



**CHARACTERISATION OF AQP4-SPECIFIC MONOCLONAL
ANTIBODIES IN NEUROMYELITIS OPTICA SPECTRUM
DISORDERS**

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submitted in partial fulfilment of the requirements for the degree of
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Declaration

I hereby declare that this thesis titled “Characterisation of AQP4-specific monoclonal antibodies in neuromyelitis optica spectrum disorders” with approximately 41,733 words excluding appendices and references is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (DPhil) at the University of Oxford. The work presented is a product of my original research with valuable assistance from others in some areas as detailed below:

Chapter	Area	Collaborator
III	Isolation of single B cells from peripheral blood and thymus samples	Dr Sudarshini Ramanathan
	Amplification of immunoglobulin gene transcripts	Dr Deniz Akdeniz
IV	Poly and autoreactivity ELISAs of monoclonal antibodies	Dr Miriam Fichtner

Figures, tables, and data adapted from work by others have been acknowledged and appropriately referenced. This study was mainly performed across two sites, the Nuffield Department of Clinical Neurosciences and the Department of Pharmacology, under the supervisions of Dr Patrick Waters, Prof Sarosh Irani, and Prof Daniel Anthony. At the time of submission, none of the work has been published elsewhere nor that it has been submitted for any other degree at this or any other university.

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List of Abbreviations

AA	: Antibiotic-antimycotic
ABTS	: 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ADCC	: Antibody-dependent cellular cytotoxicity
ADEM	: Acute disseminated encephalomyelitis
AMPA	: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASC	: Antibody secreting cell
AQP4	: Aquaporin-4
BBB	: Blood-brain barrier
BCR	: B cell receptor
Bmax	: Maximum binding
BSA	: Bovine serum albumin
CBA	: Cell-based assay
CDC	: Complement-dependent cytotoxicity
CDR	: Complementarity determining region
CFS	: Cerebrospinal fluid
CHO	: Chinese hamster ovary
CNS	: Central nervous system
CPEC	: Circular polymerase extension cloning
CSR	: Class-switch recombination
CST	: Corticosteroids
D	: Diversity
D2R	: Dopamine 2 receptor
DAPI	: 4',6-diamidino-2-phenylindole
DIV	: Day in vitro
DMEM	: Dulbecco's modified Eagle's Medium
DPPX	: Dipeptidyl-peptidase-like protein 6
dsDNA	: Double-stranded DNA
DTT	: Dithiothreitol
EAAT2	: Excitatory amino acid transporter-2
EAE	: Experimental autoimmune encephalomyelitis
EC50	: Half-maximal concentration
ELISA	: Enzyme-linked immunosorbent assay
FACS	: Fluorescence-activated cell sorting
FBS	: Foetal bovine serum
FI	: Fluorescence intensity
FR	: Framework region
GABA _B R	: γ -aminobutyric acid type B receptor
GC	: Germinal centre
GFAP	: Glial fibrillary acidic protein
HBSS	: Hanks' Balanced Salt Solution
HCDR	: Heavy-chain complementarity-determining region
HEK	: Human embryonic kidney
Hep-2	: Human epithelial type-2
HEPES	: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IgG	: Immunoglobulin G
IgH	: Immunoglobulin heavy chain
IgJ	: Immunoglobulin joining chain
IgL	: Immunoglobulin light chain

IgLON5	: Immunoglobulin-like cell adhesion molecule 5
IgM	: Immunoglobulin M
IIFA	: Indirect immunofluorescence assay
IMDM	: Iscove's Modified Dulbecco's Medium
J	: Joining
K _D	: Equilibrium dissociation constant
LB	: Luria Bertani
LCDR	: Light-chain complementarity-determining region
LDS	: Lithium dodecyl sulphate
LETM	: Longitudinally extensive transverse myelitis
LGI1	: Leucine-rich glioma-inactivated 1
LPS	: Lipopolysaccharide
mAbs	: Monoclonal antibodies
MAC	: Membrane attack complex
MBP	: Myelin basic protein
MDNeg	: Mutated double negative
Mem	: Memory
mGluR5	: Metabotropic glutamate receptor 5
MMF	: Mycophenolate mofetil
MN	: Mature naïve
MOG	: Myelin oligodendrocyte glycoprotein
MRI	: Magnetic resonance imaging
MS	: Multiple sclerosis
MTX	: Methotrexate
N/A	: Not applicable
NEA	: Non-essential amino acids
NEm	: New emigrant
NK	: Natural killer
NMDAR	: N-methyl-D-aspartate receptor
NMOSD	: Neuromyelitis optica spectrum disorders
NT	: Not tested
OAPs	: Orthogonal arrays of particles
OD	: Optical density
PAGE	: Polyacrylamide gel electrophoresis
PBMC	: Peripheral blood mononuclear cell
PBS	: Phosphate-buffered saline
PCR	: Polymerase chain reaction
PEI	: Polyethyleneimine
PES	: Polyether sulfone
PFA	: Paraformaldehyde
PLL	: Poly-L-lysine hydrobromide
PNS	: Paraneoplastic neurological syndromes
PTPN22	: Protein tyrosine phosphatase non-receptor type 22
PVDF	: Polyvinylidene fluoride
RA	: Rheumatoid arthritis
ROUT	: Robust regression and outlier removal
SLE	: Systemic lupus erythematosus
SHM	: Somatic hypermutations
T1D	: Type-1 diabetes
TBE	: Tris-borate-EDTA

TC : Thymic cell
TMB : Tetramethylbenzidine
UCA : Unmutated common ancestor
UDNeg : Unmutated double negative
V : Variable

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Abstract

Neuromyelitis optica spectrum disorders (NMOSD) is among a small but growing group of inflammatory conditions of the central nervous system (CNS) associated with autoantibodies of the IgG class against aquaporin-4 (AQP4), a principal water channel protein in astrocytes. Activation of the classical complement pathway by AQP4-IgG has been thought to initiate the formation of NMOSD lesions. However, clinical and histopathological data suggest the existence of AQP4-IgM and its potential contribution to the disease pathogenesis.

Our first aim was to investigate how early AQP4 specificity emerges along the B cell development. To achieve this, we performed isolation and single B cell culture from subsets found in four peripheral blood samples and a thymic cell sample of four AQP4-seropositive NMOSD patients as well as two peripheral blood samples of two age-matched healthy controls. One of the patients unexpectedly carried a protein tyrosine phosphatase non-receptor type 22 (PTPN22) risk allele which has been shown to interfere with the establishment of B cell tolerance. Nevertheless, we found significantly higher frequencies of AQP4-specific new emigrant B cells in the PTPN22 risk allele-negative patients than healthy controls (0.48% vs 0.1%, $p < 0.05$), suggesting a defective central tolerance checkpoint in NMOSD independent to the PTPN22 gene polymorphisms. This frequency was doubled (1.15%) in the PTPN22 risk allele-positive patient. AQP4-specific B cells were absent in the compartments downstream of the new emigrant in healthy controls and were still detected in the NMOSD patients albeit in reducing frequencies, indicating that the peripheral B cell tolerance may be partially functional in NMOSD.

Characterisation of the immunoglobulin genes revealed a trend towards IgHV2 being overrepresented in the AQP4-specific B cell repertoire. Their heavy chain complementarity determining region 3s (HCDR3s) were typically long and composed of positively charged and hydrophobic amino acids. Additionally, there was a biased usage of the lambda light chain and

downstream IgJK gene segments in those bearing the kappa light chain, suggesting that extensive receptor editing is one of the mechanisms to establish tolerance to AQP4 in NMOSD.

To gain a deeper understanding on the evolution of binding affinity to AQP4, we generated monoclonal antibodies (mAbs) from the isolated single B cells from different B cell subsets and characterise their binding kinetics. The unmutated mAbs demonstrated AQP4 binding exclusively as IgM whereas the mutated counterparts bound the antigen as both IgM and IgG1 antibodies. Characterisation of cell-based assay (CBA) endpoint titres and binding kinetics with all mAbs tested as IgM to allow cross-subsets comparisons revealed a sequential increase in affinity in the new emigrant, mature naïve, and mutated subsets, i.e., mutated double-negative and memory. Furthermore, the unmutated common ancestors (UCAs) of the mutated mAbs were shown to retain their ability to bind AQP4 albeit with reducing affinity, providing evidence that mature AQP4-specific B cells may emerge from the activation of early B cells with relatively lower initial affinity for AQP4 augmented by somatic hypermutations.

Using an optimised *in vitro* complement-dependent cytotoxicity (CDC) assay, we reported that AQP4-IgM had the capability to induce complement deposition on the surface membrane of AQP4 M23-expressing cells *in vitro*. The half-maximal concentration (EC50) values of AQP4-IgM from different subsets were relatively diverse and did not necessarily reflect the stage of B cell development. To further explore the link between B cell maturation and CDC potential, we assessed the ability of the UCAs to fix complement. Reduced CDC activities were observed in all UCA-IgMs in comparison to the corresponding mutated parental IgM mAbs, suggesting that somatic hypermutations are essential in enhancing CDC activity of individual AQP4-specific mAb lineages.

To examine the pathogenic potential of AQP4-IgM antibodies *in vivo*, we developed an animal model employing intracerebral co-injections of AQP4-IgM mAbs and human complement in rats without pre-existing neuroinflammatory conditions. Brain lesions with

features resembling human NMOSD including loss of AQP4 and GFAP immunoreactivity, deposition of human IgM and activated complement products, and neutrophil infiltration were successfully reproduced by co-injections of AQP4-IgM mAbs and human complement. This pathology was most pronounced in rats receiving the mutated subset, followed sequentially by the UCA and the unmutated mAb subsets. These results suggest that AQP4-IgM antibodies may contribute to the formation of NMOSD lesions through activation of the classical complement pathway. Their pathogenic potential is further improved by incorporation of somatic hypermutations.

In summary, defective central and partially functional peripheral B cell tolerance checkpoints may contribute to the early emergence of AQP4-specific B cells in this disease. The associated immunoglobulin gene features may be a cause or consequence of failure in establishing B cell tolerance. Affinity-matured and highly pathogenic AQP4-specific antibodies may originate from early B cells with relatively lower initial affinity to AQP4. Lastly, AQP4-IgM antibodies display classical complement activity *in vitro* and *in vivo* and thus, may play a role in the formation of lesions in NMOSD.

1 Chapter I: Introduction

1.1 Autoimmune neurological diseases

1.1.1 Defining autoimmune diseases

The Witebsky's postulates were introduced in 1957 as a set of logical approaches to define the autoimmune aetiology of a human disease¹. The original postulates dictated that an autoimmune response must be recognised as either a humoral or cell-mediated immune reaction against a well-identified antigen target. Additionally, an analogous response which results in a similar pathology must be reproduced in an experimental animal. In the light of burgeoning knowledge on molecular biology and hybridoma technology in the early 1990s, the stringent criteria were revisited². It was proposed that three lines of evidence can be gathered to establish an autoimmune disease: a) direct evidence from transfer of pathogenic immune effectors; b) indirect evidence from animal models; and c) circumstantial evidence from clinical cues including association with other known autoimmune diseases, lymphocytic infiltration of target organ(s), association with particular major histocompatibility complex (MHC) molecule(s), and favourable response to immunosuppressive therapies.

1.1.2 Autoantibodies in neurological diseases

Within the past two decades, more than 10 novel autoantibodies directed against proteins expressed in the central nervous system (CNS) have been described and characterised. These numbers are expected to increase due to the routine implementation of robust diagnostic assays such as cell-based assays (CBAs) and immunoassays using rodent neuroglial cultures and brain sections in current clinical practice. The unique staining pattern and subcellular localisation of autoreactivity sometimes suggests the yet to be identified targets which may become part of future diagnostic panels.

Autoantibodies targeting CNS intracellular (nuclear or cytoplasmic) antigens are traditionally associated with the presence of an underlying malignancy³. A widely accepted

view is that these antibodies arise from a misdirected immune response against neuroglial proteins which are ectopically expressed in tumour cells. The antibodies themselves are believed to be non-pathogenic, but they serve as excellent biomarkers in classical paraneoplastic neurological syndromes (PNSs)⁴. The neurological conditions seen in classical PNSs, for example in those associated with Hu, Ma, and Yo antibodies, are primarily caused by cytotoxic T cell-mediated immunity³. Consequently, affected patients tend to respond poorly to B cell-depleting therapies⁵.

Antibodies may also target neuroglial cell surface proteins. Upon binding to their native targets, this group of antibodies can trigger deleterious effects which include receptor cross-linking and internalisation, complement fixation, or direct alterations of target antigens' functions⁶. The resulting clinical syndromes are generally responsive to immunotherapies that reduce the production and availability of circulating antibodies⁷, signifying the key role of humoral immune effectors in the disease pathogenesis. Additionally, malignancy association in antibody-mediated neurological diseases is relatively rare, except in γ -aminobutyric acid type B receptor (GABA_BR)⁸, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)⁹, and to a lesser extent, N-methyl-D-aspartate receptor (NMDAR) encephalitis¹⁰. Well-characterised surface protein-targeting CNS autoantibodies in chronological order of their discoveries are summarised in Table 1.1 (reviewed in Ref⁶).

Antibody target	Year of discovery	Clinical syndromes	Mode of pathogenicity
mGluR1	2000	Cerebellar ataxia	Alteration of protein functions
AQP4	2004	NMOSD	CDC, ADCC
NMDAR	2007	Encephalitis, psychiatric symptoms, amnesia, seizures, dysautonomia	Receptor internalisation, alteration of receptor functions
Glycine receptor	2008	Encephalomyelitis, rigidity, myoclonus, seizures, stiff person syndrome	Receptor internalisation, alteration of receptor functions
MOG	2009	Optic neuritis, myelitis, ADEM	Alteration of protein functions
AMPA receptor	2009	Limbic encephalitis, seizures, memory loss	Receptor internalisation
LGI1	2010	Limbic encephalitis, faciobrachial dystonic seizures, neuromyotonia	Alteration of receptor functions
CASPR2	2010	LGI1-like syndromes, neuromyotonia, Morvan's syndrome, neuropathic pain	Alteration of receptor functions
GABA _B receptor	2010	Limbic encephalitis, seizures, memory loss	Alteration of receptor functions
mGluR5	2011	Limbic encephalitis, confusion, memory loss, psychosis	Reduction of membrane protein expression
D2R	2012	Parkinsonism, chorea, psychosis, dystonia	Receptor internalisation
DNER	2012	Cerebellar ataxia	Alteration of receptor functions
DPPX	2013	Confusion, hallucination, prodromal diarrhoea, memory loss, hyperexcitability	Reduction of membrane protein expression
GABA _A receptor	2014	Seizures, status epilepticus, psychosis	Reduction of receptor numbers
IgLON5	2014	Parasomnia, sleep apnoea, cognitive impairment, gait abnormalities	Reduction of membrane protein expression
SEZ6L2	2014	Cerebellar ataxia, retinopathy, hypomimia, bradykinesia, postural instability	Alteration of receptor functions
Neurexin 3 α	2016	Confusion, seizures, loss of consciousness, orofacial dyskinesia	Reduction of membrane protein expression
Septin 5	2018	Cerebellar ataxia, ocular movement abnormalities	Unknown

Table 1.1. List of neuroglial surface protein-targeting autoantibodies and their known mechanisms of pathogenicity.

