glycolipid.

Similarly, it was also possible that sulfatide interacts with its surrounding environment *in vivo* in such a way that the sulfate group is obscured from immunogenic exposure. The molecule would therefore resemble GalC, to which the mouse would have already developed tolerance thus preventing a robust antibody response.

Another possibility is that tolerance is not actually a factor in antibody generation. As mentioned in Chapter 6, recent work within our laboratory has suggested that internalisation may be one of the main mechanisms for the removal of circulating anti-ganglioside antibodies (M Cunningham, unpublished observations). This has yet to be shown with anti-sulfatide antibodies, but it is possible that the glycolipid does not internalise as readily as gangliosides. This would account for the lack of difference between the CST-/- and CST+/+ mice, as neither would be able to remove anti-sulfatide antibodies from circulation.

Aside from glycolipid repertoire, this chapter also explored the effects of different genetic backgrounds on antibody generation. It found that DBA mice were more effective hosts in producing anti-sulfatide antibodies than both the CST+/+ and CST-/- mice that were raised on a C57/B6 background. This suggested that the genetic background of mice is a more important factor in selecting hosts for antibody generation than glycolipid expression.

Previous studies supported this finding, as they have found that C57/B6 mice have defective secondary immune responses compared to DBA mice (Morokata et al., 1999; Pan et al., 2004). This suggests that, although the C57/B6 strain is useful for producing transgenic mice, it is not well suited for inducing antibody production.

Despite these issues, the immunisation studies still led to the development of several novel anti-sulfatide antibodies. These antibodies are characterised in the subsequent chapter, through the use of solid phase assays and *ex vivo* preparations, to determine their roles in both health and disease.

9 CHARACTERISATION OF ANTI-SULFATIDE ANTIBODIES

9.1 Introduction

The liposome immunisations of the previous chapters led to the successful creation of several anti-sulfatide antibodies. Whilst initial studies demonstrated that these antibodies bound to sulfatide and associated complexes, a more thorough characterisation was required to establish the binding patterns of the antibodies both *in vitro* and *ex vivo* to elucidate their roles in disease. This chapter therefore explores this characterisation through analysis of the antibodies binding behaviours to solid phase assays, cells, fixed tissue sections and live tissue preparations.

9.2 Results

9.2.1 Combinatorial Glycoarray

Initial studies analysed the binding patterns of the antibodies against a series of glycolipid complexes printed using the combinatorial glycoarray. The results of these experiments confirmed that the antibodies all bound to sulfatide but indicated that there were distinct differences in their fine specificities (Figure 9.1).

Closer examination of their binding patterns indicated that the antibodies fell into three discrete groups, which varied according to their sulfatide complex binding capabilities. The first group of antibodies included GAME-M2, GAME-M6 and GAME-M7 and were found to bind to the single Chol and sulfatide antigens alongside associated complexes.

The binding signals to the sulfatide complexes were of a particular interest as they indicated that the antibodies were capable of binding sulfatide regardless of its local microenvironment. Certain complexes, such as those containing GD1a

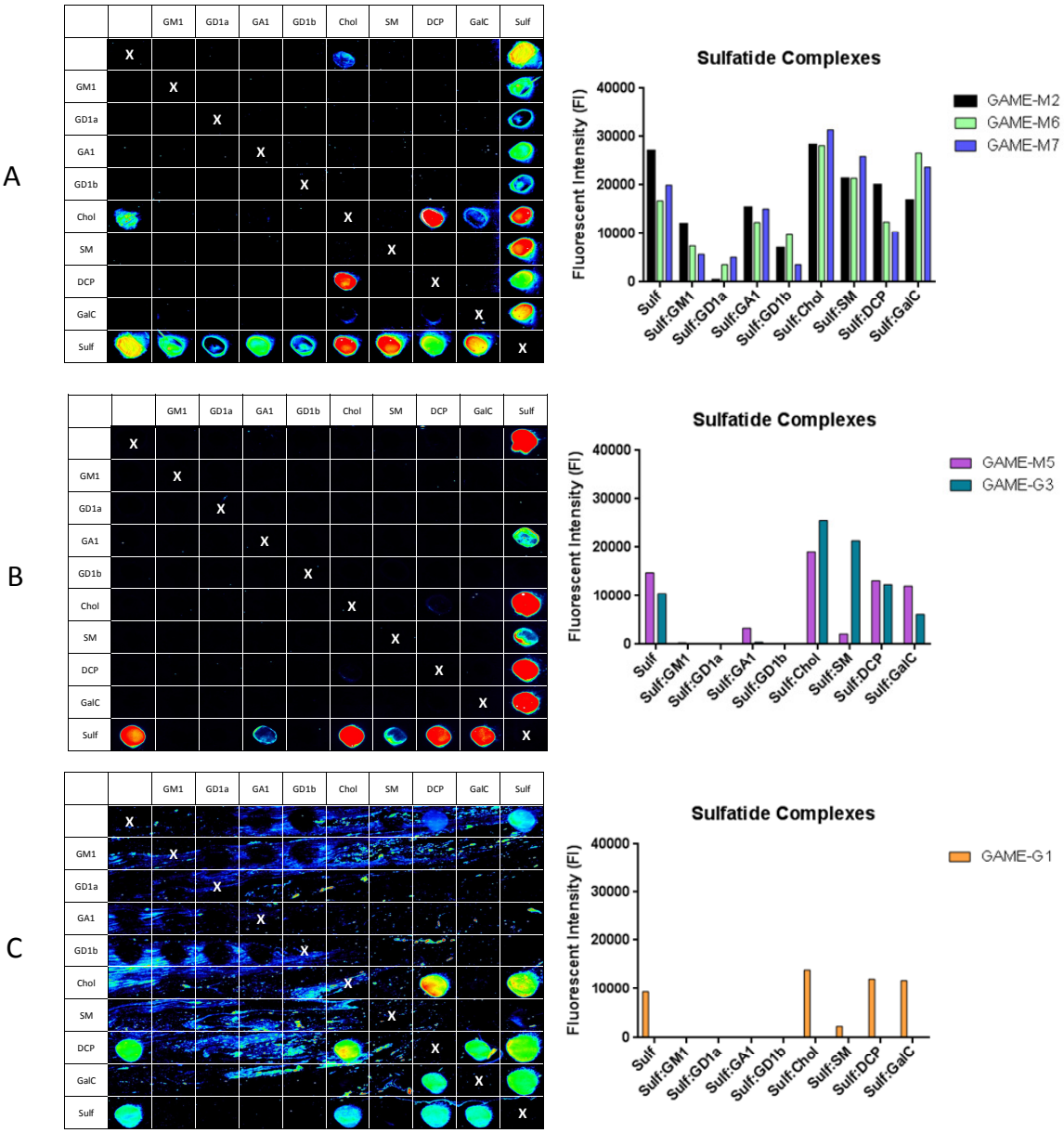


Figure 9.1 – Anti-Sulfatide mAb Characterisation on Combinatorial Glycoarray

Representative blots indicate the binding patterns of the antibodies to various lipids and complexes printed using the combinatorial glycoarray. A: This group included GAME-M2, GAME-M6 and GAME-M7. These antibodies bound to Chol as well as Chol:GalC and Chol:DCP complexes. Binding was also detected to sulfatide and all sulfatide complexes. These signals varied depending upon the lipid but no complexes were found to abolish antibody binding. B: This group included GAME-M5 and GAME-G3. These antibodies only bound to sulfatide and sulfatide complexes. Binding was inhibited by sulfatide:SM and sulfatide:GA1 and was abolished by sulfatide:GM1, sulfatide:GD1a and sulfatide:GD1a. C: This group comprised solely of GAME-G1. This antibody bound to sulfatide and DCP and a few complexes. Binding was abolished in both antigens by complexes containing gangliosides or SM.

and GD1b, did inhibit antibody binding; however, no complexes were found to completely abolish the binding signal altogether.

In addition to the sulfatide complexes, binding was also detected to Chol:DCP and Chol:GalC complexes but this was thought to lack relevance from a biological perspective as Chol binding was inhibited in the presence of most other lipids.

The second group of antibodies included GAME-M5 and GAME-G3 and were found to bind solely to sulfatide and associated complexes. Unlike the first group of antibodies, binding was substantially weaker against sulfatide:GA1 and sulfatide:SM complexes and was completely abolished against sulfatide:GM1, sulfatide:GD1a and sulfatide:GD1b complexes. This suggested that the length of the ganglioside headgroup may interfere with the antibodies abilities to access the binding epitopes on the sulfatide molecules.

The final antibody group comprised solely of GAME-G1 and was found to bind to both the single DCP and sulfatide antigens alongside a limited number of complexes. Binding was completely abolished when either antigen was in complex with gangliosides or SM, which suggested that the antibody may be incapable of binding to a biological membrane as these lipids are abundantly expressed throughout the body (Pike, 2003).

To ensure that no potential antigens were being overlooked, additional arrays were created that contained a series of other lipids (data not shown). These included GM3, GD3, DPPC, PC, SGPG, and LM1. No binding was detected to any of these single antigens or to their complexes except those containing sulfatide. The binding signals detected against these sulfatide complexes were found to be comparable to those detected against the single glycolipid species.

9.2.2 Cell Characterisation

9.2.2.1 Oligodendrocyte Progenitor Cells (OPCs)

Oligodendrocyte progenitor cells (OPCs) are a type of glial cell that populate the CNS before differentiating into myelinating oligodendrocytes (O'Rourke et al., 2014). Sulfatide is highly expressed in oligodendrocytes at every stage of development but its presence is used specifically as a marker of OPCs in the

adult brain (Lee, 2001; Reynolds & Hardy, 1997). These cells were therefore deemed to be best suited for determining the binding capabilities of the anti-sulfatide antibodies to biological membranes. As such, the antibodies were probed against rat OPCs as per Section 2.12.3.1.

Unfortunately quantification of the results was not possible due to the irregular binding of the OPC markers NG2 and PLP. As a result, only representative images were taken (Figure 9.2).

Despite these issues, the majority of the antibodies were found to bind well to OPCs, with GAME-G3 and GAME-M7 producing the strongest binding signals. The only antibody that was not found to bind was GAME-G1. It was postulated that this may be related to the findings of the array screening, which showed that the antibody was unable to bind sulfatide in the presence of particular lipids.

9.2.2.2 Schwann Cells

Schwann cells perform a similar supportive role as oligodendrocytes in the nervous system but are restricted to the PNS (Nave & Werner, 2014). As with the OPCs, the cells express high levels of sulfatide (Mirsky et al., 1990), which suggested that they may also be targets of the anti-sulfatide antibodies.

To confirm this, Schwann cells were grown over a period of several days and probed with the antibodies as per Sections 2.12.1 and 2.12.2. In addition to those with a normal distribution of sulfatide, cells were also taken from CST-/- mice to confirm that the antibodies were targeting the glycolipid and not another sulfated protein or lipid. Unfortunately it was difficult to produce enough cells for screening all of the antibodies, so only GAME-M2 was screened against cells from both genotypes (Figure 9.3).

The results from this experiment confirmed that the antibody was primarily targeting sulfatide as the CST+/+ cells produced a median signal of 243 FI units. This was substantially higher than the signals detected to CST-/- cells, which had a median signal of 0 FI units. Although very low levels of antibody deposition were detected in these cells, it was attributed to non-specific background binding.