ADCC, antibody-dependent cellular cytotoxicity; ADEM, acute disseminated encephalomyelitis; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AQP4, aquaporin 4; CDC, complement-dependent cytotoxicity; DPPX, dipeptidyl-peptidase-like protein 6; DNER, Delta/Notch-like epidermal growth factor-related receptor; D2R, dopamine 2 receptor; GABA, γ -aminobutyric acid;

IgLON5, immunoglobulin-like cell adhesion molecule 5; LGII, leucine-rich glioma-inactivated 1; mGluR5, metabotropic glutamate receptor 5; MOG, myelin oligodendrocyte glycoprotein; NMDAR, N-methyl-d-aspartate receptor; NMOSD, neuromyelitis optica spectrum disorders; SEZ6L2, seizure-related 6 homolog like 2

1.2 Neuromyelitis optica spectrum disorders (NMOSD)

1.2.1 Evolution of diagnostic criteria

Neuromyelitis optica spectrum disorders (NMOSD) is among a small but growing group of autoimmune conditions affecting the central nervous system (CNS) which was previously considered as a variant of multiple sclerosis (MS). The first observations of this disease entity were written in a publication by Eugene Devic in 1895¹¹. He described a 45-year-old female patient presenting with an acute-onset paralysis accompanied by progressive impairment of sensory functions of the lower extremities. Within a few weeks, profound bilateral visual loss was noted. At autopsy, necrotic lesions were found in both the spinal cord and optic nerves, revealing the possible organic cause of the clinical symptoms. Devic proposed the term *neuro-myélite optique aiguë* to define these clinico-anatomic findings. The dual localisation of the syndrome begged the question of whether it might be governed by a single pathological process occurring relatively simultaneously at segments of the cord and optic nerves despite the absence of obvious anatomical lesions connecting the two.

The past two decades have immensely revolutionised our understanding of NMO as a clinical phenomenon in its own right. A study by Lennon and colleagues at the Mayo Clinic in 2004 discovered an autoantibody found in 73% of patients with classical NMO¹². This “NMO-IgG” was rarely present in sera from patients with MS, other myelopathies, optic neuropathies, and miscellaneous disorders including myasthenia gravis (MG) and Sjogren’s syndrome among others. Using an indirect immunofluorescence assay (IIFA) with a substrate of mouse tissues, NMO-IgG was shown to selectively target aquaporin-4 (AQP4)¹³, a water channel protein that is predominantly expressed on astrocyte end-foot processes at the blood-brain

barrier (BBB). The term AQP4-IgG has since been used and this autoantibody has served as a pathogenic biomarker in the majority (76%) of NMO cases¹⁴.

In Lennon's study, patients were recruited according to the 1999 diagnostic criteria of NMO which comprised 3 absolute requirements: a) optic neuritis; b) acute myelitis; and c) no evidence of clinical disease in other regions of the brain¹⁵. Additionally, one major (a negative brain MRI, a spinal MRI showing longitudinally extensive transverse myelitis, or a cerebrospinal fluid pleocytosis) or two minor (a bilateral optic neuritis, a severe optic neuritis with visual acuity <20/200, or a severe weakness in one or more limbs) supportive criteria had to be fulfilled to establish the diagnosis of NMO. The limitations of these criteria were soon exposed as some cases which otherwise would be classified as typical NMO also demonstrated MRI lesions in other parts of the CNS, such as the cerebral peduncles and medulla¹⁶.

Using a new cohort of patients, Wingerchuk and colleagues reported that 14.6% of NMO patients had experienced extra-optic-spinal CNS symptoms at some point prior to diagnosis¹⁶. Additionally, the detection of serum AQP4-IgG was shown to be 76% sensitive and 94% specific for NMO¹⁶. In combination with at least one of two neuroimaging findings which included a longitudinally extensive spinal cord lesion or a brain MRI atypical for MS, serum AQP4-IgG seropositivity was 99% sensitive and 90% specific for NMO¹⁶. To appreciate the wider spectrum of the disease and the significance of serum AQP4-IgG status, the 2006 revised diagnostic criteria was proposed. The updates removed the prior restriction of CNS involvement and incorporated AQP4-IgG serology among the supportive criteria.

The concept of NMOSD emerged a year later after the introduction of the 2006 revised criteria¹⁷. Recognition of the remarkable specificity of AQP4-IgG assays led to the proposal of NMO as a spectrum of phenotypes associated with AQP4-IgG seropositivity which could present as its historical form of both optic neuritis and transverse myelitis, or exist in a more limited clinical manifestation of either one of them. The authors also highlighted the possible

coexistence of NMO and other systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjogren's syndrome. Other cardinal features of the disease consistent with lesions at the area postrema and hypothalamic periventricular zones, i.e., refractory hiccups and vomiting, were also acknowledged.

Building on the expanded concept of NMO, the diagnostic criteria were again refined in 2015 (Table 1.2)¹⁸. The term NMO is formally subsumed into the unifying term of NMOSD, which is stratified further according to AQP4-IgG serology status. Evidence of a clinical episode, including any of the six core clinical criteria is now adequate for the diagnosis of NMOSD in the setting of a positive serum AQP4-IgG. Presentation of any of the two classical forms of NMO is no longer an absolute requirement. Furthermore, the diagnosis of "NMOSD without AQP4-IgG" has become possible in a suspected patient when serology testing is negative or unavailable, provided that the more stringent clinical and neuroimaging criteria are met.

<i>Diagnostic criteria for NMOSD with AQP4-IgG</i>
1. At least 1 core clinical characteristic 2. Positive test for AQP4-IgG using best available detection method (cell-based assay strongly recommended) 3. Exclusion of alternative diagnosis
<i>Diagnosis criteria for NMOSD without AQP4-IgG or NMOSD with unknown AQP4-IgG status</i>
1. At least 2 core clinical characteristics occurring as a result of one or more clinical attacks and meeting all of the following requirements: a. At least 1 core clinical characteristic must be optic neuritis, acute myelitis with LETM, or area postrema syndrome b. Dissemination in space (2 or more different core clinical characteristics) c. Fulfilment of additional MRI requirements, as applicable 2. Negative tests for AQP4-IgG using best available detection method, or testing unavailable 3. Exclusion of alternative diagnoses
<i>Core clinical characteristics</i>
1. Optic neuritis 2. Acute myelitis 3. Area postrema syndrome: episode of otherwise unexplained hiccups or nausea and vomiting 4. Acute brainstem syndrome 5. Symptomatic narcolepsy or acute diencephalic clinical syndrome with NMOSD-typical diencephalic MRI lesions 6. Symptomatic cerebral syndrome with NMOSD-typical brain lesions
<i>Additional MRI requirements for NMOSD without AQP4-IgG and NMOSD with unknown AQP4-IgG status</i>
1. Acute optic neuritis: requires brain MRI showing (a) normal findings or only nonspecific white matter lesions, OR (b) optic nerve MRI with T2-hyperintense lesion or T1-weighted gadolinium-enhancing lesion extending over >1/2 optic nerve length or involving optic chiasm 2. Acute myelitis: requires associated intramedullary MRI lesion extending over ≥ 3 contiguous segments (LETM) OR ≥ 3 contiguous segments of focal spinal cord atrophy in patients with history compatible with acute myelitis 3. Area postrema syndrome: requires associated dorsal medulla/area postrema lesions 4. Acute brainstem syndrome: requires associated periependymal brainstem lesions

Table 1.2. Adapted from the 2015 international consensus NMOSD diagnostic criteria for adult patients.

AQP4, aquaporin-4; IgG, immunoglobulin G; LETM, longitudinally extensive transverse myelitis; MRI, magnetic resonance imaging; NMOSD, neuromyelitis optica spectrum disorders. (Ref¹⁸)

As detection of AQP4-IgG has become essential for diagnostic and therapeutic purposes, several laboratory assays have been developed over the years. A review on the diagnostic performance of three methods which include ELISA, tissue-based (TBA), and cell-based assays (CBA) has shown that CBA is the most superior of all with a mean sensitivity and specificity of 76% and 99%, respectively¹⁴. The ELISA method placed second with regard

to sensitivity (65%) and last for specificity (97%)¹⁴. Despite the increased abundance and accessibility of antigen, the substrate used in the ELISA test does not possess the same conformational structure as it presents within the cell membrane. This may explain the relatively low performance of the test compared to CBA which preserves the natural, three-dimensional shapes of the antigen. Additionally, while, on average, the TBA is less sensitive (59%) and specific (98%) than CBA¹⁴, fields undergoing extensive research may benefit from this approach. The availability of vastly different antigenic substrates provides a suitable platform for high-throughput screens and the discovery of potentially novel autoantibodies.

Further advances in defining NMOSD have considerable implications for research and clinical practice. The use of a combination of clinical, serology, and neuroimaging characteristics has now clearly distinguished NMOSD from the highly heterogeneous group of inflammatory demyelinating disorders of the CNS including MS whilst, at the same time, recognising the various presentations of NMOSD itself. The validity and reproducibility of basic research and clinical trials can be preserved by inclusion of properly identified participants. More importantly, treatment strategies for acute attacks have been reported to be different between NMOSD and MS. Certain MS immunotherapies appear to be ineffective for NMOSD^{19,20}, signifying an imperative of early, accurate diagnosis.

1.2.2 AQP4 as an antigen target

Similar to other members of the aquaporin family, AQP4 monomers are composed of six helical, transmembrane domains and two short helical segments surrounding a narrow aqueous pore (Figure 1.1a)²¹. Alternative splicing of AQP4 transcripts produces two major isoforms of the protein, a longer M1 (323 aa) with translation initiation at methionine-1 and a 22-amino-acid shorter M23 (301 aa) with translation initiation at methionine-23²². Both isoforms are present in mammalian brains with M23 being approximately 3-fold more abundantly expressed than M1²³. As other aquaporins, endogenous AQP4 monomers assemble

as tetramers. Uniquely, freeze-fracture electron microscopy images revealed that AQP4 tetramers further aggregate to form regularly-arranged, supramolecular intramembrane particles, also termed orthogonal arrays of particles (OAPs) (Figure 1.1b)²⁴. The membrane organisation and size of OAPs are regulated by interaction between the two AQP4 isoforms. On its own, AQP4-M23 produces large assemblies with a morphology similar to a raft with abundant cross-bridges whereas AQP4-M1 homotetramers are present as singlets. The OAP-limiting effect of AQP4-M1 is apparent when the two isoforms are co-expressed; the formed OAPs are of intermediate sizes, mimicking those constitutively expressed in astrocytes²⁵.

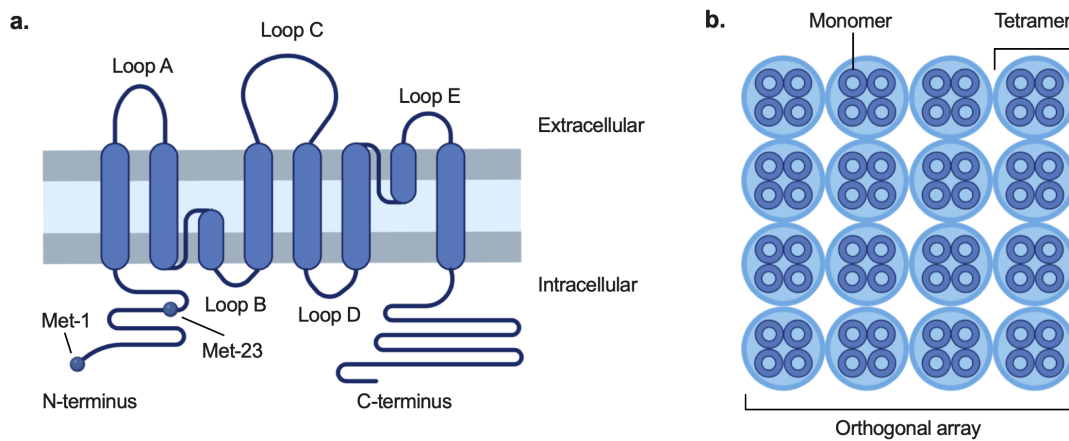


Figure 1.1. Structure and surface membrane organisation of AQP4.

a. Proposed topological structure of AQP4. Each monomer is composed of 6 helical, transmembrane domains and 2 short helical segments. To isoforms of AQP4, M1 (met-1) and M23 (met-23), differ in their intracellular N-terminus but otherwise possess identical extracellular domains; **b.** Schematic illustration showing membrane organisation of AQP4 as orthogonal arrays of particles (OAPs).

Several studies have reported that AQP4-IgG from patient serum preferentially binds to cells expressing AQP4-M23 than AQP4-M1^{26,27}. A more detailed evaluation of binding affinity utilising both polyclonal serum AQP4-IgG and monoclonal AQP4-IgG generated from clonally expanded plasma cells from CSF of NMOSD patients showed a wide variation in binding, from comparable binding to both isoforms to exclusive binding to AQP4-M23²⁷. Binding measurements in cells transfected with varying M23:M1 ratios or AQP4-M23 mutants which lack the capability to form OAPs further revealed that the differential binding of AQP4-IgG to M23 versus M1 is due to OAP assemblies rather than to differences in the two isoforms’

N termini²⁷. It is thought that the greater binding affinity of AQP4-IgG to OAP than non-OAP associated AQP4 is a result of structural changes in the AQP4 epitope(s) upon array formation, and not due to the opposing mechanisms of IgG binding (monovalent binding on separate tetramers versus bivalent cross-linking on OAPs)^{27,28}.

The tissue distribution of AQP4 is tied to its main biological role as a specialised water transport channel. In addition to its strong expression in the CNS, AQP4 is also present in the epithelial cells lining the collecting duct in the kidney, the airways, the stomach (parietal cells), most secretory glands, and skeletal muscles²⁹. In the CNS, AQP4 is enriched in astrocytes in the brain, spinal cord, and optic nerves with polarised expression at the end-foot processes in contact with blood vessels³⁰. Similarly, ependymal cells at the site of CSF production in the brain ventricles and Muller cells facing the vitreous body and retinal blood vessels also have abundant AQP4³¹.

Knowledge about AQP4 physiology is mainly gained from studies on AQP4 knockout mice. At homeostasis, mice with loss-of-function mutation of AQP4 exhibit normal appearance, growth, and general neuromuscular functions³² albeit partial impairment in the signalling pathways of several sensory organs³¹. Following application of various stresses, AQP4 knockout mice show a slight reduction in urine-concentrating ability due to decreased water permeability in the renal collecting duct³². In the CNS, the absence of AQP4 expression leads to improvement of brain oedema after water intoxication and in a mouse model of ischemic stroke³³. This positive effect can be attributed to reduced water entry into cells, thereby alleviating cytotoxic oedema due to cell swelling in both circumstances. In contrast, AQP4 deficiency is proven to be detrimental in mouse models of brain tumours and obstructive hydrocephalus^{34,35}. The lack of AQP4 hinders efficient removal of excess interstitial water in these two conditions, resulting in vasogenic brain oedema.

1.2.3 Evidence of AQP4-IgG pathogenicity

Clinical and pathological observations suggest the pathogenicity of antibodies specific to AQP4 in NMOSD: 1) antibody-depleting therapies have been proven to ameliorate acute attacks in NMOSD patients^{36,37}; 2) MRI studies demonstrated localisation of CNS lesions at sites with high AQP4 expression, such as the brain ventricles³⁸; and 3) histopathological examinations of active CNS lesions showed loss of AQP4 immunoreactivity and deposition of IgG and IgM, co-localising with activated complement products in a vasulocentric pattern which corresponds to the tissue distribution of AQP4^{39,40}.

AQP4-IgG are predominantly IgG1, an antibody subclass with a strong complement response. In the presence of human complement, binding of AQP4-IgG to the extracellular domain of AQP4 on transfected HEK293 and Chinese hamster ovary (CHO) cells as well as mouse cortical astrocyte cultures induced complement-dependent cytotoxicity (CDC)^{41,42}. Activated complement products, or C9neo, were clearly visualised on the plasma membrane of target cells and were absent on cells exposed to control IgG or heat-inactivated human complement. Consistent with the distinctive pattern of AQP4-IgG binding to AQP4 in individual tetramers and OAPs, CDC has also been shown to be dependent on AQP4 array assembly. Polyclonal serum and monoclonal AQP4-IgG exhibited a significantly greater cytotoxic capacity in cells expressing OAP-forming AQP4-M23 compared to AQP4-M1 or AQP4-M23 with OAP-disrupting mutations⁴³. Super resolution microscopy studies demonstrated that optimally arranged epitopes on AQP4 OAPs facilitate clustering of multiple IgGs⁴⁴. Through antibody Fc-Fc interactions, the clustered IgGs serve as a multimeric platform which subsequently increases multivalent engagement of C1q, the first component of the classical complement pathway, and efficiently initiates its activation^{44,45}.

The pathogenicity of AQP4-IgG is substantiated by data from animal studies. Intraperitoneal administration of serum AQP4-IgG worsened CNS inflammation in rats with

experimental autoimmune encephalomyelitis (EAE)⁴⁶ or those pre-treated with Freund's adjuvant⁴⁷. The acknowledged weakness of these models is that the pre-existing neuroinflammation confounds further interpretation. Compelling evidence that AQP4-IgG is pathogenic comes from co-injections of AQP4-IgG and human complement directly into the brains of wild-type mice⁴⁸. The histological features of human NMOSD-like lesions such as, loss of AQP4 and glial fibrillary acidic protein (GFAP), perivascular complement deposition, immune cell infiltration, and demyelination were successfully reproduced. A similar pathology was observed in rats receiving intracerebral injection of AQP4-IgG, even without human complement⁴⁹. The differing requirement of supplementation with an external source of complement between the two rodent models has been thought to be due to the inherently weaker activity of mouse immune effectors^{50,51} and the presence of unknown complement inhibitor(s) in mouse serum⁵¹.

Other molecular outcomes of IgG binding to AQP4 has been described. AQP4-IgG initiated antibody-dependent cellular cytotoxicity (ADCC) in mouse cortical astrocyte cultures when natural killer (NK) cells were present⁵². Administration of AQP4-IgG with altered CDC effector function in wild-type rats resulted in brain lesions characterised by AQP4 loss with intact GFAP and myelin basic protein (MBP) immunoreactivity, also called penumbra, suggesting a complement-independent mechanism⁴⁹. The penumbra was absent with AQP4-IgG lacking ADCC function.

There is conflicting evidence on the ability of AQP4-IgG in inducing internalisation of membrane-bound AQP4. Initial studies suggested that AQP4 on transfected cells was internalised, together with excitatory amino acid transporter-2 (EAAT2), following AQP4-IgG binding^{41,53}. The resulting cytotoxicity from impaired extracellular glutamate uptake after neuroexcitation, termed glutamate excitotoxicity, was proposed as a possible mechanism underlying oligodendrocyte injury and demyelination in NMOSD. However, there was little to

no internalisation of endogenously expressed AQP4 or EAAT2 in mouse cortical astrocytes and mouse brains following exposure to AQP4-IgG⁴². These results have challenged the significance of this pathogenic mechanism *in vivo*.

Astrocyte death has been thought to release molecular stress signals which leads to recruitment of immune cells to the site of injury. Infiltrating immune cells are dominated by granulocytes, particularly neutrophils and eosinophils, as well as macrophages³⁹. Evidence from mouse models⁵⁴ and clinical reports⁵⁵ suggests the role of degranulating neutrophils during the development of early lesions. The presence of eosinophils, on the other hand, is intriguing as this type of immune cells are most commonly seen in allergic reactions and responses to parasitic infections⁵⁶. Generation of free radicals and eosinophil-derived neurotoxins might potentially amplify the inflammatory response in NMOSD.

In addition to the data obtained from numerous *in vitro* and *in vivo* studies as mentioned above, clinical trials involving Eculizumab and Ravulizumab demonstrated a significant reduction of attack frequency and an improvement of clinical disability measures in patients with severe NMOSD^{57,58}. Eculizumab is therapeutic monoclonal IgG which neutralises the complement protein C5 and ultimately prevents generation of the lytic membrane attack complex (MAC) C5b-9. Substitutions of 4 amino acids in the Eculizumab frame resulted in the creation of Ravulizumab, a structurally related humanised monoclonal IgG with a prolonged half-life and extended C5 inhibition duration⁵⁹. Based on these lines of evidence, CDC has been widely cited as the key initiator by which NMOSD lesions develop. The cascade of events leading from IgG binding to AQP4 to tissue destruction and formation of NMOSD lesions are summarised in Figure 1.2.

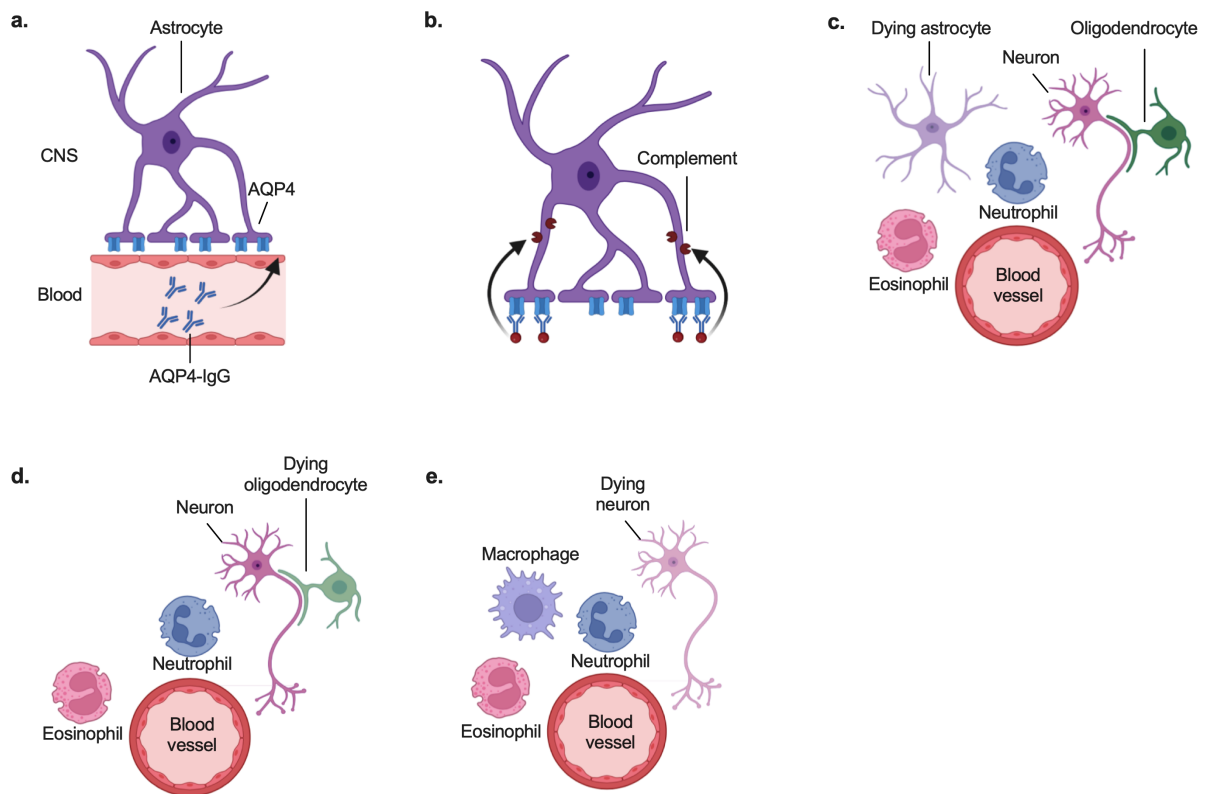


Figure 1.2. Development of CNS lesions in NMOSD.

a. AQP4-IgG antibodies in the blood reach their target antigen by some yet to be identified mechanisms; **b.** AQP4-IgG binding to AQP4 on astrocytic end foot processes activates the classical complement pathway with subsequent deposition of membrane attack complexes (MACs); **c.** Astrocyte death releases pro-inflammatory factors which trigger infiltration of neutrophils and eosinophils into the site of injury; **d.** Loss of astrocytes results in demyelination and axonal degeneration, possibly due to glutamate excitotoxicity of bystander oligodendrocytes; and **e.** Widespread infiltration of macrophages and tissue necrosis characterise late-stage NMOSD lesions. Reproduced with modifications and permissions from Ref⁶⁰.

1.3 B cell immunobiology in NMOSD

1.3.1 General principle of B cell development

Differentiation of multipotent hematopoietic stem cells into common lymphoid progenitor cells and subsequently into pro-B cells occurs in the bone marrow. During the pro-B cell stage, random rearrangement of the variable (V), diversity (D), and joining (J) immunoglobulin gene segments of the heavy chain locus is initiated⁶¹. A successful attempt to produce an intact μ heavy chain will allow developing B cells to enter the pre-B cell stage. As

the light-chain locus has not yet rearranged, two 'surrogate' proteins ($\lambda 5$ and VpreB) which have a structural resemblance to the light chain will be paired with the μ chain to form the pre-B cell receptor (BCR). The assembly of a pre-BCR signals indicates that a productive rearrangement has been made. This is then followed by rearrangement of the V and J gene segments of the light chain locus which, as opposed to the heavy chain counterpart, lacks the D gene segment. This process takes place in only one chromosome at a time, although repeated rearrangements can occur in the same chromosome should it fail to produce a functional light chain. Rearrangements in the second chromosome is initiated when all possible combinations have been exhausted. Additionally, in mice and humans, the κ light chain locus tends to rearrange before the λ locus^{62,63}. Pairing of a productive light chain with the previously rearranged μ chain will be expressed as surface membrane IgM which indicates the progression of a pre-B cell to an immature B cell.

The expressed BCR is then tested for reactivity to self-antigens. Production of autoreactive B cells is minimised via mechanisms of central tolerance which include receptor editing and induction of apoptosis, anergy, and immunological ignorance⁶⁴. The fate of autoreactive B cells upon counter-selection is believed to be dependent on the strength of interaction between the BCR and self-antigens. Those which pass to the periphery as new emigrant B cells will further exposed to a peripheral tolerance checkpoint⁶⁴. Encounter with sufficient quantities of self-antigens in the periphery will prompt elimination or inactivation of autoreactive B cells through similar mechanisms in the bone marrow. Otherwise, B cells continue to the mature naïve B cell stage. When a naïve B cell first recognises its cognate antigen in the secondary lymphoid organs, it migrates from the primary lymphoid follicles to the follicle border to receive T cell help through linked-recognition⁶⁵. Some activated B cells will continue to proliferate and differentiate into short-lived plasma cells which mainly secrete IgM antibodies. The remaining B cells will enter germinal centre reactions which involve

somatic hypermutations (SHM) and class-switch recombination (CSR) to become either IgG⁺ switched memory B cells or long-lived plasma cells. Although this process allows diversification and improvement of antigen affinity of BCRs, reactivity to self-antigens can also be inadvertently generated. It is believed that a third tolerance checkpoint takes place to remove B cells which recognise self-antigens expressed in the germinal centre micro-environment⁶⁶. Figure 1.3 illustrates the well-established central and peripheral tolerance checkpoints along the B cell developmental stages.

IgD⁻ CD27⁻ B cells, also termed double-negative (DN) B cells, have been shown to play a role in the immunobiology of several autoimmune diseases including SLE. To date, four subtypes of DN B cells have been identified: DN1, 2, 3 and 4. DN1 B cells constitutes the majority of the DN B cell population in healthy individuals and are thought to serve as precursors to switched memory B cells. Of all, DN2 B cells are the most well-characterised. Recent studies suggest that this subpopulation may develop through an extrafollicular response, differentiate into short-lived antibody secreting cells (ASCs), and contribute to autoimmunity. DN3 and DN4 B cells are yet to be fully characterised.

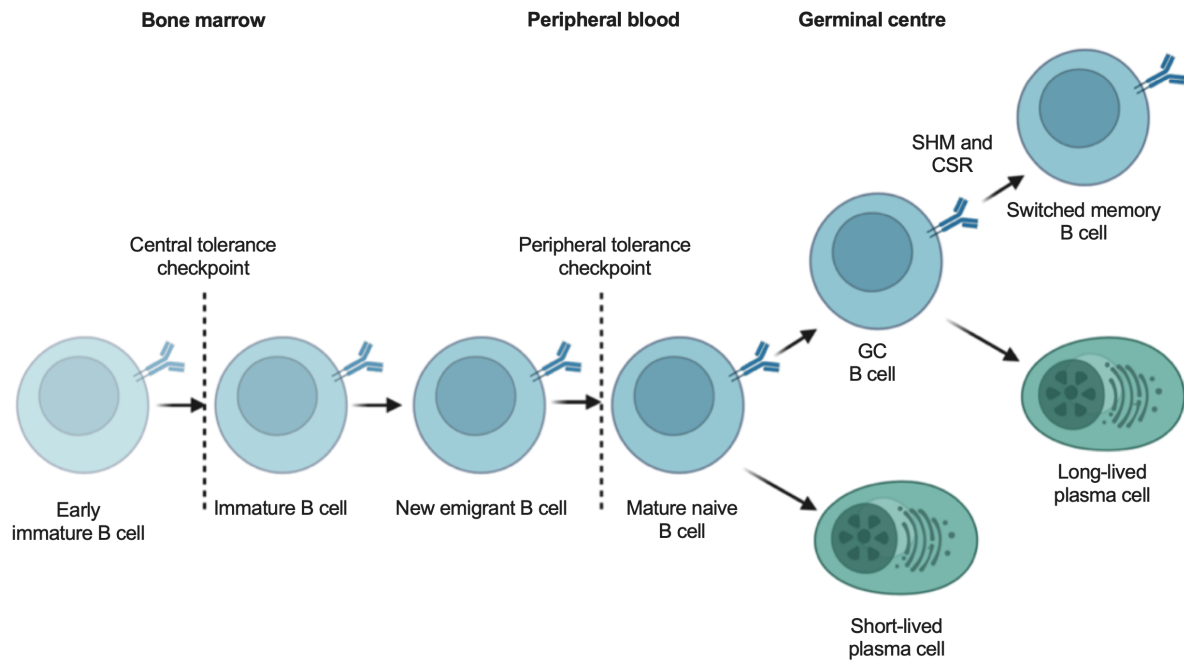


Figure 1.3. B cell development and tolerance checkpoints.

Differentiation of B cells from common lymphoid progenitor takes place in the bone marrow. During early B cell development, rearrangement of the immunoglobulin genes in the heavy and light chain locus produces a surface membrane IgM which acts as a B cell receptor (BCR). In early immature B cells, the expressed BCR is tested for reactivity to self-antigens via mechanisms known as central tolerance. B cells which pass this tolerance checkpoint become immature B cells and egress to the periphery as new emigrant B cells. Autoreactive new emigrant B cells are further removed by peripheral tolerance checkpoint before transitioning into mature naïve B cells. In the secondary lymphoid organs, activation of mature naïve B cells by their cognate antigen will induce some of them to differentiate into short-lived plasma cells. The remaining activated B cells enter germinal centre (GC) reactions which involve somatic hypermutation (SHM) and class-switch recombination (CSR) to become class-switched memory B cells and long-lived plasma cells

1.3.2 Proposed origin of AQP4 specificity in NMOSD

Insights into the possible aetiology of human autoimmune diseases are mostly gained from studies comparing the frequency of autoreactive B cells at each developmental stage in affected patients and healthy individuals⁶⁷. This approach employs single B cell isolation, immunoglobulin gene amplification, cloning, and generation of monoclonal antibodies (mAbs). The generated mAbs are subjected to reactivity testing via enzyme-linked immunosorbent assays (ELISAs) against major macromolecules namely, double-stranded DNA (dsDNA), insulin, and lipopolysaccharide (LPS) as well as human epithelial type-2 (Hep-2) cell lysate as a surrogate for nuclear antigens⁶⁷. The terms “polyreactive” has been

consistently used throughout the literature to refer to simultaneous reactivities against dsDNA, insulin, and LPS whereas “autoreactive” is used interchangeably with Hep2-reactive.