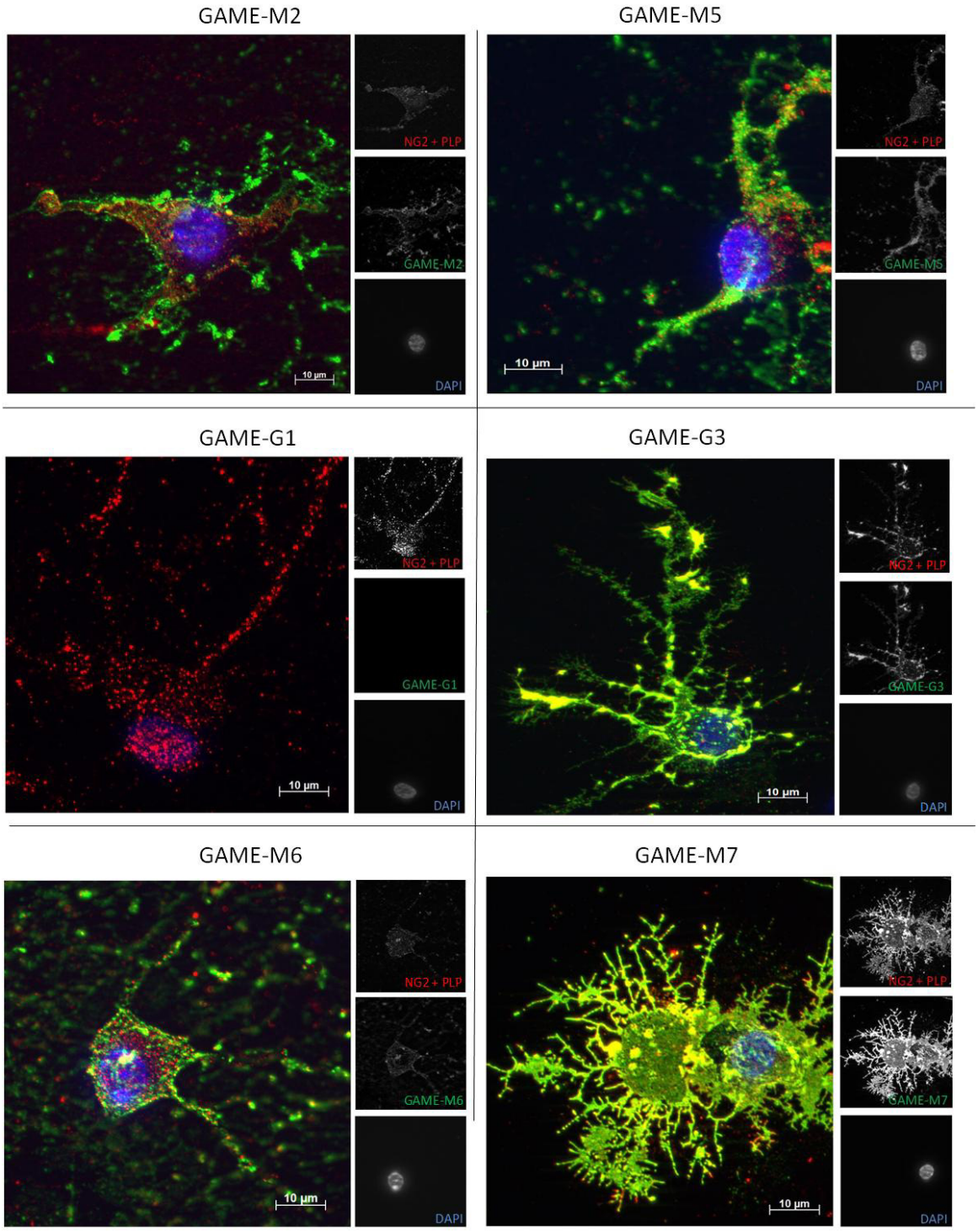


Figure 9.2 – Anti-sulfatide mAb binding to OPCs

The anti-sulfatide antibodies were probed against rat OPCs. Representative images indicate the relative binding patterns of each antibody. Quantitative analysis was not performed. The strongest binding signals were detected with GAME-G3 and GAME-M7. Binding was also detected to GAME-M2, GAME-M5 and GAME-M6. No GAME-G1 binding was detected to the OPCs whatsoever (n=2).

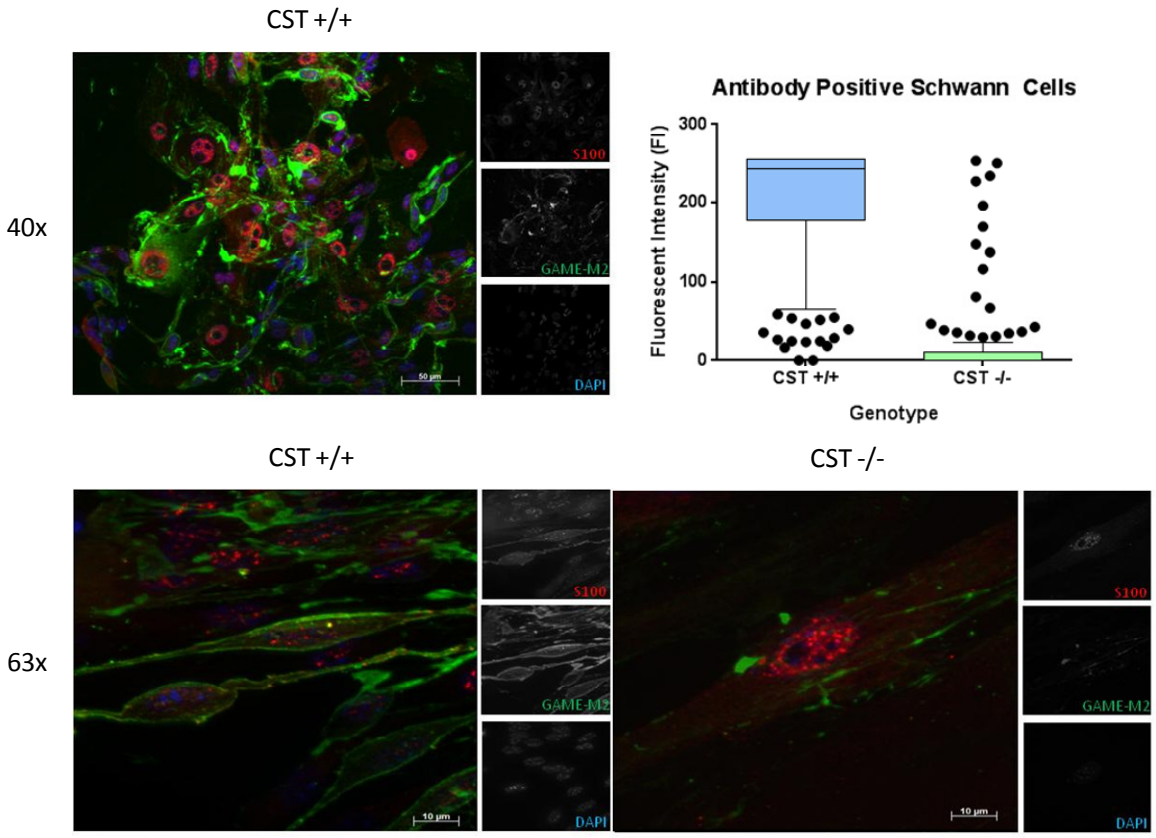


Figure 9.3 – Anti-sulfatide mAb binding to Schwann Cells

GAME-M2 was probed against Schwann cells grown from CST+/+ and CST-/-mice. Representative images of the CST+/+ tissue at 40x magnification and both the CST+/+ and CST-/- at 63x magnification indicate the relative binding of GAME-M2. The antibody was found to bind substantially better to the cells from CST+/+ mice producing a median signal of 243 FI units compared to a median signal of 0 FI units in the CST-/- cells. This confirmed that the antibody was binding to sulfatide and not any other target (n=1).

9.2.3 Sciatic Nerve Sections

Whilst characterising the antibodies cell binding behaviours was useful in determining how they bound to biological membranes, it was not particularly informative in ascertaining how they interacted with real tissue. To address this, an experiment was performed in which the antibodies were screened against transverse sections of sciatic nerve as per Section 2.13.2.2. As this nerve is myelinated it was hypothesised that it would contain an abundance of sulfatide, which would lead to high levels of antibody binding.

As the combinatorial glycoarray screening had established that complex ganglioside, such as GD1a and GD1b, inhibited or abolished antibody binding, the

decision was taken to include tissue from GalNAc T-/- mice. It was thought that the absence of complex gangliosides in this tissue would increase access to the sulfatide binding epitope, which would lead to improved antibody binding.

Similarly, tissue from CST-/- mice was also included in these studies to act as a negative control. As the previous experiments had suggested that the antibodies bound solely to sulfatide and associated complexes, it was hypothesised that no antibody binding would be detected in these tissues.

The results of the experiment confirmed that all of the anti-sulfatide antibodies, except GAME-G1, bound to the endoneurium and adaxonal myelin of the CST+/+ and GalNAc T-/- tissue (Figure 9.4). Unfortunately quantification of the results was not possible; however, from an observational perspective there did not appear to be a substantial difference between the two tissues types.

As predicted very little binding was detected in the CST-/- tissue, except with the preeminent anti-sulfatide antibody, O4, which was found to bind weakly to the endoneurium (data not shown).

9.2.4 *Ex vivo* screening of anti-sulfatide mAbs

Although the sciatic nerve sections demonstrated the tissue binding capabilities of the anti-sulfatide antibodies, they were not representative of the natural biological environment and, as such, were not indicative of how the antibodies would bind within the body. To determine this, *ex vivo* preparations of TS were created and probed with the monoclonal antibodies as per Section 2.13.1.

9.2.4.1 CST+/+ vs CST-/- tissue

Despite the evidence from the previous experiments that the antibodies bound solely to sulfatide, it was necessary to employ both CST+/+ and CST-/- *ex vivo* preparations in this study, to confirm that the antibodies did not bind to another antigen in live tissue.

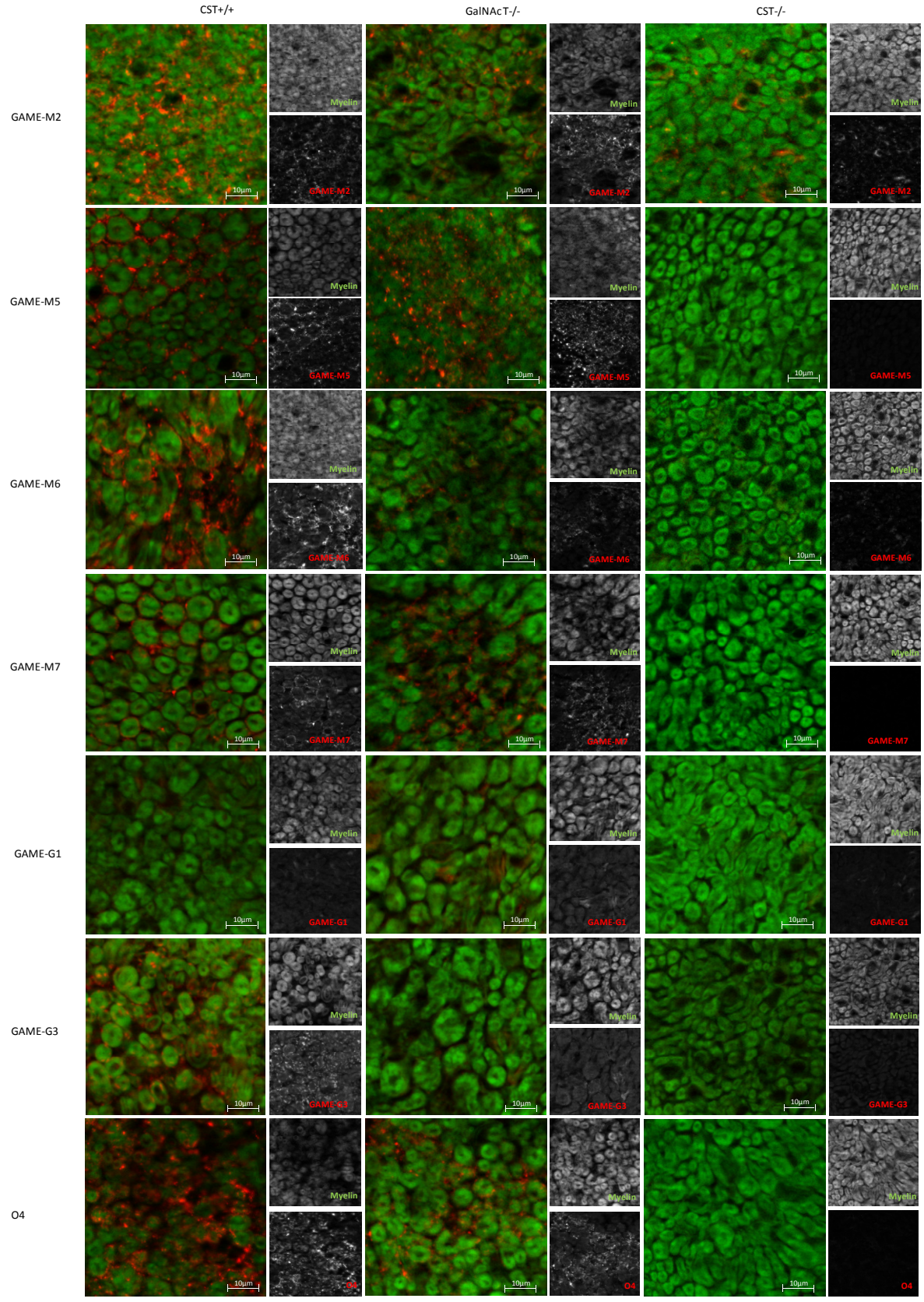


Figure 9.4 – Anti-sulfatide mAb binding to sciatic nerve sections

The new anti-sulfatide mAbs and O4 were probed against sciatic nerve sections. All the antibodies except GAME-G1 were found to bind to the adaxonal myelin in both the CST+/+ and GalNAc T-/- tissue. Very little binding was detected in the CST-/- tissue with any of the antibodies except O4, which weakly bound to the endoneurium.

Based upon the previous findings and the knowledge that sulfatide is enriched in Schwann cells (Mirsky et al., 1990), it was hypothesised that the antibodies would bind to the Schwann cell plasmalemma in a similar manner as GAME-M1; however, this was not found to be the case.