Earlier work demonstrated that 55.2% and 75.9% of all antibodies derived from early immature B cells displayed poly and autoreactivity, respectively⁶⁷. These frequencies decreased in the immature stage, remained constant in newly emigrated B cells, and further reduced in the naïve B cell compartment⁶⁷. These observations suggest that under physiologic circumstances, most poly and autoreactive B cells are counterselected at two discrete checkpoints: a) between the early immature and immature B cell stage in the bone marrow; and b) between the new emigrant and mature naïve B cell stage in the periphery. Elevated frequencies of poly and autoreactive new emigrant and mature naïve B cells compared to healthy donors were reported in patients with SLE⁶⁸, rheumatoid arthritis (RA)⁶⁹, type-1 diabetes (T1D)⁷⁰, Sjogren’s syndrome⁷¹, and NMOSD⁷². Based on this, it has been thought that defects in both central and peripheral B cell tolerance checkpoints lead to substantial increase in the proportion of autoreactive naïve B cells in these patients which may predispose them to develop autoimmunity.

The fidelity of the B cell tolerance checkpoints in NMOSD has been specifically investigated in one recent study⁷². A total of 130 antibodies were generated from 3 NMOSD patients with AQP4-IgG seropositivity and compared to 733 antibodies from 15 healthy donors. Consistent with what is shown in other autoimmune diseases, higher frequencies of poly and autoreactive new emigrant and mature naïve B cells were found in NMOSD patients than controls. To study the origin of pathogenic AQP4-IgG secreting B cells, 6 somatically mutated AQP4-IgG were reverted to their presumed unmutated germline precursors based on the IMGT reference library, expressed as IgG antibodies, and tested for both poly and autoreactivity as well as AQP4 specificity. Interestingly, it was demonstrated that all revertants lost their ability to recognise AQP4. Meanwhile, 33.3 % and 66.7% of them were poly and

autoreactive, respectively⁷². The authors concluded that early B cell tolerance defects may allow accumulation of non AQP4-specific but poly/autoreactive naïve B cell in the peripheral immune repertoire of NMOSD patients. These B cells may acquire AQP4 specificity following somatic hypermutation and subsequently secrete high-affinity, pathogenic AQP4 antibodies.

1.4 Gaps of knowledge

The significance of AQP4-IgM in NMOSD has long been underappreciated. An initial study reported that patient serum and CSF completely lacked AQP4-IgM as tested by live cell-based assays (CBAs)⁴¹. This finding is discrepant with histopathological data from 9 autopsy cases of clinically confirmed NMOSD⁴⁰. Active lesions were shown to have abundant deposits of immunoglobulins, most of which was IgM rather than IgG^{39,40}. It has been suggested that the presence of excess IgG would outcompete IgM for antigen binding during immunoassays, thus causing false negative results in the prior study. Depletion of IgG by immunoprecipitation prior to testing subsequently demonstrated the existence of AQP4 antibodies of the IgM class in almost 10% of NMOSD patients⁷³.

Second, IgM is considered a more potent complement activator than IgG^{74,75}. This unique effector function is inherent in the structure of IgM polymers. Upon antigen binding, polymeric IgM antibodies undergo conformational changes which expose its multiple Fc regions for binding to C1q globular heads⁷⁶. In contrast, there is only one Fc region per IgG molecule available for C1q binding. Consequently, two or more IgG molecules within 30-40 nm of each other are required to achieve the minimum binding requirements for C1q activation⁴⁵. Considering that CDC is thought to be the key mechanism in NMOSD, it is therefore conceivable that AQP4-IgM antibodies play a role in the disease pathogenesis.

Third, a clinical study involving deep cervical lymph node aspirates and peripheral blood from 63 NMOSD patients suggested that clinical relapses were associated with generation of AQP4-IgM⁷⁷. IgM is normally the first antibody to be produced in a humoral

immune response. The most likely source of these antibodies could be AQP4-specific naïve B cells that manage to escape early immune tolerance to become short-lived plasma cells following germinal centre reactions in a primary immune response. Alternatively, these IgM-secreting cells may also be derived from AQP4-specific memory B cells that were generated from past immune responses against AQP4.

Lastly, a number of pre- and post-germinal centre B cells isolated from 6 NMOSD patients were shown to produce AQP4-IgG in vitro under select culture conditions⁷⁸. AQP4-IgG antibodies were detected in supernatants from the post-germinal centre, switched (CD19⁺ CD27⁺ IgD⁻) and unswitched (CD19⁺ CD27⁺ IgD⁺) memory B cell wells. Surprisingly, antigen-inexperienced naïve B cells (CD19⁺ CD27⁻ IgD⁺) were also found to have the capacity to secrete IgGs which were reactive to AQP4. Although the presence of AQP4-IgM was not evaluated, these antibodies likely also exist, considering that immunoglobulin molecules expressed by naïve B cells are typically of the IgM class. Nevertheless, these observations suggest that AQP4 specificity may emerge as early as the mature naïve B cell stage due to pre-germinal centre loss of immune tolerance.

1.5 Study aims

Based on the rationale described in the above section, this DPhil project aimed to:

1. Investigate how early AQP4 specificity emerges within B cell lineages whilst at the same time, evaluate the integrity of central and peripheral B cell tolerance checkpoints in NMOSD;
2. Characterise the immunoglobulin genes repertoire of AQP4-specific B cells isolated from NMOSD patients;
3. Characterise the evolution of binding affinity of AQP4-specific monoclonal IgM and IgG to AQP4 across B cell lineages; and

4. Characterise the *in vitro* and *in vivo* pathogenic potential concerning complement-dependent cytotoxicity of AQP4-IgM.

2 Chapter II: Materials and Methods

2.1 Materials

The materials used in this study are listed below:

<i>Cell culture reagents</i>	<i>Company</i>	<i>Catalogue #</i>
Antibiotic-antimycotic	Gibco	15240062
β -mercaptoethanol	Gibco	21985023
Bovine serum albumin	Sigma-Aldrich	A9647
Dulbecco's modified eagle medium, high glucose	Thermo Scientific	11965092
Dulbecco's modified eagle medium, high glucose, HEPES, no phenol red	Thermo Scientific	21063029
Dulbecco's phosphate-buffered saline, no calcium, no magnesium	Thermo Scientific	14190144
Foetal bovine serum	Thermo Scientific	16000044
GlutaMAX	Gibco	35050061
Hanks' Balanced Salt Solution	Gibco	14025092
Iscove's modified Dulbecco's medium	Gibco	21056023
Minimum essential medium, non-essential amino acids	Gibco	11140050
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, HEPES	Gibco	15630080
Poly-L-lysine hydrobromide	Sigma-Aldrich	P1399
R848	Enzo Life Sciences	ALX-420-038
Recombinant human BAFF	R&D Systems	2149-BF
Recombinant human IL-2	PeproTech	200-02
Recombinant human IL-4	PeproTech	200-04
Recombinant human IL-21	PeproTech	200-21
RPMI 1640	Gibco	32404014
Sodium pyruvate	Gibco	11360070
Trypan blue	Sigma-Aldrich	93595
Trypsin-EDTA, 0.5%, no phenol red	Gibco	15400054

<i>Flow cytometry and immunofluorescence staining reagents</i>	<i>Company</i>	<i>Catalogue #</i>
4'6-diaminidino-2-penylindole, DAPI	BD Biosciences	564907
Alamar Blue cell viability reagent	Thermo Scientific	DAL1025
Bovine serum albumin	Sigma-Aldrich	A9647
Fluorescence mounting medium	Agilent Dako	S3023
Formaldehyde, 36% w/v	Taab	F003
Goat anti-human IgG (H+L), Alexa Fluor 488	Thermo Scientific	A-11013
Goat anti-human IgG (H+L), Alexa Fluor 594	Thermo Scientific	A-11014
Goat anti-human IgM (heavy chain), Alexa Fluor 488	Thermo Scientific	A-21215
Goat anti-human IgM (heavy chain), Alexa Fluor 594	Thermo Scientific	A-21216
Goat anti-rabbit IgG (H+L), Alexa Fluor 594	Thermo Scientific	A-11012

Mouse monoclonal anti-human CD3, Pacific Blue, clone UCHT1	BioLegend	300418
Mouse monoclonal anti-human CD10, Phycoerythrin/Dazzle 594, clone HI10a	BioLegend	312227
Mouse monoclonal anti-human CD19, Allophycocyanin-Cy7, clone SJ25C1	BD Biosciences	348794
Mouse monoclonal anti-human CD20, Brilliant Violet 711, clone 2H7	BioLegend	302341
Mouse monoclonal anti-human CD27, Brilliant Violet 605, clone O323	BioLegend	302829
Mouse monoclonal anti-human IgA, Phycoerythrin, clone IS11-8E10	Miltenyi Biotec	130-093-128
Mouse monoclonal anti-human IgD, Fluorescein, clone IA6-2	BD Biosciences	555778
Mouse monoclonal anti-human IgM, Alexa Fluor 647, clone MHM-88	BioLegend	314535
Mouse monoclonal anti-human IgG, Alexa Fluor 700, clone G18-145	BD Biosciences	561296
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, HEPES powder	Sigma-Aldrich	H3375
Normal mouse serum	Sigma-Aldrich	NS03L
Poly-L-lysine hydrobromide	Sigma-Aldrich	P1399
Triton X-100	Sigma-Aldrich	X100

<i>Molecular biology reagents</i>	<i>Company</i>	<i>Catalogue #</i>
5-alpha competent <i>Escherichia coli</i>	New England Biolabs	C2987I
Agarose, UltraPure	Thermo-Scientific	16500
CutSmart buffer	New England Biolabs	B7204
Dithiothreitol	Thermo Scientific	A39255
DNA ladder, 1 kb	Promega	G754A
dNTP mix	Promega	U1511
Gel loading dye, purple	New England Biolabs	B7024S
Glycerol	Sigma-Aldrich	G5516
GoTaq G2 Green Master Mix	Promega	M7822
Monarch DNA Gel Extraction Kit	New England Biolabs	T1020
IGEPAL CA-630	Sigma-Aldrich	I8896
Luria Bertani broth powder (Miller)	Sigma-Aldrich	L3522
Luria Bertani broth with agar powder (Miller)	Sigma-Aldrich	L3147
Nuclease-free water	Fisher BioReagents	BP2484
QIAGEN Plasmid Maxi Kit	QIAGEN	12162
QIAprep Spin Miniprep Kit	QIAGEN	27104
Random primer mix	New England Biolabs	S1330S
Restriction enzyme, AgeI	New England Biolabs	R3552
Restriction enzyme, BsiWI	New England Biolabs	R3553
Restriction enzyme, SalI	New England Biolabs	R3138
Restriction enzyme, XhoI	New England Biolabs	R0146
RNasin ribonuclease inhibitor	Promega	N2111
SOC outgrowth media	New England Biolabs	B9020S
SYBR Safe DNA gel stain	Thermo Scientific	S33102

SuperScript IV reaction buffer	Invitrogen	18090050B
SuperScript IV reverse transcriptase	Invitrogen	18090010
T4 DNA ligase	New England Biolabs	M0202S
T4 DNA ligase buffer	New England Biolabs	B0202S
Tris-borate-EDTA buffer	Thermo Scientific	B52
Tris-EDTA buffer solution pH 8.0	Sigma-Aldrich	93283

<i>Monoclonal antibody expression and immunoblotting reagents</i>	<i>Company</i>	<i>Catalogue #</i>
Ammonium sulphate solution	Thermo Scientific	45216
Goat anti-human IgG, Fcγ, HRP-conjugated	Jackson ImmunoResearch	109-035-008
Goat anti-human IgM, Fc ₅ μ, HRP-conjugated	Jackson ImmunoResearch	109-035-129
Imperial Protein Stain	Thermo Scientific	24615
MOPS-SDS running buffer	Invitrogen	NP0001
NuPAGE 4-12% Bis-Tris gradient gel	Invitrogen	NP0322BOX
NuPAGE antioxidant	Invitrogen	NP0005
NuPAGE lithium dodecyl sulphate (LDS) sample buffer	Invitrogen	NP0007
Nutridoma-SP	Roche	11011375001
PageRuler Plus prestained protein ladder, 10-250 kD	Thermo Scientific	26619
Pierce IgG elution buffer	Thermo Scientific	21004
Protein G Sepharose 4 Fast Flow	Cytiva	17061801
Purified human IgG	Sigma-Aldrich	I4506
Purified human IgM	Sigma-Aldrich	I8260
Skim milk	Millipore	70116
SuperSignal West Pico PLUS chemiluminescent substrate	Thermo Scientific	34580
Tris, 1M pH 8.0.	Millipore	648314
Tris-buffered saline, Tween 20 buffer, pH7.2	Thermo Scientific	28360

<i>ELISA reagents</i>	<i>Company</i>	<i>Catalogue #</i>
2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)	Bio-Rad	1721064
Double-stranded DNA	Sigma-Aldrich	D8515
Human IgM-specific ELISA kit	Thermo Scientific	88-50620-88
Lipopolysaccharide	Sigma-Aldrich	L2630
Recombinant human insulin	Fitzgerald Industries	30-A151
Sulfuric acid	Sigma-Aldrich	258105
QUANTA Lite ANA ELISA kit	INOVA Diagnostics	708750

<i>Animal model and Immunohistochemistry reagents</i>	<i>Company</i>	<i>Catalogue #</i>
3,3'-Diaminobenzidine tetrahydrochloride hydrate	Sigma-Aldrich	D5637

Chromium (III) potassium sulphate dodecahydrate	Sigma-Aldrich	243361
Citric acid	Sigma-Aldrich	791725
Cresyl violet acetate	Sigma-Aldrich	C5042
DPX mounting medium	Sigma-Aldrich	06522
Ethanol absolute	Fisher Chemical	E/0665DF/17
Gelatine	Sigma-Aldrich	G1890
Goat anti-human IgG (H+L), biotinylated	Vector Laboratories	BA-3000-15
Goat anti-human IgM (heavy chain), biotinylated	Vector Laboratories	BA-3020-05
Goat anti-rabbit IgG (H+L), biotinylated	Vector Laboratories	BA-1000-15
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	H3393
Hydrogen peroxide solution, 30% (w/w)	Sigma-Aldrich	H1009
Isopentane	VWR	24872.323
Normal goat serum	Vector Laboratories	S-1000-20
OCT cryostat sectioning medium	Tissue-Tek	4583
Paraformaldehyde powder	Sigma-Aldrich	158127
Rabbit polyclonal anti-human AQP4	Santa Cruz Biotechnology	sc-20812
Rabbit polyclonal anti-bovine GFAP	Agilent Dako	Z0334
Rabbit polyclonal anti-human C5b-9	Abcam	Ab55811
Rabbit polyclonal anti-rat neutrophil	In house-produced	N/A
Rat, Wistar (CrI:WI)	Charles River UK	003
Sodium chloride	Sigma-Aldrich	S9888
Sucrose	Sigma-Aldrich	S0389
VECTASTAIN Elite ABC-HRP Kit, Peroxidase (Standard)	Vector Laboratories	PK-6100
Xylene	Sigma-Aldrich	214736

<i>Software and algorithms</i>	<i>Company</i>	<i>Catalogue #</i>
Volocity	Quorum Technologies Inc	Volocity 7
Zen	ZEISS	ZEN 3.8
Fiji	NIH	Fiji.sc
Whole image scanner	Leica Biosystems	Whole image scanner
GraphPad Prism	GraphPad software	Version 9.5.1
BioRender	BioRender	biorender.com
IMGT	IMGT	imgt.org

<i>General laboratory products</i>	<i>Company</i>	<i>Catalogue #</i>
Amicon ultra-0.5 centrifugal filter unit, 30 kD MWCO	Merck	UFC503096
Amicon ultra-0.5 centrifugal filter unit, 100 kD MWCO	Merck	UFC510096
Bijou tube	SLS	CON7504
Cell counting chamber slides	Thermo Scientific	C10228

Cell culture plates, 6-well, 24-well	SLS	4489, 4490
Cell culture microplates, polystyrene, 96-well, flat/round/V bottom	Corning	3596, 3799, 3894
Cell culture microplates, 96-well, flat bottom, chimney well	Greiner Bio-One	655090
Circular coverslips	VWR	631-0149
Conical/falcon tubes, 15 mL, 50 mL	Greiner Bio-One	188271-N, G210261
Cover glass	Fisherbrand	1230-3148
Cryostat blades	Leica Biosystems	1403584496
Cryostat brushes	SLS	BRU2050, BRU2056, BRU2058
Filter tips, 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL	SLS	PIP7920, PIP5618, PIP7860, PIP7940, PIP7952
Glass Pasteur pipette	VWR	612-1702
Glass slides	VWR	631-0909
Lobind tubes, protein 1.5 mL	Eppendorf	0030108116
Lobind tubes, DNA 1.5 mL	Eppendorf	0030108035
Microcentrifuge tubes, 1.5 mL	Appleton Woods	KC126
Multichannel pipettes, 5-100 µL, 100-300 µL	Sartorius	LH-729230, LH-729240
Non-treated microplates, polypropylene, 96-well, flat/round bottom	Corning	3364, 3355
Pipettes, 5 mL, 10 mL, 25 mL, 50 mL	SLS	4487, 4488, 4489, 4490
Polyether sulfone filter system, 500 mL, 0.2 µm	SLS	431097
Syringe filters, 0.2 µm	Sartorius	10730792
U-shaped canted neck cell culture flask with vent cap, 75 cm ² , 175 cm ²	Corning	430641U, 431080

<i>Equipment</i>	<i>Company</i>	<i>Catalogue #</i>
pH meter	Mettler Toledo	SevenExcellence S400-Std-Kit
Scale	Mettler Toledo	Balance XPR204S
Countess 3 FL automated cell counter	Thermo Scientific	AMQAF2000
Fluorescence microscope	Leica Biosystems	Leica DM1000
Inverted microscope	Evident	CKX53
Confocal microscope	Zeiss	LSM 980
Microplate reader	BMG Labtech	FLUOstar OMEGA
Cryostat	Leica Biosystems	Leica CM1860

2.2 Methods

This section describes the general methods used throughout this study whereas procedures pertaining to a specific set of experiments are detailed at the beginning of the corresponding chapter. A summary of the stages starting from PBMC and thymic cells extraction, leading to monoclonal IgM and IgG production is shown below (Figure 2.1). All solutions were made with autoclaved and deionised distilled water (dH₂O) and the pH was measured using a SevenExcellence S400-Std-Kit benchtop pH meter (Mettler Toledo). Chemicals and powders were weighed on an XPR204S benchtop analytical balance (Mettler Toledo). All reagents are of analytical grade unless stated otherwise.