The majority of the antibodies were instead found to bind specifically to a Schwann cell that lay on the periphery of the NMJ (Figures 9.5 and 9.6). The subsequent characterisation of this cell found that it was the terminal myelinating Schwann cell, which it shall be referred to as from hereon in.

Quantification was performed on these results by counting the number of antibody bound cells in a total of 100 NMJs. The total number was then expressed as a percentage. Examination of this data found that GAME-M2, GAME-M5, GAME-M6, GAME-M7 and GAME-G3 all bound to a significantly higher number of cells in the CST+/+ tissue compared to the CST-/- tissue (Fisher's exact test, $P<0.0001$). In fact, the antibodies were not found to bind to the CST-/- tissue whatsoever, confirming that they were only targeting sulfatide.

Neither GAME-G1 nor O4 were found to bind the terminal myelinating Schwann cells in either tissue type, which suggested that the antibodies were unable to access their binding epitopes on these cells.

9.2.4.2 GalNAc T+/+ vs GalNAc T-/- Tissue

As the previous studies had demonstrated that the anti-sulfatide antibodies were inhibited by certain complex gangliosides, an experiment was derived in which the antibodies were probed against both GalNAc T+/+ and GalNAc T-/- tissue. It was hypothesised that the antibodies would bind to a higher number of terminal myelinating Schwann cells in the GalNAc T-/- tissue, as the absence of these gangliosides would better expose the sulfatide binding epitope.

The results of the experiment, however, did not fully support this hypothesis (Figures 9.7 and 9.8). Whilst GAME-M2 and GAME-G3 were found to bind to a significantly higher number of cells in the GalNAc T-/- tissue, the number of GAME-M5 and GAME-M6 bound cells did not appear to differ significantly between either tissue type (Fisher's exact test, $P<0.01$).

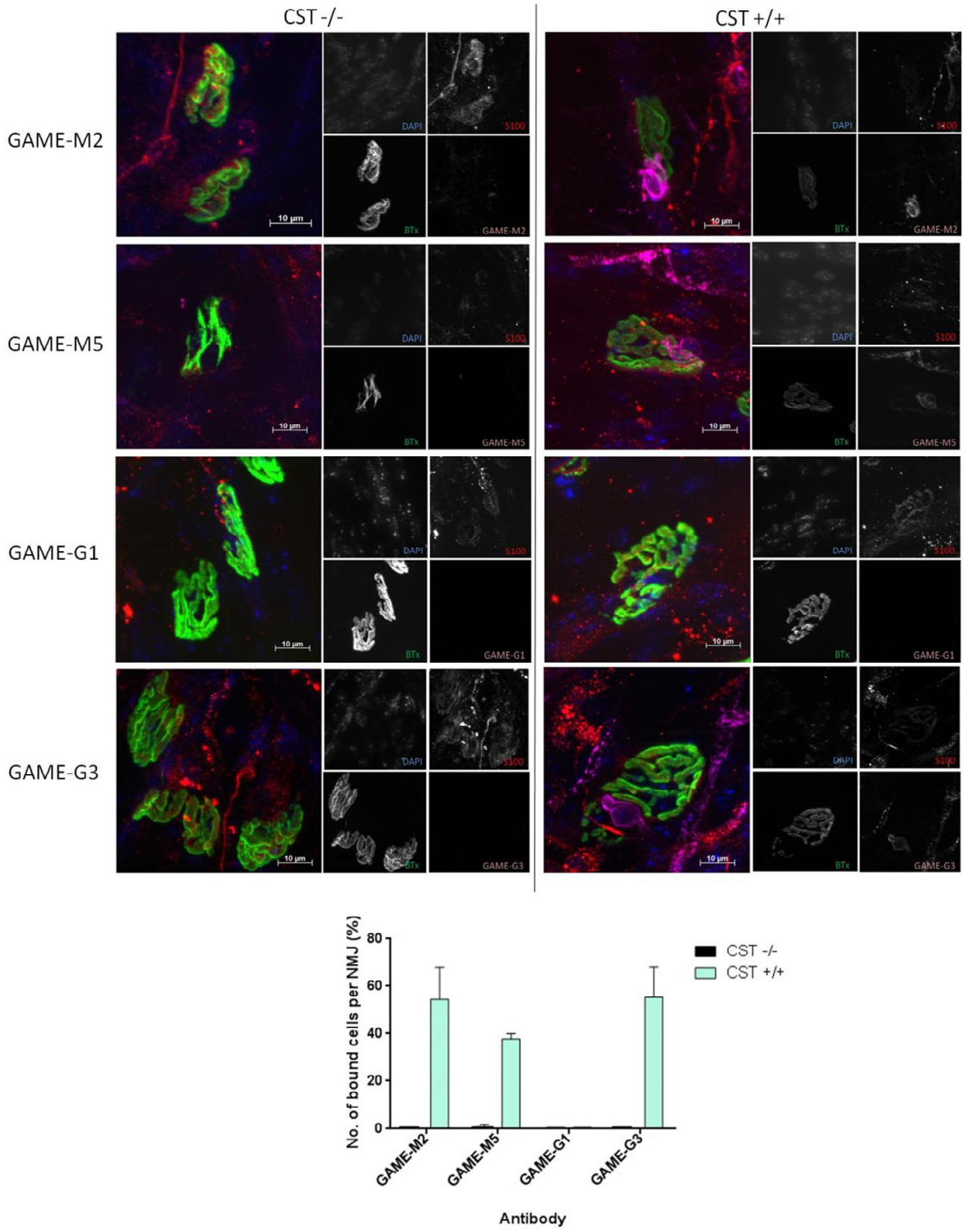


Figure 9.5 – Anti-sulfatide mAb binding to CST+/+ and CST-/- *ex vivo* preparations.

GAME-M2, GAME-M5, GAME-G1 and GAME-G3 were probed against ex vivo preparations of CST+/+ and CST-/- tissue. The antibodies were found to bind to a Schwann cell on the periphery of the NMJ (arrows). 100 NMJs were examined for each tissue section and the number of bound cells were counted and expressed as a percentage. GAME-M2, GAME-M5 and GAME-G3 were found to bind to these cells significantly higher in CST+/+ tissue compared to CST-/- tissue. (Fisher's exact test, $P<0.0001$) (n=2) GAME-G1 was not found to bind the cell in either tissue type.

GAME-M7 actually had the opposite effect and bound to a higher number of cells in the GalNAc T+/+ tissue (Fisher's exact test, P<0.001). This suggested that the antibody preferentially bound to sulfatide molecules that were in a complex

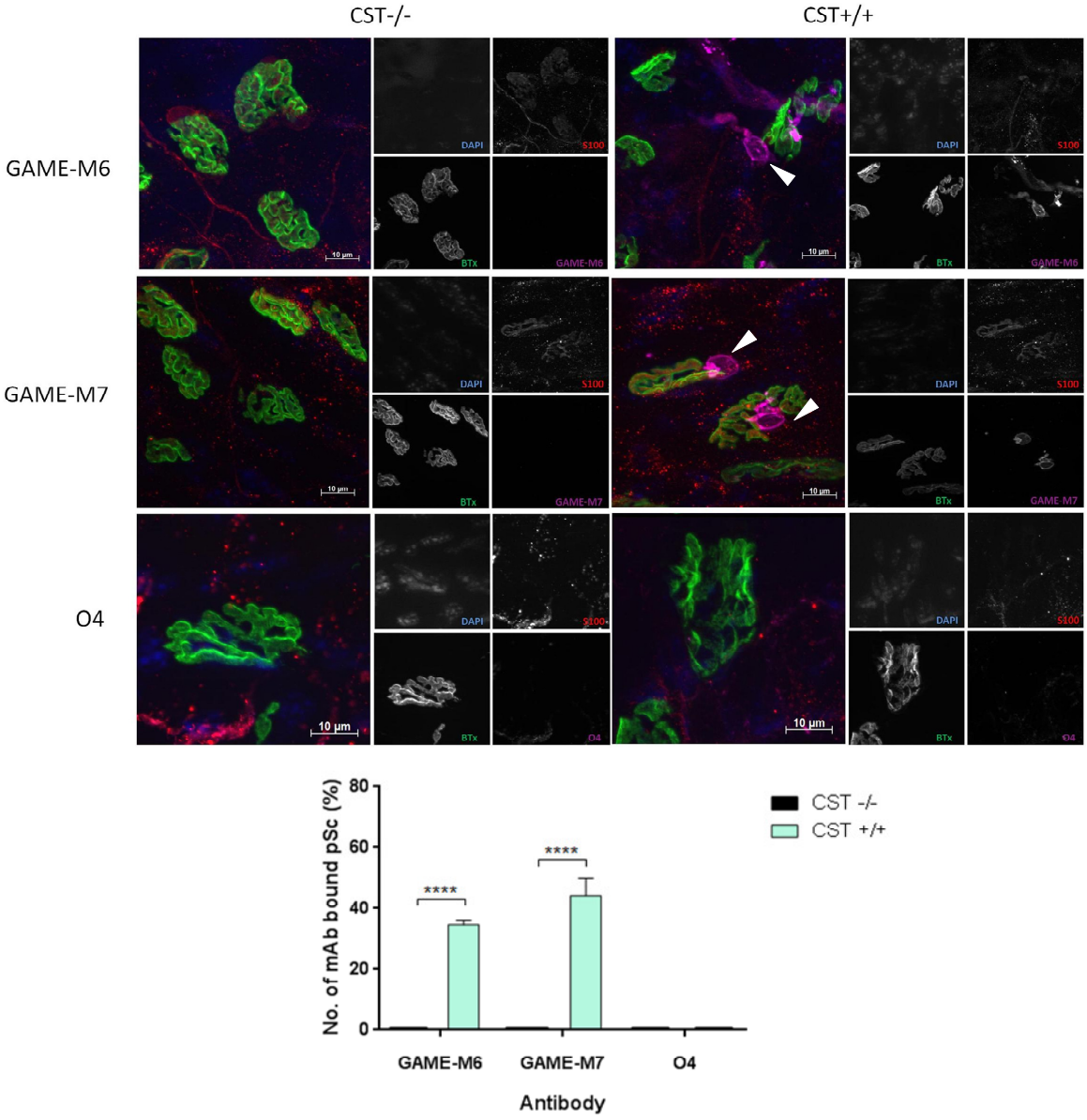


Figure 9.6 - Anti-sulfatide mAb binding to CST+/+ and CST-/- *ex vivo* preparations.

GAME-M6, GAME-M7 and O4 were probed against ex vivo preparations of CST+/+ and CST-/-. The antibodies were found to bind to a Schwann cell on the periphery of the NMJ (arrows). 100 NMJs were examined for each tissue section and the number of bound cells were counted and expressed as a percentage. GAME-M6 and GAME-M7 were found to bind to these cells significantly higher in CST+/+ tissue compared to CST-/- tissue. (Fisher's exact test, P<0.0001) (n=2) O4 was not found to bind the cell in either tissue type.

ganglioside rich microenvironment, contrary to the findings of the combinatorial glycoarray screening.

GAME-G1, as demonstrated previously, was incapable of binding to either tissue type, suggesting that the sulfatide molecule was being obscured by another, non-ganglioside, lipid.

Taken together, this data suggested that the biological binding patterns of the antibodies were more complex than they appeared using the combinatorial glycoarray. Future studies may therefore wish to examine the binding patterns of these antibodies against a range of transgenic mice to further clarify how complex gangliosides impact antibody binding. Further to this, it may also be worthwhile to repeat the experiment to confirm these initial findings, as it was only performed twice within this study due to tissue availability.

9.2.5 Terminal Myelinating Schwann Cell Characterisation

The specific binding of the anti-sulfatide antibodies to the terminal myelinating Schwann cell has, to our knowledge, not been demonstrated previously. This novel finding was therefore particularly interesting from an autoimmune neuropathy perspective, as it is currently unclear what targets these antibodies bind in tissue and how they bring about neurological injury.

The properties of this cell were therefore characterised to ascertain whether the antibodies were capable of specifically injuring it. This was carried out by employing one of the anti-sulfatide antibodies to act as a cell marker, followed by the application of a series of protein or lipid markers that indicated different cellular subtypes. Due to the abundance of available antibody stock, GAME-M7 was selected for this role.