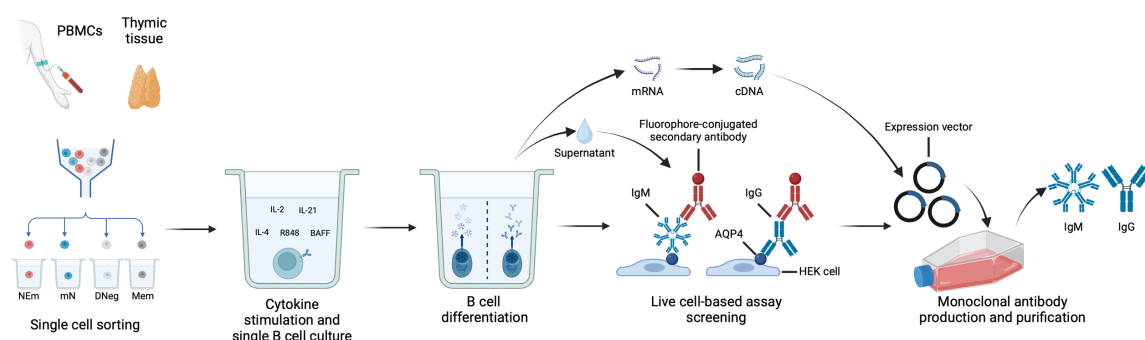


Figure 2.1. A schematic illustration of the stages from PBMC and thymic cells extraction to monoclonal IgM and IgG production.

2.2.1 Single B cell isolation, culture, and differentiation

2.2.1.1 Culture and maintenance of MS40L feeder cells

MS40L cells are a mouse stromal cell line which has been engineered to express human CD40L. A frozen aliquot of non-irradiated MS40L cells were removed from liquid nitrogen, rapidly thawed in a 37°C water bath, transferred to a 15-mL falcon tube, and centrifuged for 300 x g for 5 min. The supernatant was aspirated and the pelleted cells were washed with 10 mL phenol red-free Iscove's Modified Dulbecco's Medium (IMDM, 21056023, Gibco) supplemented with 10% foetal bovine serum (FBS, 16000044, Thermo Scientific), 1% antibiotic-antimycotic containing 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25

µg/mL amphotericin B (AA, 15240062, Gibco), and 55 µM β-mercaptoethanol (21985023, Gibco). Cells were plated in a T75 flask (430641U, Corning) in the above MS40L culture media for 3 days at 37°C and 5% CO₂. Cells were subcultured twice weekly for maintenance.

2.2.1.2 Preparation of MS40L feeder cells for B cell culture

A T75 flask with confluent MS40L cells was washed twice with 10 mL of Dulbecco phosphate-buffered saline (DPBS, 14190144, Thermo Scientific) and the adherent cells were detached with 5 mL 0.5% trypsin-EDTA (15400054, Gibco) for 5 min. Pre-warmed MS40L culture media (10 mL) was added to quench the reaction. Cell suspension was centrifuged at 300 x g for 5 min, the supernatant was decanted, and the pelleted cells were resuspended in 10 mL of B cell culture media made up of phenol red-free RPMI 1640 media (32404014, Gibco) supplemented with 10% FBS (16000044, Thermo Scientific), 1% AA (15240062, Gibco), 1% GlutaMAX (35050061, Gibco), 1% N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, 15630080, Gibco), 1% MEM non-essential amino acids (MEM NEA, 11140050, Gibco), 1% sodium pyruvate (11360070, Gibco), and 0.1% β-mercaptoethanol (21985023, Gibco). Cell number and viability was determined by taking 10 µL of cell suspension, mixing it with 0.4% Trypan Blue solution (93595, Sigma-Aldrich) at a 1:1 ratio, and loading 10 µL of the mixture on each side of a chamber slide (C10228, Thermo Scientific) to be analysed with an automated cell counter (AMQAF2000, Thermo Scientific). MS40L cells were then seeded at 1×10^3 cells/well of a flat-bottom 96-well plate in 100 µL of B cell culture media as feeder cells for subsequent B cell cultures.

2.2.1.3 Flow cytometry

Frozen cryovials of PBMCs and thymic cells were extracted from 4 AQP4-seropositive NMOSD patients and 2 age-matched healthy donors, rapidly thawed in a 37°C water bath, transferred to a 15-ml falcon tube, and centrifuged at 300 x g for 5 min. The pelleted cells were

washed, adjusted to a concentration of $1-5 \times 10^6$ cells/mL in ice cold FACS buffer containing DPBS (14190144, Thermo Scientific) and 0.1% bovine serum albumin (BSA, A9647, Sigma-Aldrich). A 100- μ L aliquot of cell suspension was transferred to a 5-mL, round-bottom falcon tube. To block non-specific binding, 100 μ L of normal mouse serum (NS03L, Sigma-Aldrich) in FACS buffer with a final concentration of 0.1% was added into each tube for 20 min on ice. Cells were pelleted by centrifugation at $300 \times g$ for 5 min and labelled with antibodies against CD3 (clone UCHT1, Pacific Blue, BioLegend), CD10 (clone HI10a, Phycoerythrin 594, BioLegend), CD19 (clone SJ25C1, Allophycocyanin-Cy7, BD Biosciences), CD20 (clone 2H7, Brilliant Violet 711, BioLegend), CD27 (clone O323, Brilliant Violet 605, BioLegend), IgA (clone IS11-8E10, Phycoerythrin, Miltenyi Biotec), IgD (clone IA6-2, Fluorescein, BD Biosciences), IgM (clone MHM-88, Alexa Fluor 647, BioLegend), and IgG (clone G18-145, Alexa Fluor 700, BD Biosciences) diluted in 200 μ L FACS buffer for 30 min on ice and protected from light. Cells were washed three times, stained with 500 ng/mL of 4',6-diaminidino-2-penylindole (DAPI, 564907, BD Biosciences) in 200 μ L FACS buffer, and analysed with a BD LSRII flow cytometer (BD Biosciences).

2.2.1.4 Fluorescence-activated cell sorting (FACS)

Following staining with the labelled primary antibody cocktail as described above, some PBMC and thymic cell suspensions were washed three times and split into 4 tubes with each tube containing a total of 4×10^6 cells in 4 mL of ice cold FACS buffer for cell-sorting experiments. Single CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁺ IgG⁼ CD10⁺ new emigrant (NEm), CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁺ IgG⁼ CD10⁻ mature naïve (MN), CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁻ IgM^{low} double negative (DNeg), and CD19⁺ CD20⁺ IgA⁻ CD27⁺ IgD⁻ IgM⁻ IgG⁺ memory (Mem) B cells were sorted with FACS Aria III cell sorter (BD Biosciences) into individual wells of flat-bottom 96-well plates which were previously seeded with MS40L cells in 200 μ L

of B cell culture media. There was a total of 10 plates for each B cell subset, or 40 plates for 4 B cell subsets, obtained from each patient or healthy control.

2.2.1.5 Single B cell culture and differentiation

Optimisation of the following single B cell culture and differentiation protocol was performed by Dr Sudarshini Ramanathan. Cultures were maintained and differentiated into antibody secreting cells (ASCs) in the presence of the following cytokines: 50 ng/mL interleukin-2 (IL-2, 200-02, PeproTech), 10 ng/mL interleukin-4 (IL-4, 200-04, PeproTech), 50 ng/mL interleukin-21 (IL-21, 200-21, PeproTech), 2.5 µg/mL R848 (ALX-420-038, Enzo Life Sciences), and 200 ng/mL BAFF. The cytokine cocktail was prepared in a total volume of 50 µL per well and added directly into 200 µL of B cell culture media in each well. Culture media was replenished every 3 days by removing 125 µL of old culture media and replacing it with 125 µL of fresh culture media supplemented with 2x cytokine concentrations.

2.2.1.6 Collection of culture supernatants for live cell-based assay (CBA)

On day 26, 200 µL of culture supernatants were collected from all wells and transferred to U-bottom, polypropylene 96-well plates. For initial target-specific antibody screening by live cell-based assay (CBA), 30 µL of supernatants from corresponding wells from 5 plates were pooled and transferred to a new plate, for example, well A1 of the pooled supernatant plate 1 comprised aliquots of supernatants from wells A1 from individual supernatant plates 1-5 with a total volume of 150 µL per well. There was a total of 8 pooled plates from 40 individual plates obtained from each patient or healthy control. Shall there be any specific binding in any of the wells of the pooled plate, a second run of live CBA was performed on the individual supernatants that made up the pooled supernatant from which the positive signal was detected.

2.2.2 *Live cell-based assay (CBA)*

2.2.2.1 Preparation of complete HEK293T growth media

A 500-mL bottle of Dulbecco's modified Eagle's Medium (DMEM, 11965092, Thermo Scientific) was obtained from a 4°C fridge and placed in a laminar flow hood. Using a pipette, 55 mL of solution was removed and replaced with 50 mL of foetal bovine serum (FBS, 16000044, Thermo Scientific) and 5 mL of antibiotic-antimycotic (AA, 15240062, Gibco) solution. The final concentrations of supplements were 10% FBS and 1% AA containing 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. The complete growth media was stored at 4°C until use.

2.2.2.2 Culture and maintenance of HEK293T cells

An aliquot of frozen, adherent HEK293T cells was retrieved from the liquid nitrogen, rapidly thawed for <5 min in a 37°C water bath, transferred to a 15-mL falcon tube, and centrifuged at 300 x g for 5 min. The supernatant was discarded and the pelleted cells were resuspended with 15 mL of pre-warmed complete growth media. Cells were transferred to a T75 flask (430641U, Corning) and grown at 37.5°C and 5% CO². At around 80% confluency, the adhering cells were detached using 5 mL 0.5% trypsin-EDTA (15400054, Gibco). Pre-warmed complete growth media (10 mL) was added to deactivate the trypsin. Cell suspension was centrifuged at 300 x g for 5 min and the supernatant was decanted. The pelleted cells were resuspended with 30 mL of fresh, pre-warmed complete growth media and expanded by seeding into T175 flasks (431080, Corning). Subculturing of cells were performed twice weekly.

2.2.2.3 Poly-L-lysine coating of coverslips and 96-well plates

A working solution of 0.01% poly-L-lysine hydrobromide (PLL) was prepared by dissolving 5 mL of 1% PLL stock solution (P1399, Sigma-Aldrich) in 495 mL of sterile PBS

(BR0014G, SLS). The mixture was added into the top container of a vacuum filter system and passed through a 0.2- μ m polyether sulfone (PES) membrane (431097, SLS). The surface of coverslips and 96-well plates were aseptically and evenly coated with 1 mL/25 cm² of the filtered PLL working solution for 5 min at room temperature (RT). Solution was then removed by aspiration and the surface was rinsed twice with distilled water and allowed to dry in a laminar flow hood for at least 2 hours before use.

2.2.2.4 Seeding of HEK293T cells

A T175 flask that was previously seeded with HEK293T cells with >80% confluency was taken from an incubator. Cells were evaluated under an inverted microscope (CKX53, Evident) to ensure viability. Culture media was aspirated, cells were washed twice with 10 mL of Dulbecco PBS (DPBS, 14190144, Thermo Scientific), and 5 mL of 0.5% trypsin-EDTA (15400054, Gibco) was added to detach cells. Trypsinisation was neutralised by adding 10 mL of pre-warmed complete growth media. Cell suspension was transferred to a 50-mL falcon tube and centrifuged at 300 x g for 5 min. The supernatant was discarded and the pelleted cells were resuspended in 10 mL of fresh, pre-warmed complete growth media. Cell number and viability was determined by taking 10 μ L of cell suspension, mixing it with 0.4% Trypan Blue solution (93595, Sigma-Aldrich) at a 1:1 ratio, and loading 10 μ L of the mixture on each side of a chamber slide (C10228, Thermo Scientific) to be analysed with an automated cell counter (AMQAF2000, Thermo Scientific). The remaining cell suspension was diluted to the desired cell concentration with complete growth media, seeded on PLL-coated coverslips in 6-well plates (900,000 cells/well) or 96-well plates (40,000 cells/well), and grown at 37°C and 5% CO₂ overnight.

2.2.2.5 Transient transfection of HEK293T cells

Plasmid DNAs were introduced into HEK293T cells 24 hours after seeding by transfection with polyethylenimine (PEI). A PEI working solution containing 5.626 $\mu\text{g}/\mu\text{L}$ of PEI was first prepared by diluting 45 mg of powdered PEI (408727, Sigma-Aldrich) in 8 mL of distilled water and adjusting the pH of the final solution to 7.2 using hydrochloric acid solution (HCl, H9892, Sigma-Aldrich). To make a transfection mixture per mL of cell culture media, 1.5 μg of a plasmid DNA and 4.2 μg of PEI (2.8:1 ratio of PEI to DNA, w/w) were added into 25 μL of serum-free DMEM and incubated for 10 min at RT. The mixture was then added into each culture well drop-wise with gentle agitation. After 6 hours of incubation at 37°C and 5% CO_2 , culture media containing the transfection mixture was aspirated and replaced with fresh complete growth media. Cells were allowed to express the transfected plasmid DNA for 24 hours prior to performing an assay.