9.2.5.1 Cell Markers

The experiments were carried out as per Section 2.13.1.5. The use of fluorescent mice in the previous antibody characterisation studies had already established that the cell was S100 positive, which indicated that it was a Schwann cell (Jessen & Mirsky, 2005; Zuo et al., 2004). To determine whether it

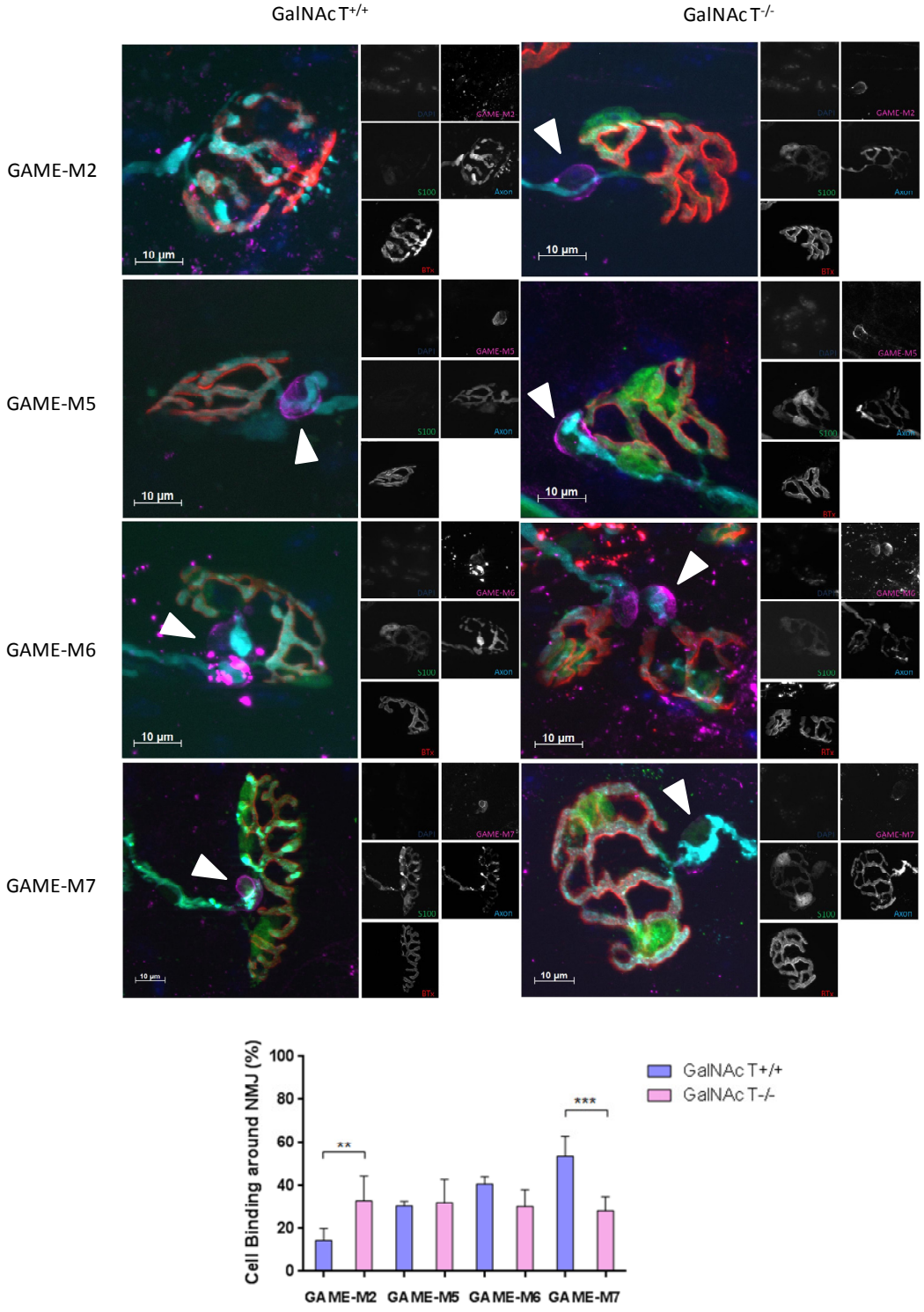


Figure 9.7 - Anti-sulfatide IgM mAb binding to GalNAcT-/- and GalNAcT+/+ *ex vivo* preparations

GAME-M2, GAME-M5, GAME-M6 and GAME-M7 were probed against ex vivo preparations of GalNAc T+/+ and GalNAc T-/-. The antibodies were found to bind to a Schwann cell on the periphery of the NMJ (arrows). 100 NMJs were examined for each tissue section and the number of bound cells were counted and expressed as a percentage. There was no difference between the tissue for GAME-M5 and GAME-M6. GAME-M2 was found to bind significantly better to the cells in the GalNAc T-/- tissue whereas GAME-M7 was found to bind significantly better to GalNAc T+/+ tissue. (Fisher's exact test, **=$p<0.01$, ***=$p<0.001$) (n=2)

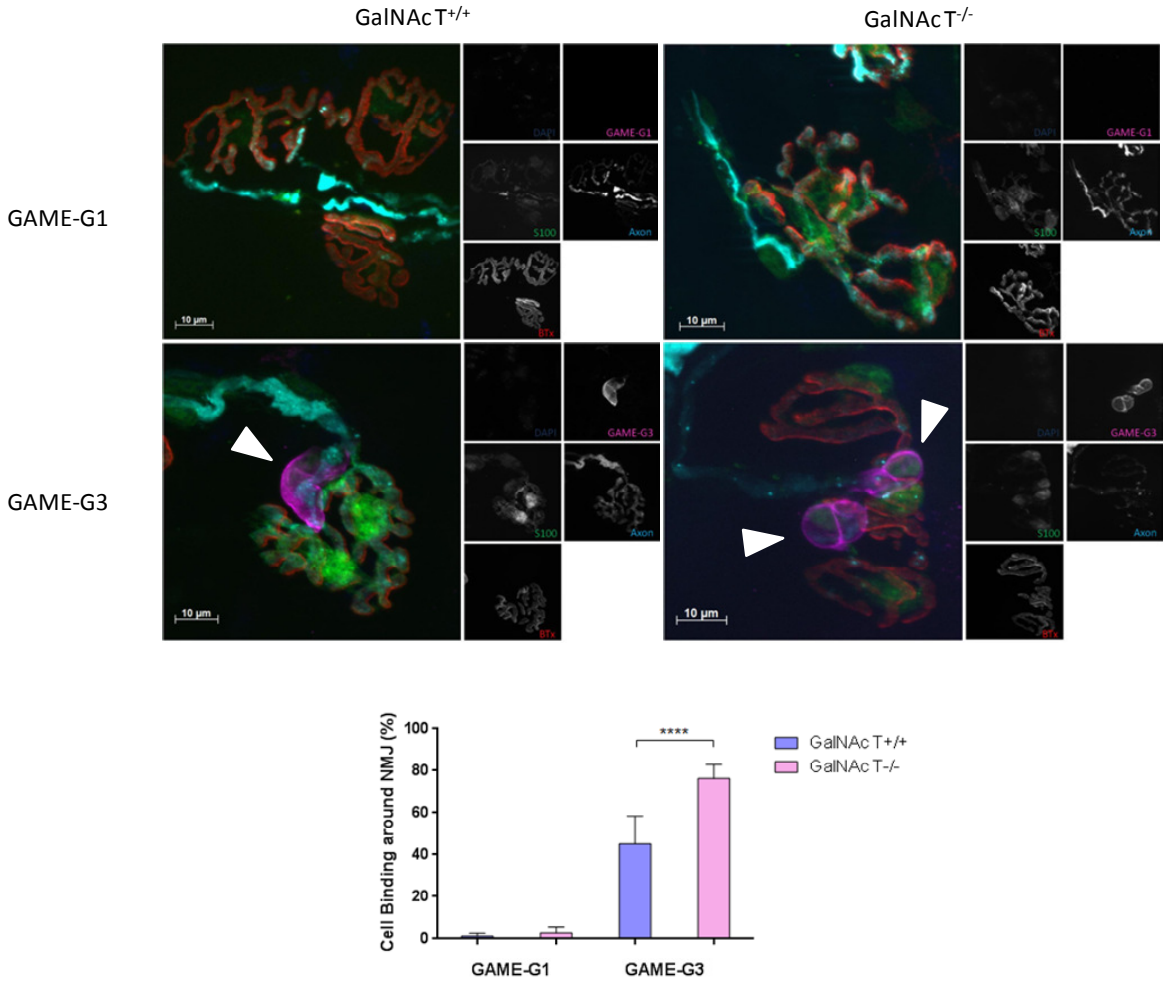


Figure 9.8 - Anti-sulfatide IgG mAb binding to GalNAcT-/- and GalNAcT+/+ *ex vivo* preparations

GAME-G1 and GAME-G3 were probed against ex vivo preparations of GalNAc T+/+ and GalNAc T-/-. The antibodies were found to bind to a Schwann cell on the periphery of the NMJ (arrows). 100 NMJs were examined for each tissue section and the number of bound cells were counted and expressed as a percentage. No binding was detected to GAME-G1 in either tissue configuration. GAME-G3 was found to bind significantly better to GalNAc T-/- tissue. (Fisher's exact test, ****=$p<0.0001$) (n=2)

was a myelinating or unmyelinating Schwann cell, it was necessary to probe the tissue with an anti-GD3 antibody called R24. Previous studies had established that this antibody specifically binds to unmyelinated cells, known as perisynaptic Schwann cells (pSc) (Halstead, Morrison, et al., 2005).

The results of the experiment showed that there was no co-localisation of R24 and GAME-M7, which clearly demonstrated that the cell was not a pSc (Figure

9.9) The cells position also substantiated this finding, as it was always present outwith the NMJ, whereas pScs would overly the junction (Kang et al., 2004).

Building upon this observation, it was hypothesised that the cell may have a role in myelination. As such, an *ex vivo* TS preparation was probed with GAME-M7 and either an anti-MAG or anti-MBP antibody (Quarles, 2007). The results of this experiment found that both MAG and MBP stained the axon leading to the NMJ but not the cell body of the terminal myelinating Schwann cell (Figure 9.10).

GAME-M7 staining was polarised towards the NMJ, however, suggesting an overlap with both the MBP and MAG proteins. It is therefore possible that the cell is responsible for myelinating the axon, although further experiments would need to be performed to confirm this hypothesis.

As discussed previously, it was thought that the cell may have a role in maintaining or repairing the nerve terminal. To examine whether this was true, the TS was probed with GAME-M7 and an anti-glial fibrillary acidic protein (GFAP) antibody to determine if it was a reactive Schwann cell (Court et al., 2008).

The results indicated that the anti-GFAP antibody stained the NMJ but was not enriched within the terminal myelinating Schwann cell itself (Figure 9.11). It was therefore concluded that the cell was not reactive. It may be worthwhile in future studies, however, to injure the nerve terminal and apply an anti-nestin antibody. If the cell was reactive it would upregulate the protein, which could then be bound by the antibody (Hayworth et al., 2006). This was not performed in these experiments due to time constraints but would be useful for confirming the anti-GFAP findings.

9.2.5.2 Incubation Conditions

As the experiments proceeded, questions were raised about whether the antibody's specific binding to the cell was a result of the blood nerve barrier (BNB). In particular, the observation that GAME-M7 was polarised towards the NMJ, suggested that, under standard conditions, the BNB may be preventing the

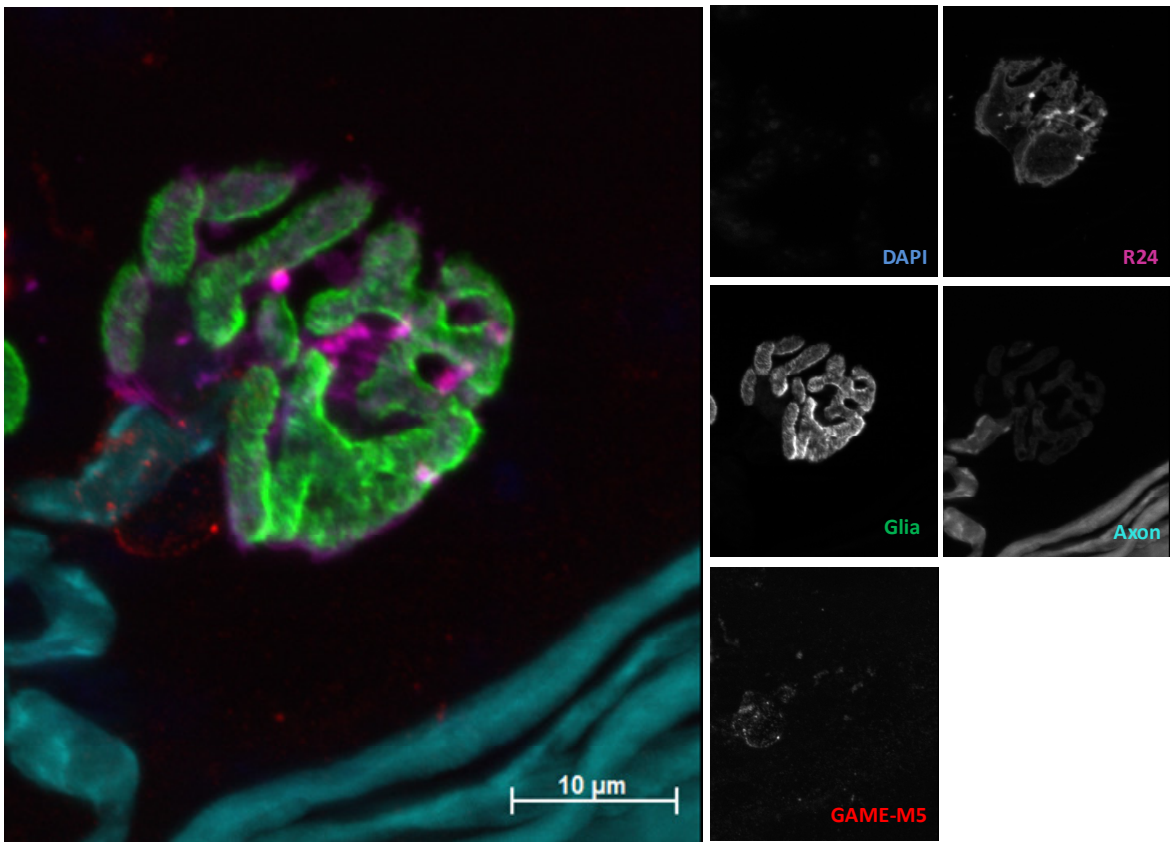


Figure 9.9 - Terminal Myelinating Schwann Cell Characterisation – perisynaptic Schwann cell exclusion

Ex vivo preparations of TS were probed with GAME-M7 and R24. R24 was found to bind specifically to GD3 on the perisynaptic Schwann cells. GAME-M7 was not found to co-localise with the R24 staining, which indicated that the cell was not a pSc.

antibody accessing other binding sites. To overcome this, changes were proposed in the incubation conditions, such as applying the antibody for a longer period of time or permeablising the tissue prior to its addition.

For the longer incubation experiment, GAME-M7 was applied to an *ex vivo* preparation of WT TS for 5 hours at 37°C. Following incubation, the tissue was imaged, permeabilised with 0.5% Triton X-100 and then re-imaged. The purpose of the permeabilisation step was to ensure that the absence of antibody binding was not due to internalisation. The results indicated little difference between the two treatments, as antibody binding was only detected on the terminal myelinating Schwann cells in both the pre and post permeabilised tissue (Figure 9.12).

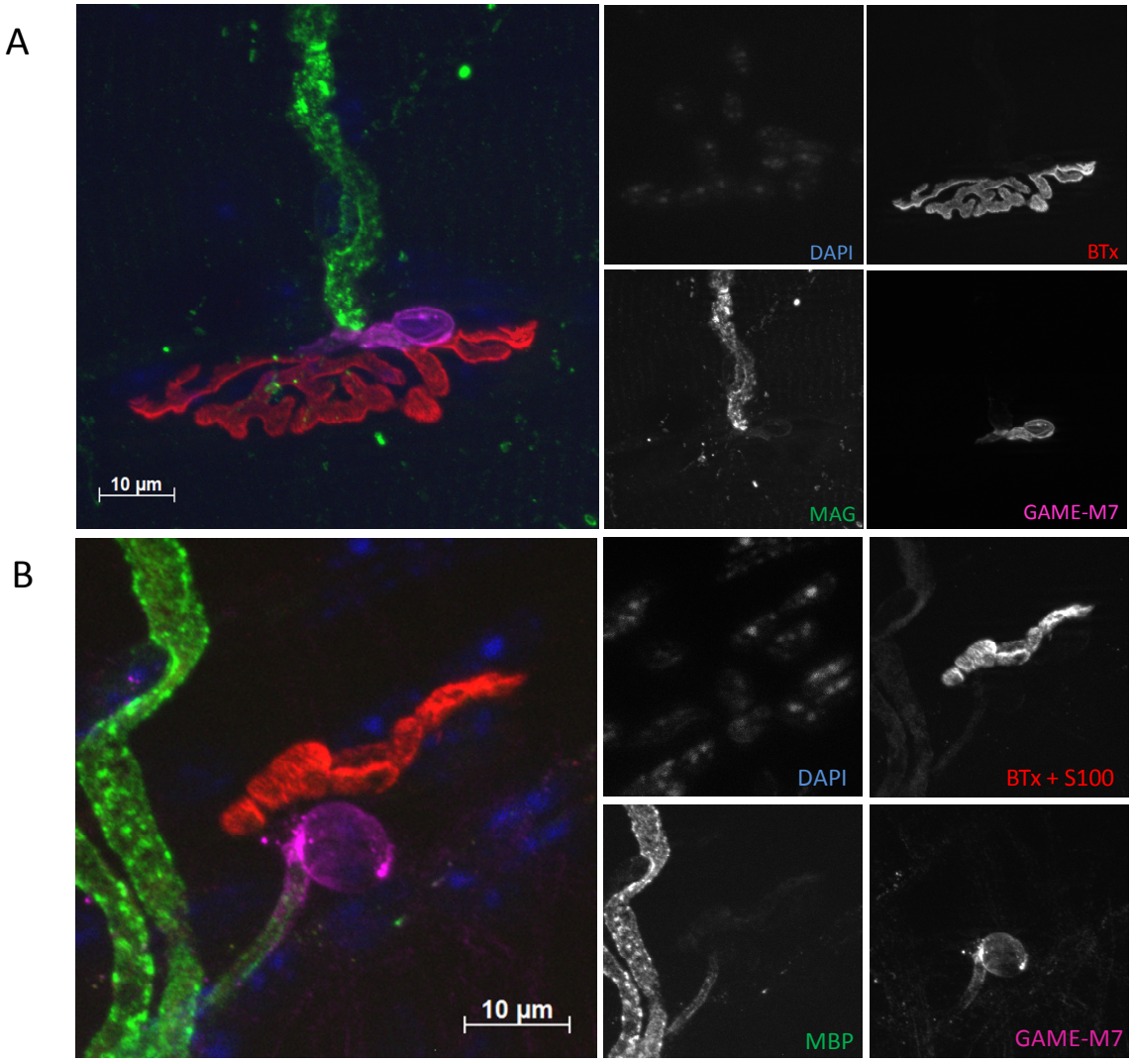


Figure 9.10 – Terminal Myelinating Schwann Cell Characterisation – Myelin Markers

Ex vivo preparations of TS were probed with GAME-M7 (100µg/ml) for 1 hour and permeabilised. Anti-MAG or Anti-MBP antibodies were then applied overnight. A: The axon was MAG positive but the cell body of the terminal myelinating Schwann cell was not. B: The axon was also MBP positive but the cell body was not. There appeared to be a degree of polarisation of sulfatide positivity towards the NMJ in both tissues.

Two possible reasons were given for these findings; either incubation times are irrelevant in overcoming the BNB or the antibody does not bind other Schwann cells.

The pre-permeabilisation experiment sought to address which of these two reasons were valid. *Ex vivo* preparations of WT TS were therefore permeabilised and probed with GAME-M7 to establish if other sites could be targeted by anti-

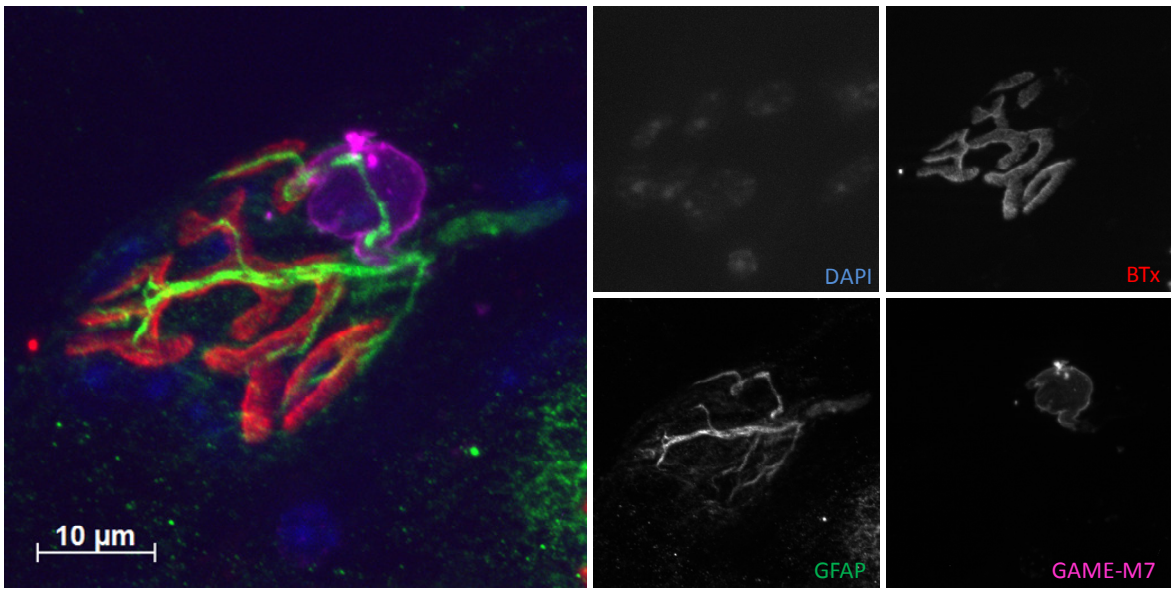


Figure 9.11 –Terminal Myelinating Schwann Cell Characterisation – GFAP

An *ex vivo* preparation of TS was probed with GAME-M7 (100µg/ml) for 1 hour and permeabilised. An anti-GFAP antibody was applied to determine if the cell was a reactive Schwann cell. The antibody stained the NMJ but did not appear to be focussed on the terminal myelinating Schwann cell, which indicated that it was not reactive.

sulfatide antibodies. It was hypothesised that if the BNB was responsible for restricting antibody access, binding would be detected on the other Schwann cells surrounding the axon.

The results of the experiment, however, disproved this hypothesis. No binding was detected on any of the other myelinating Schwann cells but binding was detected on the pSc and their processes (Figure 9.13). This was unexpected as the pSc had been demonstrated to lack sulfatide. Although this binding behaviour was only detected in a few of the NMJ, a possible explanation was that the permeabilisation process had damaged the tissue.

Previous research on the NMJ had established that when the axon is damaged, the terminal myelinating Schwann cell will extend processes into the junction to aid in repair (Brill et al., 2011). This appears to explain the observations from this experiment and corroborates the belief that the cell was responsible for maintaining and repairing the nerve terminal.

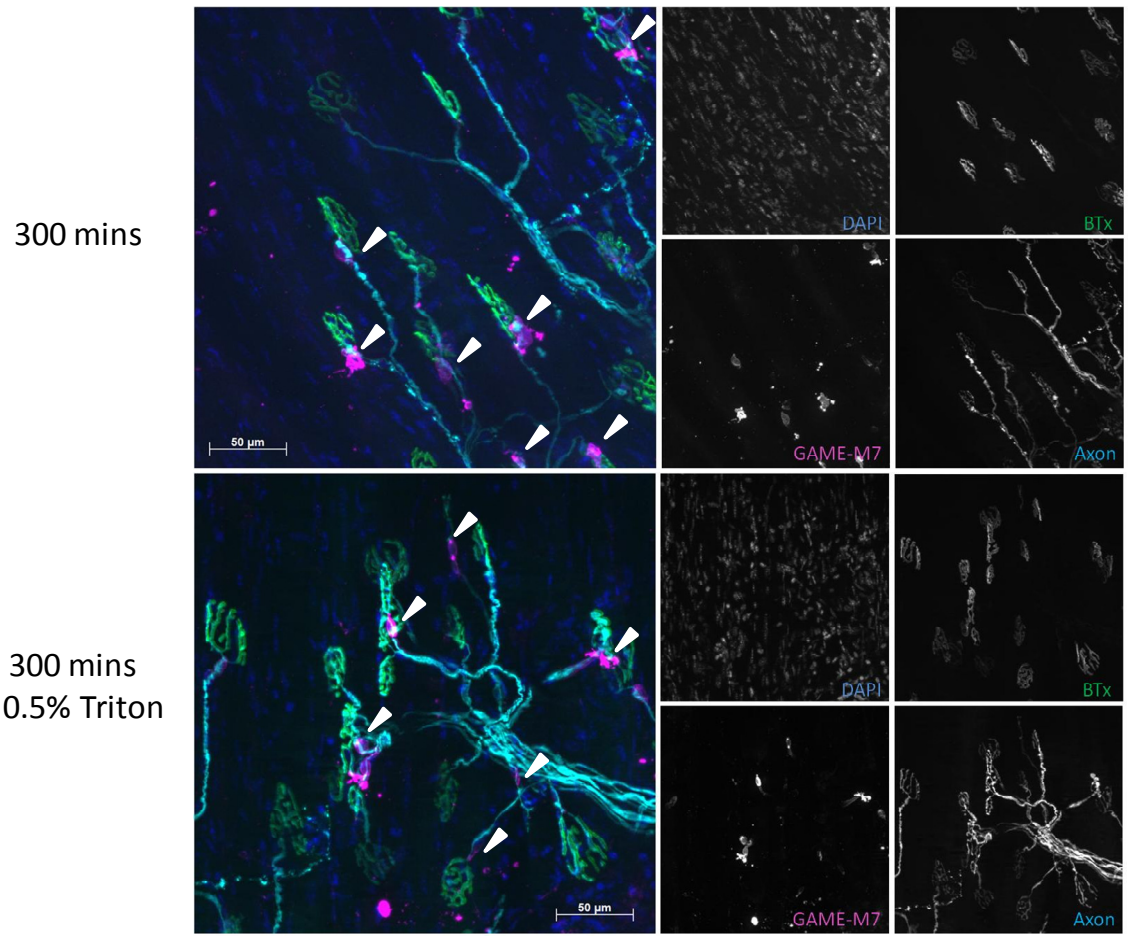


Figure 9.12 – Longer Incubations of GAME-M7 with *ex vivo* preparations of WT TS.