2.2.2.6 Live cell-based assay (CBA)

To perform live CBAs, culture plates previously seeded with transiently transfected HEK293T cells were removed from an incubator and washed twice with DPBS (14190144, Thermo Scientific). Serial dilutions of serum samples, monoclonal antibodies, or commercial antibodies against surface membrane proteins were directly added onto live cells in blocking buffer containing serum-free DMEM (11965092, Thermo Scientific), 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, H3375, Sigma-Aldrich), and 1% bovine serum albumin (BSA, A9647, Sigma-Aldrich) for 1 hour at RT with gentle agitation. The total reaction volumes were 250 μL per coverslip placed in a 24-well plate and 50 μL per well of a 96-well plate. Cells were then rinsed twice with wash buffer made up of DMEM and 20 mM HEPES, and fixed with 4% paraformaldehyde (PFA, F003, Taab) in PBS for 10 min. After two rounds of washing, a fluorescence-conjugated secondary antibody diluted 1:750 in blocking buffer was applied for 45 min with gentle shaking. Cells were washed twice with PBS

before mounting on a glass slide (631-0909, VWR) in fluorescence mounting medium (S3023, Agilent Dako) supplemented with DAPI (1:1000, 564907, BD Biosciences). For 96-well plate CBAs, a drop of mounting medium/DAPI was added directly into each well.

2.2.2.7 Visual inspection of specific binding

Mounted coverslips and 96-well plates were visualised using a fluorescence (Leica DM1000, Leica Biosystems), inverted (CKX53, Evident), or confocal microscope (LSM 980, Zeiss) with the associated imaging software, namely, Volocity 7 (Quorum Technologies Inc.) and Zen 3.8 (ZEISS). Specific binding was evaluated visually and scored semi-quantitatively from 0-4 with scores less than 1 considered negative, 1 being the lowest positive score with dim but widespread binding detected, and 4 the highest level of bright binding observed.

2.2.2.8 Measurement of fluorescence intensity (FI)

For some experiments, fluorescence intensity (FI) was measured with a microplate reader (FLUOstar OMEGA, BMG Labtech) with the following settings as a quantitative readout for specific binding: mode, fluorescence endpoint; gain, 4000; well scan, spiral average; diameter, 6mm; optic, bottom optic; setting time, 0.2s; number of flashes, 20. FI values were normalised against values from wells incubated with secondary antibody only. Binding curves were generated by fitting data to a one site – specific binding model.

2.2.3 ***Generation of monoclonal antibodies (mAbs)***

2.2.3.1 Reverse-transcription polymerase chain reaction (RT-PCR)

After collecting culture supernatants from the differentiated and stimulated single B cell cultures, 30 μ L RNA lysis solution containing 10 mM dithiothreitol (DTT, A39255, Thermo Scientific) and 40 units RNasin ribonuclease inhibitor (N2111, Promega) in Tris-EDTA buffer pH 8.0 (93283, Sigma-Aldrich) was added into each single B cell culture well to isolate RNA. RNAs were transferred into a 96-well PCR plate and incubated with 2.5 μ L

random primer mix (35 μ M hexamers, 25 μ M dT₂₃VN, 1 mM dNTPs, S1330S, New England Biolabs), 0.25 μ L CH5 universal immunoglobulin primer (5'-3' sequence, CCTCTCACCAACTTTCTTGTCC), and 1.75 μ L RNase-free water (BP2484, Fisher BioReagents) per well for 5 min at 65°C in a thermocycler followed by further incubation on ice for 10 min. Reverse transcription to generate the cDNA was performed by adding 10 μ L 5x SuperScript IV reaction buffer (18090050B, Invitrogen), 2.5 μ L 10 mM dNTP mix (U1511, Promega), 0.5 μ L 25% IGEPAL CA-630 (I8896, Sigma-Aldrich), 1 μ L SuperScript IV reverse transcriptase (18090010, Invitrogen), 0.5 μ L RNasin ribonuclease inhibitor, and 1 μ L 0.1 mM DTT in a total reaction volume of 50 μ L per well. Plates were incubated at 25°C for 10 min, 50°C for 60 min, and 85°C for 10 min in a thermocycler.

2.2.3.2 Amplification of immunoglobulin transcripts (PCR-1)

Immunoglobulin heavy (Ig γ and Ig μ) and light (Ig κ , Ig λ) chain transcripts were amplified by two rounds of PCRs (PCR-1 and -2). PCR-1 was performed by adding 2 μ L of cDNA into a PCR-1 master mix containing 10 μ L GoTaq G2 Green Master Mix (M7822, Promega), 2 μ L leader primer mix (see Appendix 1 for list of primers), and 6 μ L nuclease-free water prepared in a 96-well PCR plate. This reaction was run for 45 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

2.2.3.3 Amplification of immunoglobulin transcripts (PCR-2)

A Circular Polymerase Extension Cloning (CPEC) forward primer mix was first prepared by mixing 14 Ig γ /Ig μ , 13 Ig κ , and 7 Ig λ primers. For PCR-2, 2 μ L of PCR-1 product was added into a PCR-2 master mix containing 10 μ L GoTaq G2 Green Master Mix (M7822, Promega), 1 μ L CPEC forward primer mix, 1 μ L specific reverse primer (see Appendix 2 for list of primers), and 6 μ L nuclease-free water in a total reaction volume of 20 μ L. PCR-2 was performed for 35 cycles at 95°C for 30 sec, 55-60°C for 30 sec, and 72°C for 60 sec.

2.2.3.4 Nucleic acid agarose gel electrophoresis

To make a 1% agarose gel, 0.5 g of UltraPure agarose (16500, Thermo Scientific) was dissolved in 50 mL of 1X Tris-borate-EDTA (TBE) buffer (B52, Thermo Scientific) in a conical flask by heating up the solution in a microwave. The solution was allowed to cool down at RT for 5 min before adding 5 μ L of SYBR Safe (S33102, Thermo Scientific) and decanting it into a casting apparatus. Once solidified, the gel was placed in a gel box filled with 1x TBE buffer. To each of the PCR-2 products (20 μ L), 4 μ L of 6x purple gel loading dye (B7024S, New England Biolabs) was added prior to loading to the wells of the gel. Electrophoresis was carried out at 90 V constant for 20-30 min. As a molecular weight standard, 10 μ L of BenchTop 1 kb DNA ladder (G754A, Promega) was loaded into a separate well.

2.2.3.5 Nucleic acid gel extraction

Gel extraction was performed using a Monarch DNA Gel Extraction Kit (T1020, New England Biolabs). DNA bands corresponded to 300-500 bp were excised using a clean scalpel, transferred to 1.5-mL microcentrifuge tubes, and incubated with 400 μ L of the supplied Gel Dissolving Buffer per 100 mg agarose at 50°C for 5-10 min until the gel slice was completely dissolved. Samples were then loaded onto spin columns, spun at 1000 x g for 1 min, and washed twice with 200 μ L DNA wash buffer. The purified DNA products were eluted with 12 μ L of DNA elution buffer and collected in 1.5-mL microcentrifuge tubes. DNA concentrations were measured using a NanoDrop One UV-Vis spectrophotometer (ND-ONE-W, Thermo Scientific). All samples were sent for sequencing prior to restriction cloning.

2.2.3.6 Preparation of linear immunoglobulin heavy and light chain vectors

Immunoglobulin heavy (Ig γ and Ig μ) and light (Ig κ , Ig λ) chain vectors were linearised by restriction digest. All vectors were cut with AgeI (R3552, New England Biolabs) in combination with Sall (R3138, New England Biolabs) for Ig γ , BsiWI (R3553, New England

Biolabs) for Ig κ , and XhoI (R0146, New England Biolabs) for Ig λ and Ig μ . In a 1.5-mL microcentrifuge tube, 2 μ g of vector plasmid, 3 μ L of AgeI, 3 μ L of Sall/BsiWI/XhoI, 5 μ L of CutSmart buffer (B7204, New England Biolabs) and nuclease-free water were mixed in a total reaction volume of 50 μ L. The mixture was incubated for 3 hours at 37.5°C. Digestion products were run on a 1% agarose gel and linear vectors with a molecular size of approximately 500 kbp were extracted and purified.

2.2.3.7 Ligation

Linear vectors and PCR-2 inserts were adjusted to a concentration of 50 ng/ μ L and 10 ng/ μ L, respectively. A ligation reaction was set up by mixing 1 μ L linear vector, 1 μ L PCR-2 insert, 1 μ L 10x T4 DNA ligase buffer (B0202S, New England Biolabs), 1 μ L T4 DNA ligase (M0202S, New England Biolabs), and 6 μ L nuclease-free water in a 1.5-mL microcentrifuge tube with a total reaction volume of 10 μ L. The mixture was incubated at 25°C for 15 min and 16°C for 45 min. After adding 10 μ L of T4 DNA ligase buffer, another round of incubation was performed at 16°C for 60 min. Ligation products were kept on ice for bacterial transformation. All inserts were cloned into both IgM and IgG backbone for downstream cross-subset comparison and analysis with reference to binding affinity and complement-dependent cytotoxicity.

2.2.3.8 Generation of unmutated common ancestors (UCAs)

Somatically mutated mAbs were reverted to their presumed unmutated germline confirmations based on the IMGT reference library. Mutated sequences were first entered into the IMGT/V-QUEST program to identify the most closely aligned germline templates. As the N (non-template) regions are not encoded by the germline genes, direct alignments against the germline sequences were practically impossible, and thus, these regions were preserved. Reversion of nucleotides were performed using Q5 Site-Directed Mutagenesis Kit (E0554S,

New England Biolabs). A mixture of 12.5 μ L of Q5 Hot Start High-Fidelity 2X Master Mix, 10 μ M forward primer, 10 μ M reverse primer, 1 μ L of DNA sequence, and 9 μ L of nuclease-free water were added into a PCR tube with a total volume of 25 μ L. PCR reactions were run for 25 cycles at 98°C for 10 sec, 50-72°C for 10-30 sec, and 72°C for 20-30 sec/kb. PCR products were subjected to KLD reaction by adding 1 μ L of the product into a mixture of 5 μ L 2X KLD reaction buffer, 1 μ L 10X KLD enzyme mix, and 3 μ L of nuclease free-water. Mixtures were incubated for 5 minutes before adding them into chemically-competent cells for transformation.

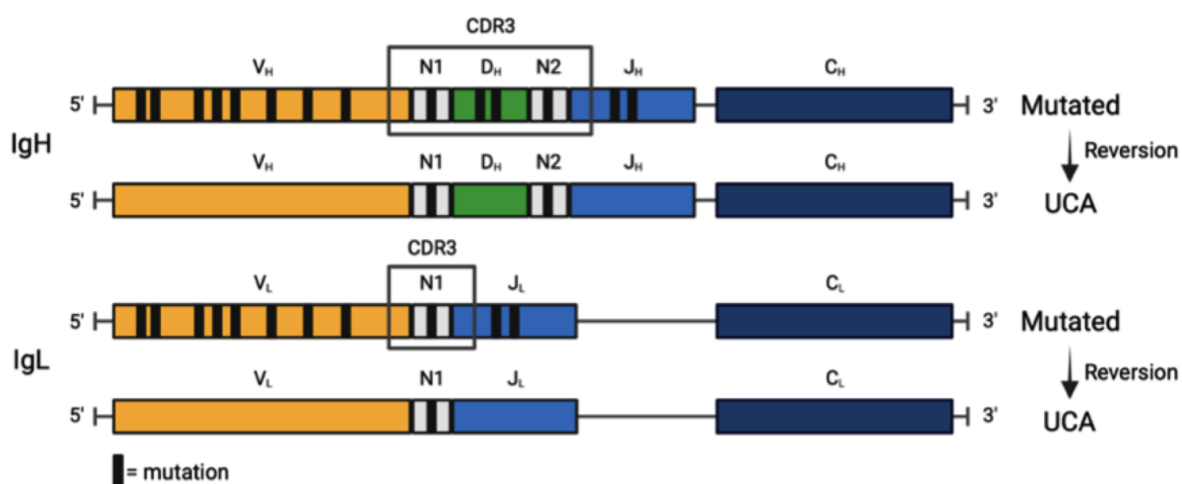


Figure 2.2. Generation of unmutated common ancestors (UCAs)

Closely aligned germline precursors were reconstructed by reverting mutations across the immunoglobulin genes of the parent mAbs. During V(D)J recombination, *N* nucleotides are randomly inserted at the junctions between the *VH-DH* and *DH-JH* segments in the rearranged heavy chain gene and the *VL-JL* segment in the rearranged light chain gene. These *N* regions were not reverted as they are not encoded by the germline immunoglobulin genes.

2.2.3.9 Preparation of selective agar plates and microbial growth media

To prepare agar plates for selective cultivation of *Escherichia coli* (*E. coli*), 40 g of Luria Bertani (LB) broth with agar powder (Miller, L3147, Sigma-Aldrich) was resuspended in 1 L of distilled water and autoclaved for 15 min at 121°C. The suspension was cooled down to 50°C prior to adding a working solution of the desired antibiotic (100 μ g/mL amoxicillin or 10 μ g/mL gentamicin) and subsequent dispensing into sterile petri dishes (10 mL/dish). To

prepare selective growth media for maintenance and propagation of *E. coli*, 25 g of LB broth powder (Miller, L3522, Sigma-Aldrich) was resuspended in 1 L of distilled water, autoclaved, and supplemented with an appropriate antibiotic as described above. Selective agar plates and growth media were stored at 4°C until use.

2.2.3.10 Transformation of *Escherichia coli*

Ligation products were transformed into 5-alpha Competent *E. coli* (C2987I, New England Biolabs). An aliquot of frozen bacterial cells was retrieved from -70°C and thawed on ice until the last ice crystals disappeared. In a 1.5-mL microcentrifuge tube, 2 µL of the ligation product was added to 20 µL of the cell mixture. The mixture was incubated on ice for 30 min, subjected to heat shock at 42°C for 30 sec, and rested on ice for 5 min without mixing. Transformed cells were recovered by incubation at 37°C for 1 hour in 150 µL SOC outgrowth media (B9020S, New England Biolabs) with vigorous shaking at 200 rpm and subsequently grown on a pre-warmed ampicillin agar plate (100 µL/plate) overnight at 37°C.

2.2.3.11 Colony polymerase chain reaction (PCR)

Correct assembly of cloned plasmid DNA was verified by colony PCR. To make a PCR master mix, 5 µL 2x GoTaq G2 Green Master Mix (M7822, Promega), 1 µL CPEC forward primer mix, and 3 µL nuclease-free water were dispensed in a PCR tube with a total reaction volume of 10 µL. A small amount of a bacterial colony from an agar plate was transferred to the PCR tube using a sterile micropipette tip and stirred to resuspend it in the master mix. For each transformation, 10-15 distinct colonies were assayed. The PCR reaction was run for 34 cycles at 98°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec. The PCR products were then loaded into a 1% agarose gel for electrophoresis with the backbone vector without an insert as a negative control. Successful cloning was indicated by the appearance of a band with the correct fragment size.

2.2.3.12 Plasmid DNA minipreparation

A liquid bacterial culture was prepared by inoculating each positive colony as identified by colony PCR into 5 mL of selective growth media made up of LB broth supplemented with 100 µg/mL ampicillin in a loosely covered, 15-ml falcon tube. Cultures were incubated overnight at 37°C with vigorous shaking at 200 rpm. Minipreparation of plasmid DNA was performed using a QIAprep Spin Miniprep Kit (27104, QIAGEN). All reagents described below were included in the kit. Bacterial cells were harvested by centrifugation at 5,400 x g for 10 min at 4°C. The supernatant was drained by inverting the tube. The pelleted bacterial cells were resuspended in 250 µL resuspension buffer (P1), transferred to a 1.5-mL microcentrifuge tube, and lysed with 250 µL lysis buffer (P2) by inverting the tube 4-6 times. The lysis reaction was stopped by adding 350 µL neutralisation buffer (N3). The cell lysate was centrifuged for 10 min at 17,900 x g. The supernatant (800 µL) was transferred to the supplied QIAprep 2.0 spin column and centrifuged at the same speed for 30-60 seconds. Endonucleases and salts were removed by washing with 500 µL buffer PB and 750 µL buffer PE, respectively. After washing, an additional round of centrifugation was performed to remove any residual wash buffer. The bound DNA on the silica matrix was eluted in 50 µL nuclease-free water and its concentration was determined using a NanoDrop One UV-Vis spectrophotometer (ND-ONE-W, Thermo Scientific). The purified plasmid DNA was submitted for Sanger sequencing to verify the insert sequence.