Ex vivo preparations of WT TS were incubated with GAME-M7 for 5 hours. Following incubation, the tissue was imaged and then permeabilised with 0.5% Triton X-100 and reimaged. Binding was only found to occur on the terminal myelinating Schwann cells (arrows) in both the pre and post permeabilised tissue.

9.2.5.3 Complement Mediated Injury

Whilst, the previous experiments confirmed that GAME-M7 was specifically targeting the terminal myelinating Schwann cell, it was still unclear if the antibody itself was able to induce an injury. To address this, a complement kill was performed in which the antibody and normal human serum (NHS) were applied to tissue, alongside a control that contained NHS only. A nucleus marker, EthD-1, was then applied and the number of positive nuclei were counted on each tissue section.

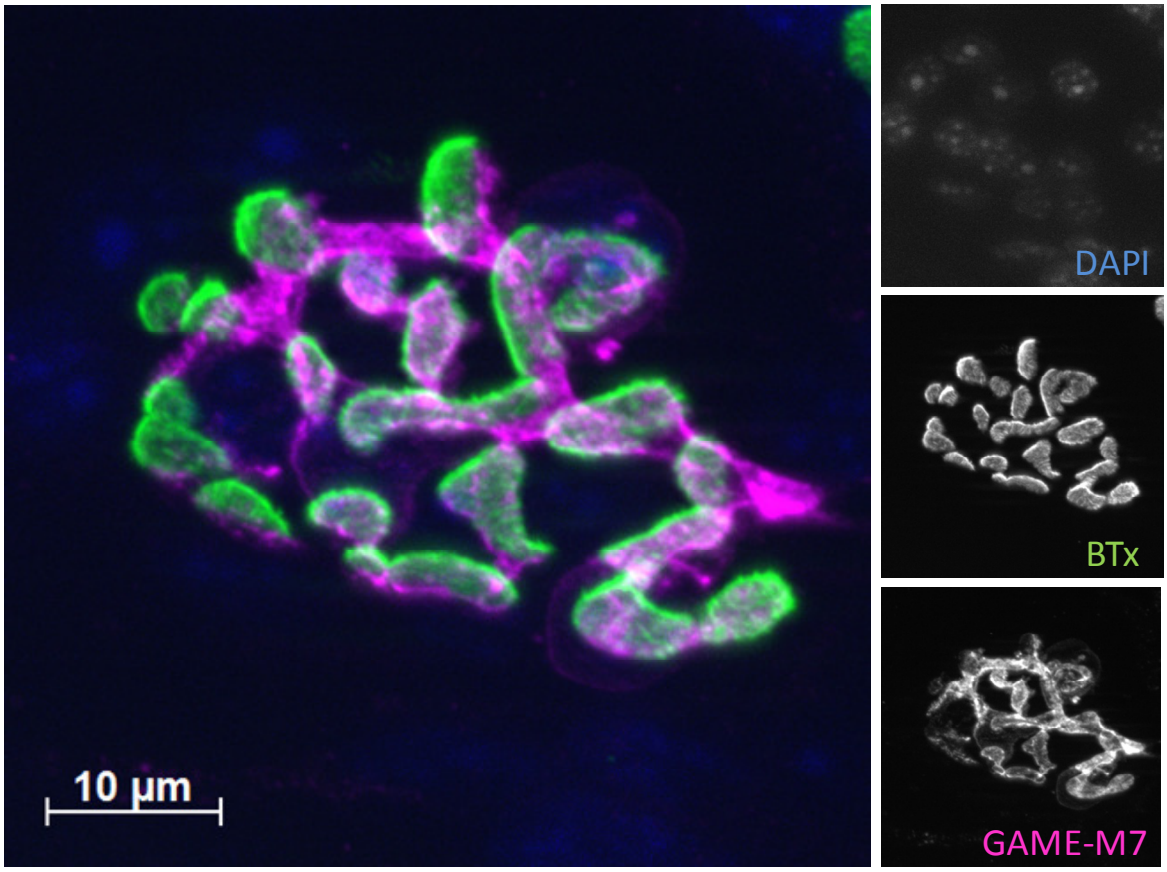


Figure 9.13 – Binding of GAME-M7 to Permeabilised Tissue

Ex vivo preparations of WT TS were light fixed in 1% PFA, followed by incubation with GAME-M7. No binding was detected to myelinated Schwann cells but binding was detected on the pSc and their processes. This only occurred infrequently in the tissue.

As GAME-M7 was not applied to the control tissue, the kink in the axon as it joined the NMJ was used to estimate the position of the terminal myelinating Schwann cell (Figure 9.14). The results of these counts indicated that GAME-M7 killed a significantly higher number of cells compared to NHS alone (Fisher's exact test, $P<0.0001$).

This data therefore confirmed that the antibody was pathogenic, which suggested that these cells may be the target of immune mediate injury in patients with high titres of anti-sulfatide antibodies. Further experiments will need to be performed to verify these findings and determine the relevance of the terminal myelinating Schwann cells in maintaining and repairing the NMJ in health and disease.

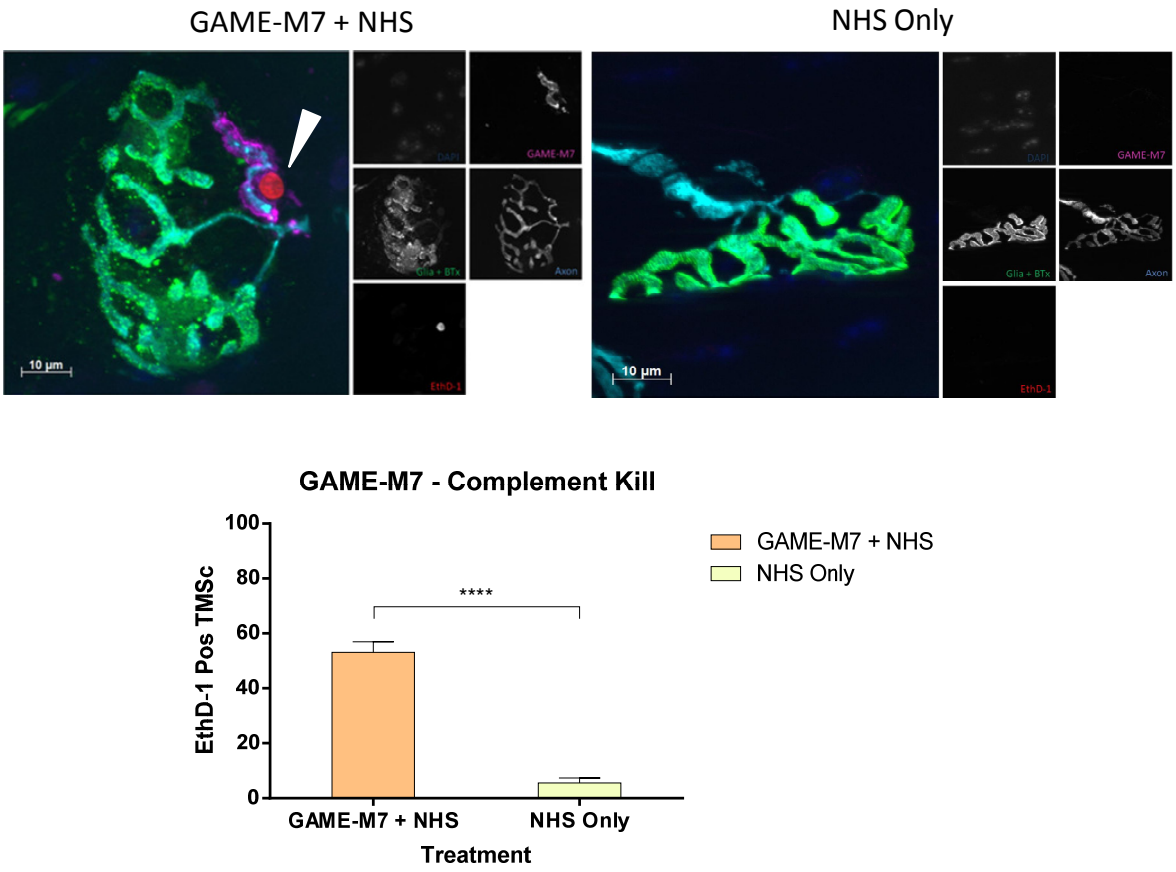


Figure 9.14 –GAME-M7 induced complement kill of Terminal Myelinating Schwann Cell

An *ex vivo* preparation of WT TS was probed with GAME-M7. Control tissue was probed with Ringer's solution alone. Normal human serum (NHS) was then applied to both tissues, followed by the application of EthD-1. As the control tissue did not contain the mAb, the kink in the axon as it joined the NMJ was used to estimate the position of the terminal myelinating Schwann cell. The number of EthD-1 positive nuclei in these positions was counted on each tissue. The experiment indicated that GAME-M7 injured a significantly higher number of terminal myelinating Schwann cells (arrow) than NHS alone (Fisher's exact test, $P<0.0001$) (n=2)

Unfortunately, these experiments could not be performed within the context of this thesis due to time restrictions but would be beneficial to furthering our understanding of the pathogenicity of anti-sulfatide antibodies.

9.3 Discussion

Although anti-sulfatide antibodies have long been associated with a wide range of demyelinating conditions (Alpa et al., 2007; Andersson et al., 2002; Ilyas,

2003; Souayah et al., 2007), little is known about their specific mechanisms of action or whether they are in fact responsible for bringing about neurological injury.

This, in part, is due to the discrepancy between animal and human studies. Whilst the preeminent mouse anti-sulfatide antibody, O4, has been demonstrated to bring about demyelination and dysmyelination in CNS cultures (Elliott et al., 2012; Rosenbluth & Moon, 2003), there has been difficulty in replicating the same findings using patient derived antibodies.

Although some researchers have shown that these antibodies bind the sulfatide enriched myelin membrane (Petratos et al., 2000), others have found that binding could not be detected in either live CNS myelin or cells from the oligodendrocyte lineage (Brennan et al., 2011).

The disparity between these findings is difficult to explain, but has been attributed to differences in the fine specificities of anti-sulfatide antibodies. O4, for example, has been shown to bind specifically to a sulfatide:SM complex, which is thought to form on the surface of the myelin sheath (Brennan et al., 2011). If human antibodies are unable to bind this same complex, then it would be unlikely that they could bind tissue, raising the possibility that O4 is not entirely representative of the antibodies that arise in neuropathies.

In an attempt to address the issue of antibody diversity, we generated several anti-sulfatide antibodies in the hope of creating one that more closely resembled those found in natural disease states. The aim of this chapter was to characterise the binding behaviours of these antibodies, through the use of both *in vitro* and *ex vivo* techniques, to establish their pathogenicity and whether they were representative of those antibodies that arise in demyelinating neuropathies.

The initial characterisation experiments focussed on establishing the antibodies binding patterns to sulfatide and associated complexes. These studies demonstrated how the binding signals of the different antibodies were affected by the presence of different glycolipids, particularly gangliosides and SM.

The inhibitory behaviour of gangliosides such as GD1a and GD1b, was similar to that observed with anti-ganglioside antibodies (Greenshields et al., 2009; Nobile-

Orazio & Gallia, 2013). This suggested that the terminal sialic acid of the ganglioside headgroup was interacting with the sulfatide molecule, in a similar manner as it did with GM1, to obscure the binding epitope.

Similarly, the presence of other gangliosides and lipids with long fatty acid chains, such as SM, would likely prevent certain antibodies accessing the sulfatide molecule, due to steric hindrance (Alving & Richards, 1977). These inhibitions varied between the different antibodies, however, with GAME-M2, GAME-M6 and GAME-M7 showing only slight inhibition with GD1a and GD1b, whilst the binding signal of GAME-G1 was completely abolished by all complex gangliosides and SM.

Unusually, the ganglioside cis-inhibitions did not directly translate into differences in tissue binding patterns. In fact the only antibody that was found to be incapable of binding most, if not all, tissue was GAME-G1. It was hypothesised that this may be related to the presence of sulfatide:SM complexes in the plasma membrane. As indicated by the combinatorial glycoarray screening, these complexes would prevent antibody binding, which is consistent with the observations made using human derived anti-sulfatide antibodies (Brennan et al., 2011). This strongly supports the theory that sulfatide:SM complexes, rather than sulfatide, are the true targets of demyelinating antibodies in tissue.

Aside from GAME-G1, the new anti-sulfatide antibodies behaved in a similar manner as O4. They were found to bind sulfatide enriched cells and tissues, including OPCs, Schwann cells and sciatic nerves (Mirsky et al., 1990; O'Rourke et al., 2014) but did not appear to differ substantially in their relative binding intensities. It was only when applied to *ex vivo* preparations of TS that the new antibodies were found to behave in a different manner.

In this tissue, they appeared to bind exclusively to a cell on the periphery of the NMJ, known as the terminal myelinating Schwann cell. In contrast, O4 was not found to bind this cell whatsoever, suggesting that the presence of an unexamined lipid or protein was inhibiting the antibody from accessing its binding epitope.

This novel finding was particularly interesting, as it suggested that more diverse anti-sulfatide antibodies existed than had previously been described.

Furthermore, the discovery that these antibodies bound exclusively to a cell in proximity to the NMJ, suggested that they may have a role in paralytic neuropathies.

To further clarify these roles, experiments were performed to characterise this cell and its properties. The use of CST-/- mice confirmed that the antibodies were specifically binding to sulfatide, whilst the use of markers proved that it was indeed a Schwann cell. Preliminary investigations into its other functions were inconclusive but suggested that it may have roles in myelinating the axon.

Examination of the literature found little mention of the cell but limited work, using laser ablation of individual Schwann cells at the NMJ, had suggested that it may function to repair the axon during Wallerian degeneration (Brill et al., 2011). Further to this, it appears that it forms tight paranodal adhesions with the axon to prevent retrograde growth of the pScs suggesting that it may also have a major role in maintaining normal NMJ morphology.

Through the use of a complement kill, it was confirmed that GAME-M7 was pathogenic and that the terminal myelinating Schwann cell can be the target of immune mediated injury. If this translates into human disease, it suggests that the cell may be targeted and injured in patients with high titres of circulating anti-sulfatide antibodies, which may result in a degree of dysfunction.

Unfortunately, time constraints limited further characterisation of both the anti-sulfatide antibodies and the terminal myelinating Schwann cell. However, the cells and particularly the antibodies hold great potential for investigating the roles of sulfatide in health and disease and should certainly be employed in future studies.

10 DISCUSSION

10.1 Main Findings

Multifocal motor neuropathy has been associated with anti-GM1 antibodies since its original conception in 1988 (Pestronk et al., 1988). Whilst the enhancing effects of accessory lipids, such as GalC and sulfated cholesterol, were noted not long after (Pestronk et al., 1997), it was not until recently that researchers began to intensify the use of ganglioside complexes in the interest of improving antibody detection (Galban-Horcajo et al., 2013; Nobile-Orazio & Gallia, 2013).

As with the original studies, these experiments found that GM1:GalC complexes were capable of greatly improving the antibody detection rate; however, an interesting observation was made regarding an antibody subset. Whereas, most antibodies bound to both the single ganglioside and the complex, this subset appeared to only be capable of binding to the latter. This led some researchers to postulate that these antibodies may be binding to a neo-epitope formed by the combination of two different lipids (Rinaldi et al., 2010).

If this hypothesis was correct, then it would suggest that a new class of antibody may exist that is complex-dependent. As serum studies detected higher levels of binding to these complexes than to GM1, it also raised the possibility that GM1:GalC complexes may be the true targets of immune mediated injury in MMN. Based upon this premise, the main aim of this thesis was to either isolate one of these antibodies from patient serum or produce one through active immunisations with mice.

As the thesis evolved, however, it became apparent that this premise may have been misguided. Analysis of the binding patterns of human monoclonal antibodies, patient sera studies and mouse immunisations indicated that what appeared to be complex-dependent antibodies were more likely low concentrations of normal antibodies that were cis-enhanced by particular lipids.

Despite not achieving its main aim, this thesis still furthered knowledge within the field of neuroimmunology through several novel findings. These included determining how the concentration of accessory lipids in complexes influences antibody binding, improvements in hybridoma production and the creation of several new anti-ganglioside and anti-sulfatide antibodies. These findings, along with their impact on current MMN research, are summarised here.

10.1.1 The binding signals of human monoclonal anti-ganglioside antibodies can be influenced by the presence of secondary lipids

The work carried out in the first sections of this thesis focussed on assessing the cis-inhibitions and cis-enhancements of several human monoclonal antibodies. Despite being employed in vast amounts of research (O'Hanlon et al., 1996, 1998; Paterson et al., 1995; Willison et al., 1994), the fine specificities of these antibodies had yet to be fully elucidated, which impacted on determining their roles in disease.

The results from these experiments confirmed many of the findings of previous research. In particular, they demonstrated that gangliosides containing terminal sialic acids, such as GD1a and GD1b, can obscure binding epitopes on the headgroup of GM1 molecules to prevent antibody binding (Greenshields et al., 2009; Nobile-Orazio et al., 2010). This finding, more than any other, may explain the restricted injury experienced in MMN.

Assuming that the monoclonal antibodies are representative of those found in patient sera, it could be postulated that the expression of GM1 in the absence of GD1a leaves certain tissue vulnerable to immune mediated attack. In contrast, a level of protection may be bestowed upon those tissues, which contain GM1:GD1a complexes, as the interaction of the two gangliosides will shield particular GM1 binding epitopes from the immune system. This will prevent certain antibodies from binding the tissue, thus preventing injury.

This theory is somewhat supported by experimental data, as it has been demonstrated that anti-GM1 antibodies preferentially target the myelin and nodes of Ranvier (NoR) in motor nerves (Gong et al., 2002; Susuki et al., 2012). However, a sufficient explanation for why these sites are targeted and other

GM1 enriched tissues are spared has yet to be elucidated. The GM1:GD1a complex theory may offer a possible explanation but requires further study to verify this assumption.

Aside from gangliosides, the antibodies binding signals were also found to be influenced by the presence of other lipids, including Chol, GalC, sulfatide, PS, SM and PC. Whilst the enhancing effect of GM1:GalC and GM1:sulfatide had been demonstrated previously (Galban-Horcajo et al., 2013, 2014; Nobile-Orazio et al., 2013), little data could be found on the abilities of the other lipids to enhance antibody binding.

Based upon their comparatively small size and their expression in plasma membranes(Brown & Rose, 1992; DeBruin et al., 2005; Pike et al., 2002; M. Simons et al., 2000), however, it was hypothesised that they improved binding signals through their interactions with the ganglioside headgroup. Evidence from previous research supported this theory, as it has been shown that the various functional groups of lipids can form hydrogen bonding networks with gangliosides to alter their configuration (Lin et al., 2008; Lingwood & Simons, 2010; Lingwood et al., 2011; Mombelli et al., 2003; Stoffel & Bosio, 1997). This results in the exposure or concealment of different binding epitopes, which can affect the binding capabilities of anti-ganglioside antibodies.

As GalC contains a higher number of hydrogen donors and acceptors than the other lipids (Hall et al., 2010), it is likely that it is able to confer a specific orientation upon a ganglioside. Certain antibodies may bind to epitopes that are optimally exposed in this orientation, which may explain the high levels of enhancements that clinical studies have found using GM1:GalC complexes.

This finding, however, cast doubt upon the theory that neo-epitope formation is responsible for antibody enhancement. In fact, the similar enhancing ability of the different accessory lipids was much more supportive of the theory of conformational modulation. If this process is in fact responsible for antibody enhancement, then it suggests that the gangliosides local microenvironment is an important factor in determining the binding abilities of anti-ganglioside antibodies and the vulnerability of tissues to immune mediated attack.

10.1.2 The concentration of accessory lipids in complexes have differing effects on antibody binding

Subsequent chapters expanded on these findings further by analysing the effects differing concentrations of accessory lipids had upon antibody binding. This is a concept that has garnered little attention in array design, which is surprising considering that the proportions of lipids will differ in tissues in relation to their function and location. The results from these studies clearly demonstrated that higher concentrations not only enhanced certain binding signals but also distinguished the binding patterns of similar looking antibodies from one another.

This led to the antibodies being classified as either concentration-dependent or concentration--independent based upon their abilities to bind ganglioside complexes containing high concentrations of accessory lipids. Under the assumption that these differences were related to the accessibility of the binding epitopes, it was hypothesised that varying the concentrations of accessory lipids had differential effects on the ganglioside headgroup orientation. This would result in the exposure of concealment of certain binding epitopes, which would alter the binding abilities of anti-ganglioside antibodies.

The mechanism behind this interaction was again thought to be conformational modulation (Harschnitz et al., 2014). High concentrations of accessory lipids would form a considerably larger hydrogen bonding network with gangliosides than lower concentrations (Hall et al., 2010). As a result, they would be able to pull the ganglioside headgroup into a position more parallel to the membrane, which would optimally expose previously hidden binding epitopes. It was assumed that the concentration-dependent antibodies bound to these epitopes, whereas the concentration-independent antibodies bound to those that were readily available when the headgroup was perpendicular to the membrane (Figure 10.1).

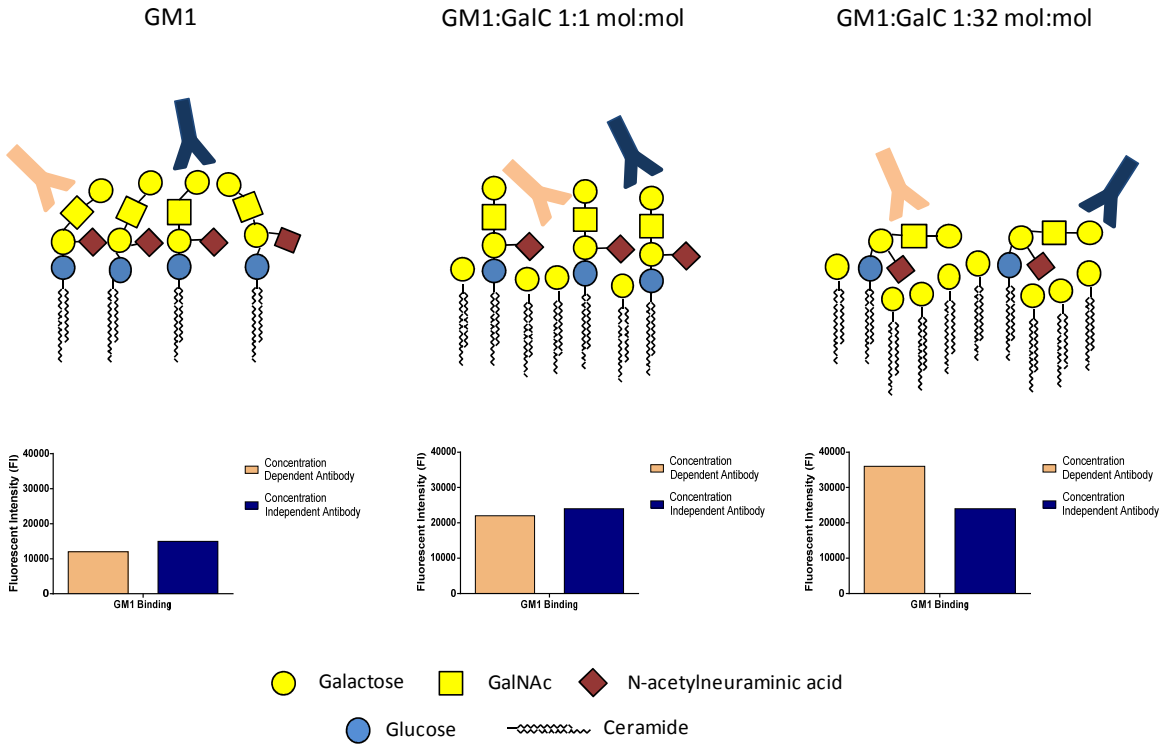


Figure 10.1 – The concentration of accessory lipids has differing effects on antibody binding

Both the concentration-dependent (orange) and concentration independent (blue) antibodies bind to different epitopes on the GM1 headgroup. When low proportions of GalC are added to the complex, the gangliosides change configuration, which improves the presentation of both binding epitopes. This enhances the binding ability of both antibodies. When the concentration is increased further, the ganglioside is pulled parallel to the membrane, better exposing the concentration-dependent epitope. As a result the binding signals of the concentration dependent antibodies increase further. In contrast, the binding signals of the concentration-independent antibodies do not change as the ganglioside epitope is already as well presented as possible.