2.2.3.13 Creating bacterial glycerol stocks

For long-term storage of plasmid DNA, 1 mL of liquid bacterial culture was transferred to a cryotube and mixed with 1 mL of 70% glycerol (G5516, Sigma-Aldrich). The glycerol stocks were stored at -80°C. To recover bacteria for future experiments, a small amount of the frozen bacteria was scraped using a micropipette tip and streaked onto a suitable selective agar plate. Grown colonies can then be picked and used for plasmid DNA preparation.

2.2.3.14 Plasmid DNA maxipreparation

To prepare a larger amount of plasmid DNA for downstream experiments, a streak of a bacterial glycerol stock was inoculated in 10 mL of 0.1% ampicillin-supplemented LB broth in a 50-mL falcon tube and incubated for 8 hours at 37°C. The initial culture was expanded by dispensing it in 100-130 mL of the selective growth media in a 3-L conical flask and grown overnight at 37°C with vigorous shaking at 200 rpm. Plasmid maxipreparation was performed using a QIAGEN Plasmid Maxi Kit (12162, QIAGEN). Overnight bacterial culture was harvested by centrifuging at 6,000 x g for 15 min at 4°C. The pelleted bacterial cells were resuspended in 8 mL buffer P1, transferred to a 50-mL falcon tube, lysed with 8 mL buffer P2 at RT for 3 min with gentle mixing. The lysis reaction was neutralised by adding 8-mL buffer S3. The cell lysate was transferred to a supplied QIAfilter cartridge and incubated for 10 min at RT. During incubation, QIAGEN Plasmid Plus spin columns were prepared and placed into a QIAvac 24 Plus vacuum manifold (19413, QIAGEN). The cell lysate was filtered by inserting a plunger into the cartridge. The cleared lysate was transferred to a new 50-mL falcon tube, mixed with 5 mL buffer BB, and loaded into the prepared spin column with a tube extender attached on the vacuum manifold. The liquid was drawn by applying approximately 300 mbar vacuum. The bound DNA was washed with 700 µL buffer ETR and 700 µL buffer PE to remove endotoxins and salts, respectively. The spin column was then placed into a 2-mL collection tube and spun at 10,000 x g for 1 min to remove residual wash buffer. The bound DNA was eluted in 400 µL nuclease-free water and its concentration was determined using a NanoDrop One UV-Vis spectrophotometer (ND-ONE-W, Thermo Scientific).

2.2.4 *Production of monoclonal antibodies*

2.2.4.1 Expression of monoclonal IgG

Adherent HEK293T cells were seeded in a T75 flask in 15 mL complete HEK293T growth media at 37°C and 5% CO₂. To make a transfection mixture per mL of culture media, 0.75 µg of each heavy (Ig γ) and light (Ig κ/λ) chain plasmid DNA and 4.2 µg of PEI were added into 25 µL of serum-free DMEM in a 1.5-mL microcentrifuge tube and incubated for 10 min at RT. At approximately 80% confluence, the transfection mixture was added into the flask with gentle agitation. After 6 hours of incubation at 37°C and 5% CO₂, culture media was discarded and replaced with 15 mL of fresh, phenol red-free DMEM supplemented with 1% Nutridoma-SP (11011375001, Roche). The culture supernatant containing secreted monoclonal IgG was collected 3-5 days post-transfection for purification.

2.2.4.2 Expression of monoclonal IgM

Adherent HEK293T cells were seeded in a T75 flask as described in the previous section and transfected with a mixture containing 0.5 µg of each heavy (Ig μ), light (Ig κ/λ), and joining (IgJ) plasmid DNA, and 4.2 µg of PEI diluted in 25 µL of serum-free DMEM per mL of culture media. The IgJ was introduced into the transfection mixture to promote the formation of pentameric IgM antibodies. Culture media containing the transfection mixture was discarded after 6 hours of incubation and replaced with 15 mL of 1% Nutridoma-SP. The culture supernatant containing secreted monoclonal IgM was collected 3-5 days post-transfection for precipitation and enrichment.

2.2.4.3 Purification of monoclonal IgG

IgG from culture supernatants was affinity purified with protein G immobilised onto a cross-linked 4% agarose beads (Protein G Sepharose 4 Fast Flow, 17061801, Cytiva). The resin was supplied in 20% ethanol with 2 IgG binding sites per ligand, ~2 mg protein G per mL resin,

and a total binding capacity of ≥ 20 mg human IgG per mL resin. To prepare the resin, 100 μ L of resin stock solution was centrifuged at 1,000 x g for 5 min, washed twice with 300 μ L PBS, and transferred to a 1.5-mL microcentrifuge tube. The resin was resuspended in 100 μ L PBS for use.

The culture supernatant was collected in a 50-mL falcon tube, centrifuged at 1,000 x g for 10 min to precipitate cell debris, and passed through 0.2 μ m filters to remove endotoxins. The pre-equilibrated protein G resin was then added into the clarified supernatant (100 μ L resin per 50 mL supernatant) and incubated overnight at 4°C with gentle rotation. The suspension was centrifuged at 1,000 x g for 10 min and the supernatant was aspirated. The precipitated resin was resuspended with 100 μ L PBS, transferred to a Pierce Micro-Spin Column with a 30- μ m polyethylene filter in a collection tube (89879, Thermo Scientific), and washed twice with 300 μ L PBS. The bound IgG was eluted by adding 300 μ L Pierce IgG elution buffer (21004, Thermo Scientific) and spinning the column at 1,000 x g for 10 min. The flow through was collected in 3-4 fractions (300 μ L each) in clean collection tubes and immediately neutralised and adjusted to approximately pH 7.3 by adding 30 μ L of 1 M Tris pH 8.0. (648314, Millipore). To obtain a highly concentrated sample, the purified IgG fractions were loaded in batches into a 500- μ L, 10 kD molecular weight cut-off centrifugal filter device (UFC503096, Merck) and centrifuged at 14,000 x g for 10 min. The flow-through was discarded and the concentrate (approximately 50 μ L) was reconstituted with 450 μ L PBS and centrifuged at 14,000 x g for 10 min. After 5 rounds of buffer exchange, the filter column was placed upside down in a clean, protein low-binding tube and centrifuged at 1,000 x g for 2 min. Concentrated IgG samples were stored at 4°C until use.

2.2.4.4 Precipitation of monoclonal IgM

The method described in this subsection was optimised by Dr Halwan Bayuangga. Monoclonal IgM in the culture supernatant was precipitated using ammonium sulphate.

Addition of a saturated ammonium sulphate solution to a protein sample reduces protein solubility by competing for water interactions. Due to its relatively high molecular weight, IgM antibodies are among the first proteins to precipitate.

The culture supernatant was harvested and clarified as described in the previous section. In a 50-mL falcon tube, 15 mL of 4.32 M ammonium sulphate solution (45216, Thermo Scientific) was slowly added to 15 mL of the clarified supernatant. The mixture was incubated overnight at 4°C and centrifuged at 3,000 x g for 20 min. After decanting the supernatant, the precipitate was reconstituted in 1 mL PBS, loaded in batches into a 500-μL, 100 kD molecular weight cut-off centrifugal filter device (UFC510096, Merck), and centrifuged at 14,000 x g for 10 min. Desalting and buffer exchange was performed by reconstituting the concentrate (approximately 50 μL) with 450 μL PBS and spinning at 14,000 x g for 10 min. This process was repeated 5 times. To recover the sample, the filter column was placed upside down in a clean, protein low-binding tube and centrifuged at 1,000 x g for 2 min. Concentrated IgM samples were stored at 4°C until use.

2.2.4.5 Quantitation of monoclonal IgG concentration

Monoclonal IgG concentrations were determined using a NanoDrop One UV-Vis spectrophotometer (ND-ONE-W, Thermo Scientific). The machine was prepared by cleaning the pedestal twice with 2 μL ultrapure water and blanking with 2 μL PBS. A 2 μL purified IgG sample was then placed on the pedestal and its absorbance at 280 nm was measured. The antibody concentration (mg/mL) was recorded directly from the display window. Alternatively, the concentration can be calculated manually using the following equation: IgG concentration (mg/mL) = ($A_{280}/E1\%$ value) x 10 mg/mL. The $E1\%$ value used in this calculation is the extinction coefficient of a 150-kD IgG molecule at 280 nm which is equal to 13.7. The sample purity was also assessed by measuring the 260/280 ratio with a value of <1 considered as free from nucleic acid contamination.

2.2.4.6 Quantitation of monoclonal IgM concentration

Addition of ammonium sulphate to culture supernatants allows precipitation of proteins by reducing their water solubility. The presence of other proteins in an IgM-enriched sample obtained using this method hinders reliable IgM quantitation by spectrophotometry. Therefore, a commercial human IgM-specific ELISA kit (88-50620-88, Thermo Scientific) was used. All reagents used in this protocol were included in the kit. As per manufacturer's instructions, a flat-bottom 96-well plate was coated with 100 μ L/well of a pre-titrated, purified anti-human IgM monoclonal antibody overnight at 4°C. The plate was washed five times with wash buffer containing 0.05% Tween-20 in PBS and blocked with 250 μ L/well of 10% BSA in the same buffer for 2 hours at RT. Two-fold serial dilutions of human IgM standard in the range of 1,000-15.6 ng/mL were prepared. IgM samples were serially diluted 1:10, 1:100, 1:1,000, and 1:10,000. IgM standards and samples were then added into the plate and incubated for 2 hours at RT. After five rounds of washing with wash buffer, the plate was incubated with 100 μ L/well of pre-titrated, HRP-conjugated anti-human IgM detection antibody (1:250) for 1 hour at RT. The plate was washed five times with wash buffer and incubated with 100 μ L/well of tetramethylbenzidine (TMB) substrate for 15 min at RT. The reaction was stopped by adding 100 μ L of 1M H₂SO₄ directly into each well. Optical density values were read using a microplate reader at 450 nm and IgM concentrations were extrapolated from a standard curve.

2.2.4.7 Polyacrylamide gel electrophoresis (PAGE)

To confirm successful expression of monoclonal IgG and IgM antibodies, samples were analysed by polyacrylamide gel electrophoresis (PAGE) under reducing and non-reducing conditions. Commercially available human IgG (I4506, Sigma-Aldrich) and IgM standards (I8260, Sigma-Aldrich) were used as controls. These standards were purified from human serum with a purity of $\geq 95\%$ as determined by high performance liquid chromatography (HPLC). For reducing PAGE, in a 1.5-mL microcentrifuge tube, 2.5 μ g of each IgG and IgM

samples and standards were mixed with 2.5 μ L of 4x NuPAGE lithium dodecyl sulphate (LDS) sample buffer (NP0007, Invitrogen) and 1 μ L of 500 mM dithiothreitol (DTT, NP0004, Invitrogen). Deionised water was added to make up a total reaction volume of 10 μ L. DTT was replaced with an equal volume of deionised water for non-reducing PAGE. The mixture was incubated at 70°C for 10 min to denature proteins and was subsequently loaded into the wells of a NuPAGE 4-12% Bis-Tris gradient gel (NP0322BOX, Invitrogen). After placing the gel cassette in a gel tank connected to a power source, the inner and outer chambers were filled with 500 mL and 800 mL of MOPS-SDS running buffer (NP0001, Invitrogen), respectively. Additionally, to maintain the reduced state of the protein samples during reducing PAGE, 500 μ L of NuPAGE antioxidant (NP0005, Invitrogen) was added to the buffer in the inner chamber. Electrophoresis was carried out at 200 V constant for 50 min. A pre-stained protein ladder with a mixture of proteins ranging from 10-250 kD was used as molecular size standards (26619, Thermo Scientific).

2.2.4.8 Coomassie Blue staining

Following electrophoresis, the gel was extracted from the cassette, washed three times with deionised water for 5 min, and immersed in 15 mL of Imperial Protein Stain (24615, Thermo Scientific) containing Coomassie dye R-250 for 1 hour at RT with gentle shaking. The solution was subsequently discarded and replaced with 250 mL deionised water to reduce background staining. Washing was performed three times for 5 min each and the gel was left in 15 mL clean deionised water overnight at RT to further remove stain from the gel and improve the visualisation of protein bands. The gel was imaged and analysed with ChemiDoc MP Imaging System (12003154, Bio-Rad).

2.2.4.9 Western Blot

Following protein separation by electrophoresis, the protein gel was extracted from the cassette and placed on an iBlot 2 Transfer Stack (IB24002, Invitrogen) with an integrated pre-activated polyvinylidene fluoride (PVDF) transfer membrane. Each transfer stack also contains a copper-coated electrode with cathode and anode buffers which are suitable for dry transfer method. Protein transfer was performed using an iBlot 2 Gel Transfer Device (IB21001, Invitrogen) at 20 V for 1 min, 23 V for 5 min, and 25 V for 2 min with a total run time of 8 min. The transfer membrane was carefully removed from the stack, washed 3 times with 10 mL 0.1% Tween-20 in tris-buffered saline pH 7.2 (TBST, 28360, Thermo Scientific) for 5 min each, and blocked with 10 mL of 5% skim milk (70166, Millipore) in TBST for 30 min at RT. To identify protein bands, the membrane was incubated with 10 mL of HRP-conjugated, goat anti-human IgG, Fc γ (109-035-008, Jackson ImmunoResearch) and IgM, Fc γ antibodies (109-035-129, Jackson ImmunoResearch) diluted 1 μ g/mL in TBST for 1 hour at RT. After three rounds of washing with 10 mL TBST for 5 min each, the membrane was incubated with a mixture of 5 mL luminol substrate solution and 5 mL H₂O₂ (34580, Thermo Scientific) for 1 min protected from light. The membrane was removed from the solution, placed in a plastic sheet protector, and imaged with ChemiDoc MP Imaging System (12003154, Bio-Rad).

2.2.5 *Poly and autoreactivity ELISA*

Screening for antibody poly and autoreactivity by ELISA was performed by Dr Miriam Fichtner from Dr Kevin O'Connor's laboratory at Yale University, USA.

2.2.5.1 Polyreactivity ELISA

Polyreactivity is defined as simultaneous reactivity to three antigens, namely, dsDNA, LPS, and insulin. To evaluate antibody polyreactivity, flat bottom, 96-well plates were coated with 100 μ L/well of 20 μ g/mL double-stranded DNA (dsDNA, D8515, Sigma-Aldrich), 10

μg/mL lipopolysaccharide (LPS, L2630, Sigma-Aldrich), or 15 μg/mL recombinant human insulin (30-A151, Fitzgerald Industries) overnight at 4°C. The plates were washed five times with 0.05% Tween-20 in PBS (PBST) and blocked with 250 μL/well of 10% BSA in PBST for 2 hours at RT. Highly reactive ED38, a monoclonal IgG generated from VpreB⁺ λ5⁺ peripheral B cells, was used as a positive control. Monoclonal IgG samples and control were serially diluted in PBS (1 μg/mL, 0.25 μg/mL, 0.063 μg/mL, and 0.016 μg/mL), added into each well in a total reaction volume of 100 μL, and incubated for 2 hours at RT. The plates were washed five times with PBST, incubated with 100 μL/well of an HRP-conjugated, goat anti-human IgG, Fcγ (109-035-008, Jackson ImmunoResearch) detection antibody diluted 1:250 in PBS for 1 hour at RT, and washed again with PBST five times. Signals were developed by adding 100 μL 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 1721064, Bio-Rad) into each well and incubating for 15 min at RT. The reaction was stopped by adding 100 μL of the supplied stop solution directly into each well. Optical density (OD) values at 405 nm were read with a microplate reader (FLUOstar OMEGA, BMG Labtech). Samples were considered positive if OD₄₀₅ at 1 μg/mL was ≥0.5.