This demonstrated that these antibodies, which appeared very similar on ELISA, behaved completely differently when the lipid concentrations of the local microenvironment were modified. This was a concept that had not been explored previously but had repercussions, not only in assessing the diversity of antibodies, but also in determining their targets *in vivo* and the vulnerability of tissues to immune mediated attack. Furthermore, it also suggested that the - standard 1:1 w:w complex configurations employed in clinical assays (Nobile-Orazio & Gallia, 2013; Rinaldi et al., 2009) may be insufficient for detecting all classes of antibody.

Although we were unable to demonstrate this using MMN patient serum ourselves, it does not mean that lipid concentration is not an important factor in antibody detection. In fact, parallel developments during the creation of this thesis showed that increasing the concentration of GalC in complexes improved the detection of anti-GM1 antibodies (Delmont et al., 2015), which corroborates this research.

Taken together, these results suggest that the binding specificities of anti-ganglioside antibodies are much more complicated than previously appreciated. The key to identifying biomarkers in patient sera therefore lies in creating diverse sets of antigens that resemble the endogenous plasma membrane as closely as possible. The work contained within this thesis and carried out within our laboratory (Halstead, unpublished data) are beginning to show the advantages of using these techniques, but researchers are going to have to increase the complexity of assays to further improve disease diagnosis.

10.1.3 Conformational modulation is responsible for antibody enhancement

Although not demonstrated at a molecular level, the results from these studies provided strong evidence that conformational modulation, rather than neo-epitope formation, was responsible for antibody enhancement. This was further confirmed through our attempts to clone a complex-dependent antibody directly from patient sera (data not shown). Whilst this attempt failed, analysis of the screening techniques indicated that antibodies that had previously been designated complex-dependent were actually standard antibodies that were cis-enhanced.

This strongly suggested that neo-epitope formation and complex-dependent antibodies were not responsible for the pathogenesis found in MMN. It is key to note, however, that this thesis did not prove this definitively. These antibodies may still exist but may require more specialised techniques to isolate and identify them.

10.1.4 Improvements in hybridoma screening aid in identifying larger numbers of antibodies

Despite not achieving our main aim of producing a complex-dependent antibody, substantial improvements were still made in hybridoma production. These were necessary as the traditional ELISA method for antibody detection (Goodyear et al., 1999) was found to be insufficient for the aims of this thesis. This was due to the limitations of the technique, which could only be used to screen one antibody isotype against one target antigen.

This was particularly problematic for high throughput screening, as the requirement to use high levels of supernatant, compounded with the quick growth rates of the hybridoma cells, made it impossible to successfully rescreen supernatant twice.

To overcome these issues, we employed the use of the lipid microarray. This technique operated on a similar principle as the combinatorial glycoarray (Rinaldi et al., 2010) but worked on a much larger scale. Whereas the combinatorial glycoarray could print multiple targets to screen 12 samples in one run, the microarray was capable of printing a higher number of targets for screening up to 320 samples.

In addition, the microarray employed the use of fluorescent antibodies as a secondary detection method, whereas ELISA used HRP-conjugated secondary antibodies. As a result the microarray was able to screen for two different antibody isotypes simultaneously, which allowed for the production of a larger number of antibodies from one mouse.

This technique also allowed for an antibody to be partially characterised prior to the cloning step of production. Antibodies could therefore be selected for cloning based upon their desired binding patterns, which could be particularly useful for quickly selecting antibodies of interest.

Overall, the improvements in screening were substantial. Through the use of the lipid microarray, supernatant could be screened quickly, larger numbers of antibodies could be detected and different isotypes could be selected. Whilst other researchers have produced similar techniques utilising their own bespoke

equipment (De Masi et al., 2005; Staudt et al., 2014), this appears to be the first time, to our knowledge, that a lipid microarray has been employed for the production of anti-carbohydrate antibodies.

10.1.5 The combination of different characterisation methods aids in determining the binding behaviours of anti-carbohydrate antibodies

Although it was not our original intention to create more antibodies against single lipid antigens, the success of the hybridoma fusions led us to produce a series of antibodies targeting gangliosides, glycolipids and sphingolipids. Subsequent studies determined the binding behaviours of these antibodies through the combination of various characterisation methods, including solid phase assays and immunofluorescence.

One of the main benefits of combining different techniques was that they could be used to reconcile differences in antibody binding patterns. For example, the anti-GM1 antibody, GAME-G2, was found to be incapable of binding tissue unless it was first treated with neuraminidase. Examination of this antibody on solid-phase assays explained this behaviour as it was demonstrated that the antibody was incapable of binding to GM1 when it was in complex with GD1a. By treating the tissue with neuraminidase, the sialic acid of GD1a was cleaved and the ganglioside was converted to GM1, thus allowing binding to take place.

This same approach aided in explaining the tissue binding patterns of the other antibodies, which helped determine their potential uses in future research. Detailed descriptions of these uses are provided within Chapters 7 and 9 but they range from localising gangliosides within the plasma membrane to determining the vulnerability of particular tissues to antibody induced injury.

10.1.6 Antibody diversity is an important factor in determining the pathogenicity of anti-sulfatide antibodies

Despite their implication in many demyelinating conditions, few monoclonal anti-sulfatide antibodies have ever been produced (Somner & Schachner, 1981). This, in part, is due to the success of the preeminent antibody, O4, which has

been shown to induce demyelination of sulfatide enriched cells and tissues both *in vitro* and *in vivo* (Elliott et al., 2012; Rosenbluth & Moon, 2003; Rosenbluth et al., 2004).

The downfall of restricting research to just one anti-sulfatide antibody, however, is that antibody diversity will not be taken into account with the results. Not all antibodies will bind tissue in the same manner, raising questions about how representative O4 is of the general anti-sulfatide population.

To address these issues, we generated a series of anti-sulfatide antibodies and probed them against various cells and tissues. As predicted, these antibodies displayed differential binding patterns, with some binding avidly to plasma membranes whilst others were incapable of binding at all.

Analysis of the antibodies binding specificities in solid phase assays indicated that they differed in their abilities to bind sulfatide in the presence of other lipids. This suggested that they may bind to different components of the sulfatide molecule and may differ from O4.

This was further confirmed through comparisons of the antibodies binding patterns in *ex vivo* preparations of mouse TS. In this tissue, the new anti-sulfatide antibodies were found to bind to the terminal myelinating Schwann cell, whereas no binding was detected with O4.

This suggested that some unknown lipid or protein in the local microenvironment of this cell was preventing O4 from accessing its binding epitope. This clearly demonstrated that the new antibodies, although similar to O4, were distinct in their ability to bind certain tissues.

This has ramifications in assessing the pathogenicity of anti-sulfatide antibodies in various neuropathies. As GAME-M7 was shown to bind and injure the cell, it could be postulated that similar antibodies may arise in patients to bring about a form of neurological dysfunction not experienced by patients with antibodies that resemble O4.

On the other side of the spectrum was the antibody GAME-G1, which was shown to be incapable of binding to any form of tissue. As discussed earlier, anti-sulfatide antibodies were isolated from the CSF of MS patients but were found to be incapable of binding sulfatide enriched cells or tissues (Brennan et al., 2011).

It is likely that GAME-G1 resembles these antibodies, as both were shown to be incapable of binding sulfatide in the presence of SM.

The absence of tissue binding antibodies could suggest that anti-sulfatide antibodies are not pathogenic in neuropathies but the work of a colleague that was produced in conjunction with this thesis may offer a possible explanation. She demonstrated that pathogenic antibodies are likely to bind tissue, illicit injury and be internalised, whereas non pathogenic antibodies will remain in the circulation (Cunningham, unpublished observations).

It is therefore possible that antibodies, such as O4 and GAME-M7 bind and injure sulfatide enriched tissue and become internalised, whereas antibodies like GAME-G1 arise but remain in the circulation as they are unable to bind the glycolipid in the plasma membrane. Further work would need to be performed to confirm this hypothesis but the fact remains that anti-sulfatide antibodies may well be major factors in immune mediated injury in a variety of neuropathies.

10.2 Future Work

10.2.1 Improvements in the reliability of biomarkers

The results within this thesis provided strong evidence that the configuration of a gangliosides local microenvironment has a large influence on the binding abilities of anti-ganglioside antibodies. This has been a concept that has been largely overlooked in neuropathy research despite ganglioside complexes being used in clinical studies since 2004 (Kaida et al., 2004).

This is due to researchers focussing on associating particular antibody enhancements with different disease subtypes and outcomes rather than the composition of the complexes themselves (Créange et al., 2014; Kaida & Kusunoki, 2010; Notturno et al., 2009). However, this is an aspect of research that must be taken into consideration if we are to improve the reliability and effectiveness of biomarkers in disease diagnosis.

Our laboratory has recently developed a lipid microarray technique, which allows serum to be screened against up to 400 separate targets simultaneously (Halstead, unpublished data). This equipment has allowed us to increase the

complexity of ganglioside complexes, by taking factors such as concentration and content into consideration.

Ideally, complexes should resemble the plasma membrane as closely as possible. To achieve this, researchers are going to have to create antigen combinations consisting of a number of different gangliosides and accessory lipids at varying concentrations. By creating antigens that closely mimic the site of injury, it is much more likely that antibodies can be detected in sera that are reliable indicators of disease.

This work, however, relies on the assumption that circulating anti-ganglioside antibodies are pathogenic. As discussed previously, a colleague's work has suggested that pathogenic antibodies bind to gangliosides, elicit injury and are removed by internalisation (Cunningham, unpublished data). The remaining antibodies would therefore be unable to bind tissue and would remain in the circulation.

If this same process is demonstrated in humans, then it would suggest that using patient serum to diagnose autoimmune neuropathies may be unreliable. Non-binding antibodies may still be generated in certain conditions and may act as indicators of disease; however, further work needs to be performed to determine the validity of anti-ganglioside antibodies in the diagnosis of autoimmune neuropathies.

10.2.2 Determining the existence of neo-epitopes and complex-dependent antibodies

The evidence from these studies provides strong support that conformational modulation is responsible for the enhancement produced by GM1:GalC complexes with MMN patient serum. However, despite the work carried out within this thesis, we were unable to demonstrate the interactions of these lipids at a molecular level.

It is still possible that gangliosides may interact with one another to form neo-epitopes, however, the abundance of these formations within the plasma membrane and their relevance in disease are unclear. The ideal method for elucidating whether antibodies could arise against these neo-epitopes would be

active immunisations with complexes consisting of covalently linked gangliosides.

These complexes have been created by groups as this thesis was developed (Mauri et al., 2012); however, no immunisations have been performed to determine whether specific complex-dependent antibodies can be produced. This would be a worthwhile pursuit as the only way to prove or disprove the existence of these antibodies is to induce their generation *in vivo*. If these antibodies could be produced and were found to bind specific sites in the plasma membrane then it would suggest that they may well arise in humans and may be responsible for the pathogenesis of various autoimmune neuropathies.

10.3 Closing Statement

Overall this thesis was able to shed some light on the complexities of anti-glycolipid antibodies. Through the analysis of their binding behaviours in solid phase assays and tissues, we were able to elucidate how the local microenvironment influences epitope access and how this affects the pathogenicity of these antibodies in disease.

Furthermore, in our attempt to isolate a complex-dependent antibody, we were able to generate 10 new monoclonal antibodies targeting various gangliosides and glycolipids. These antibodies, particularly those targeting sulfatide, may be very useful in furthering our understanding of immune mediate attack in different neuropathies.

Most importantly, this thesis demonstrated that diversity is an important factor in understanding the binding behaviours of different antibodies. This is a concept that is only truly beginning to be appreciated but has implications in understanding the roles that these antibodies have in both health and disease.

List of Publications

Papers

Yao D, McGonigal R, Barrie JA, Cappell J, Cunningham ME, Meehan GR, Fewou SN, Edgar JM, Rowan E, Ohmi Y, Furukawa K, Furukawa K, Brophy PJ, Willison HJ (2014) Neuronal Expression of GalNAc Transferase Is Sufficient to Prevent the Age-Related Neurodegenerative Phenotype of Complex Ganglioside-Deficient Mice. J Neurosci 34:880-891.

Delmont E, Robb H, Davidson A, Halstead S, Yao D, Meehan GR, Willison HJ (2015) Prospective study comparing enzyme-linked immunosorbent assay and glycoarray assay to detect antiglycolipid antibodies in a routine diagnostic neuroimmunology laboratory setting. Clin Exp Neuroimm 6:175-182.

Abstracts

Meehan GR, Galban-Horcajo F, Halstead S, Barrie J, Willison HJ (2013). *Generation of anti-GM1 complex antibodies in an active immunisation mouse model*. PNS Biennial meeting, Saint Malo, June 29-July 3, 2013. J.PNS 18:S1-S131.

Yao D, Rinaldi S, Meehan GR, Barrie J, McGonigal R, Greenshields K, Cunningham M, Fewou S, Cappell J, Furukawa K, Brophy P, Willison HJ (2012). *Axonal expression of gangliosides in ganglioside deficient mice facilitates an active immunisation model of acute motor neuropathy*. PNS/INC congress, Rotterdam, June 24-27, 2012.

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