2.2.5.2 Autoreactivity ELISA

Autoreactivity is defined as reactivity to Hep-2 cell lysate. Semi quantitative detection of autoreactive antibodies was performed using commercially available QUANTA Lite ANA ELISA plates pre-coated with human epithelial type 2 (Hep-2) cell lysate (708750, INOVA Diagnostics). Highly reactive ED38, a monoclonal IgG generated from VpreB⁺ λ5⁺ peripheral B cells, was used as a positive control. Monoclonal IgG samples and control were serially diluted in PBS (1 μg/mL, 0.25 μg/mL, 0.063 μg/mL, and 0.016 μg/mL), added into each well in a total reaction volume of 100 μL, and incubated for 30 min at RT. The wells were washed five times with 250 μL/well of 0.05% Tween-20 in PBS (PBST), incubated with 100 μL/well of an HRP-conjugated, goat anti-human IgG, Fcγ (109-035-008, Jackson ImmunoResearch)

detection antibody diluted 1:250 in PBS for 1 hour at RT, and washed again with 250 μ L/well PBST five times. Signals were developed by adding 100 μ L 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 1721064, Bio-Rad) into each well and incubating for 15 min at RT. The reaction was stopped by adding 100 μ L of the supplied stop solution directly into each well. Optical density (OD) values at 405 nm were read with a microplate reader (FLUOstar OMEGA, BMG Labtech). Samples were considered positive if OD₄₀₅ at 1 μ g/mL was ≥ 0.5 .

2.2.6 Complement-dependent cytotoxicity (CDC) assay

2.2.6.1 Seeding of HEK293S stable cells

Aquaporin-4 (AQP4) stable cell line had been generated previously by Dr Patrick Waters by transfecting HEK293S cells with an untagged human AQP4-M23 plasmid containing a geneticin resistance gene. Stable expression over generations was achieved by growing cells in growth media supplemented with 200 μ g/mL geneticin (10131027, Gibco) and sorting by flow cytometry the brightest 10% of cells.

A frozen aliquot of the stable cells was retrieved from the liquid nitrogen, rapidly thawed for <5 min in a 37°C water bath, transferred to a 15-mL falcon tube, and centrifuged at 300 x g for 5 min. The supernatant was discarded and the pelleted cells were resuspended in 15 mL selective growth media made up of DMEM supplemented with 10% FBS and 200 μ g/mL geneticin. The cells were expanded by plating in a T75 flask at 37°C and 5% CO₂.

At 80% confluence, the growth media was aspirated and 5 mL of 0.5% trypsin-EDTA was added to detach the cells. The reaction was neutralised by adding 10 mL growth media. The cell suspension was transferred to a 15-mL falcon tube and centrifuged at 300 x g for 5 min. The pelleted cells were resuspended in growth media and adjusted to a concentration of 2 x 10⁵ cells/mL from which 100 μ L was seeded into each well of a PLL-coated, flat bottom, 96-well plate.

2.2.6.2 Preparation of human complement

Peripheral bloods from healthy volunteers were collected in glass vacutainers containing the active anticoagulant tri-sodium citrate with citric acid and dextrose (364816, BD). Samples were centrifuged at 1,000 x g for 10 min and supernatants were aliquoted and immediately stored at -80°C to preserve complement activity until use.

2.2.6.3 Complement-dependent cytotoxicity (CDC) assay

After carefully removing the growth media, cells were washed twice with 100 µL/well of phenol red-free DMEM (21063029, Thermo Scientific). Cells were then incubated with serial dilutions of heat-inactivated sera or mAbs in phenol red-free DMEM and 10% human complement (v/v) in a total volume of 100 µL/well for 24 hours at 37°C and 5% CO₂. The following day, the culture media was discarded and the cells were washed with 100 µL/well of phenol red-free DMEM. Cell viability was assessed by incubating cells with 20% Alamar Blue reagent (DAL1100, Invitrogen) in 100 µL phenol red-free DMEM for 1 hour at 37°C and 5% CO₂. Resazurin, the active ingredient of Alamar Blue reagent, is cell-permeable, non-toxic, and non-fluorescent. In live cells, this compound is reduced to resorufin, a highly fluorescent metabolite. Resorufin fluorescence intensities (FIs) were recorded with a microplate reader (FLUOstar OMEGA, BMG Labtech) at 560/590 nm excitation/emission wavelengths as a measure of cell viability which is indirectly proportional to antibody cytotoxicity. Complete lysis control (100%) was determined by lysing cells with 1% Triton X-100 (Sigma-Aldrich) whereas no lysis control or background (0%) was obtained from cells incubated with an equivalent concentration of healthy serum or isotype control antibody and human complement. Cytotoxicity (%) was calculated as $(FI_{\text{experimental well}} - FI_{\text{background}}) / (FI_{100\% \text{ lysis}} - FI_{\text{background}}) \times 100\%$. Data were fitted to a sigmoidal, four-parameter logistic (4PL) regression model to generate killing curves.

2.2.7 Astrocyte indirect immunofluorescence assay (IIFA)

2.2.7.1 Isolation and culture of rat primary astrocytes

Brains from E28 Sprague Dawley rat pups (Envigo) were carefully isolated and dissected (performed by Dr Hannah Fox). After removal of the brain stem and olfactory bulbs, the cortices were peeled and the meninges were removed to minimise contamination of cultures by fibroblasts. Cortices were cut into small pieces under sterile conditions, transferred to a 15-mL Falcon tube, and washed twice with 10 mL Hanks' Balanced Salt Solution (HBSS, 14025092, Gibco). Tissues were dissociated with 2.5% trypsin-EDTA for 30 min at 37°C with occasional shaking, centrifuged at 300 x g for 5 min, and the supernatant was discarded. The pelleted tissue was resuspended with 10 mL astrocyte culture media made up of DMEM supplemented with 10% FBS and 1% AA and triturated to obtain a single cell suspension using a 10 mL pipette twice, a 5-mL pipette twice, and a 1000-μL tip twice. Single cells were counted and seeded as a mixed cortical culture at a density of 10-15 x 10⁶ cells in 15 mL culture media per T75 flask. Culture media was changed at day in vitro (DIV) 2 and every 3 days thereafter.

At DIV 7, the flask was placed on an orbital shaker at 180 rpm for 30 min to remove the overlaying microglia. The supernatant was discarded, the astrocyte layer was rinsed twice with 10 mL PBS and detached with 5 mL of 0.5% trypsin-EDTA for 5 min at 37°C. Culture media (10 mL) was added and cell suspension was centrifuged at 300 x g for 5 min. The supernatant was decanted and the pelleted cells were resuspended in 15 mL culture media and seeded as an enriched astrocyte culture in a new PLL-coated T75 flask. Media change was performed every 3 days. At DIV 14, or DIV 21 post-split, astrocytes were ready for downstream experiments.

2.2.7.2 Astrocyte indirect immunofluorescence assay (IIFA)

Mature astrocytes were seeded on PLL-coated coverslips in 24-well plates at 20,000 cells/ well in 250 μ L astrocyte culture media and grown at 37°C and 5% CO₂. On day 3, culture supernatant was aspirated and cells were rinsed with twice with 250 μ L wash buffer (DMEM and 20 mM HEPES). Serum samples or mAbs diluted in blocking buffer (DMEM, 20 mM HEPES, and 1% BSA) were added into each well with a total volume of 250 μ L. Incubation was performed for 1 hour at RT with gentle agitation. After rinsing twice with 250 μ L wash buffer, cells were fixed with 250 μ L of 4% PFA in PBS for 10 min at RT and permeabilised with 250 μ L of 0.1% Triton X-100 in PBS for 10 min at RT. Cells were rinse twice with 250 μ L PBS and incubated with a rabbit anti-bovine GFAP (cross-reactive to rat GFAP, Z0334, Agilent Dako) diluted 1:1000 in 250 μ L blocking buffer for 45 min at RT to identify astrocytes. After two rounds of washing with 250 μ L PBS, specific binding was detected by incubating cells with a cocktail of fluorescence-conjugated, goat anti-human IgG or IgM and goat anti-rabbit IgG secondary antibodies diluted 1:750 in 250 μ L blocking buffer for 45 min at RT. Cells were rinsed twice with 250 μ L PBS before mounting the coverslip on a glass slide in fluorescence mounting medium supplemented with DAPI (1:1000). Coverslips were observed under a fluorescence (Leica DM1000, Leica Biosystems) or confocal microscope (LSM 980, Zeiss) and imaged with Volocity 7 (Quorum Technologies Inc.) or Zen 3.8 (ZEISS) imaging software.

2.2.8 *Rat brain indirect immunofluorescence assay (IIFA)*

2.2.8.1 Tissue preparation

Wild type, 8-12 weeks old, male Wistar rats were purchased from Charles River UK. Non-perfused brains were carefully dissected, transferred into a 15-mL falcon tube, rinsed twice with 10 mL phosphate buffer pH 7.4, and lightly fixed with 10 mL of 4% PFA in PBS

for 1 hour on ice. Brains were immersed in 10 mL of 30% sucrose solution for 2 days at 4°C, embedded in OCT cryostat sectioning medium (4583, Tissue-Tek), snap frozen in isopentane (24872.323, VWR) on dry ice, and stored at -80°C until use.

2.2.8.2 Rat brain indirect immunofluorescence assay (IIFA)

Tissue block was sectioned in the coronal plane at 10 µm thickness with a Leica CM1860 cryostat (Leica Biosystems). Brain sections were mounted on glass slides, air dried for 30 min at RT, and rehydrated in PBS. Sections were blocked with 10% normal goat serum (S-1000-20, Vector Laboratories) in PBS for 30 min at RT and incubated with serum samples or mAbs diluted in 1% normal goat serum in PBS overnight at 4°C. After two rounds of washing with PBS for 5 min each, a fluorescence-conjugated, goat anti-human IgG or IgM was added on the sections for 45 min at RT. Sections were washed twice with PBS for 5 min each before applying DAPI diluted 1:1000 in PBS for 10 min at RT. Immunofluorescence was visualised with a fluorescence (Leica DM1000, Leica Biosystems) or confocal microscope (LSM 980, Zeiss) and the images were captured with Volocity 7 (Quorum Technologies Inc.) or Zen 3.8 (ZEISS) imaging software.

2.2.9 *Animal model*

2.2.9.1 Animals

Male wild type Wistar rats, 8-12 weeks of age, were obtained from Charles River UK, housed in a standard 12-hour light-dark cycle, and given *ad libitum* access to food and water. Experiments were carried out at Professor Daniel Anthony's laboratory (Experimental Neuropathology Group) at the Department of Pharmacology with the approval of the University of Oxford's Animal Welfare and Ethical Review Board (AWERB) under project license (PPL) number P996B4A4E. All work carried out was compliant with the UK Animals (Scientific Procedures) Act 1986 and ARRIVE guidelines at all times.

2.2.9.2 Intracerebral injections

Surgical procedures were performed with assistance from Prof Daniel Anthony and Ms Jaezah Azman. Rats were induced with 2% isoflurane at an oxygen flow rate of 2 L/min, mounted on a stereotaxic frame (Stoelting Co.), and maintained with 1.5% isoflurane at 1.5 L/min oxygen during the entire duration of surgical procedure. A small midline scalp incision was made under an operating microscope and a burr hole was drilled in the exposed bone at 1 mm anterior and 3 mm lateral to bregma. Using a finely drawn glass microcapillary (21-180-12, Drummond Scientific), a mixture of 10 µg monoclonal IgG or IgM and 10% human complement (v/v) in 2 µL PBS was injected into the left striatum at 4 mm ventral of the cortical surface. Injections were performed gently over 5 min to minimise tissue damage due to the injection alone. Rats injected with the same amount of an isotype control antibody and human complement were used as a control. The incision wound was closed with a 3-0 vycril suture (Ethicon) and dressed appropriately. Rats were transferred to a warming cabinet and returned to the cage as soon as they regained their righting reflex.

2.2.9.3 Preparation of saline-heparin solution

To prepare saline-heparin solution for perfusion, 9 g NaCl (S9888, Sigma-Aldrich) was dissolved in 1 L distilled water. Heparin sodium salt from porcine intestinal mucosa (5,000 U, H3393, Sigma-Aldrich) was then added to the saline solution. Solution was stored at 4°C until use.

2.2.9.4 Preparation of gelatinised glass slides

Glass slides (631-0909, VWR) were placed in metal slide racks, washed with 96% ethanol for 30-60 sec, and dried for 1 hour at 37°C. Gelatine solution was prepared by dissolving 2.5 g gelatine (G1890, Sigma-Aldrich) in 500 mL pre-warmed distilled water. The solution was stirred with a magnetic stirrer and allowed to cool down to approximately 40°C

before adding 0.25 g chromium (III) potassium sulphate dodecahydrate (243361, Sigma-Aldrich). Dried slides were dipped into the gelatine solution for 60 sec, re-dried overnight at 37°C, and stored at RT before use.

2.2.9.5 Tissue collection and preparation

Rats were anaesthetised with 2% isoflurane/oxygen at 2 L/min, and perfused with 0.9% saline-heparin solution to flush the blood out of the circulation followed by freshly prepared 4% PFA in PBS through the left cardiac ventricle. Brains were harvested, post-fixed in 4% PFA in PBS overnight at 4°C, immersed in 30% sucrose solution for 2 days at 4°C, embedded in OCT cryostat sectioning medium, snap frozen in pre-chilled isopentane (792632, Sigma-Aldrich) on dry ice, and stored at -80°C until ready for sectioning. The tissue block was cut coronally at 10 µm thickness and serial sections covering a length of 150 µm anterior and posterior to the injection site were collected on gelatinised glass slides.

2.2.9.6 Immunohistochemistry

Tissue sections were removed from -80°C, air-dried, and rehydrated in PBS. To remove crosslinks created by formalin fixation, heat-induced antigen retrieval was performed by incubating the sections in 10 mM citric acid pH 6.0 (791725, Sigma-Aldrich) for 3 min at 95°C. After two rounds of washing with PBS for 5 min each, endogenous hydrogen peroxidase activity was quenched by incubating the sections in 1% H₂O₂ solution (H1009, Sigma-Aldrich) for 10 min at RT with gentle agitation. Sections were washed twice for 5 min each with PBS and then blocked with 10% normal goat serum in PBS for 30 min at RT to reduce background staining. Primary antibodies diluted in 1% normal goat serum in PBS were applied overnight at 4°C. The sections were washed twice in PBS for 5 min and incubated with a biotinylated secondary antibody diluted 1:200 in 1% normal goat serum in PBS for 2 hours at RT. During incubation with the secondary antibody, a working solution of avidin-biotinylated HRP

complex (ABC, PK-6100, Vector Laboratories) was prepared by mixing avidin (1:100) and biotinylated HRP (1:100) in PBS and incubating the mixture for 30 min before use. After two rounds of washing with PBS for 5 min each, the sections were incubated with the prepared ABC solution for 30 min at RT. Specific staining was visualised by incubating the sections with 1 mg/mL 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB, D5637, Sigma-Aldrich) and 0.03% H₂O₂ in PBS. When the desired signal intensity was achieved, the sections were washed twice with PBS and twice with distilled water for 5 min each. To counterstain nuclei, the sections were dipped in 1% cresyl violet stain solution (C5042, Sigma-Aldrich) for 3-5 min. The sections were then dehydrated through washing in graded ethanol solutions (80% once, 95% once, and 100% twice for 30 sec each), cleared twice in xylene (214736, Sigma-Aldrich) for 5 min each, mounted on gelatinised glass slide with DPX mounting medium (06522, Sigma-Aldrich), and air dried for 48 hours prior to analysis.

2.2.9.7 Image processing

Tissue sections were scanned with a whole image scanner (Leica Biosystems) connected to a brightfield microscope (Leica DM1000, Leica Biosystems) at 20x objective lens magnification under constant illumination. Photomicrographs were converted into 16-bit grayscale images on ImageJ and thresholded to highlight areas of interest.

2.2.10 *Statistical analysis*

All statistical analyses were performed using GraphPad Prism version 9.5.1. Data were presented as mean $\pm$ standard error of the mean (SEM) unless otherwise stated. To assess whether data were drawn from a normal distribution, the D'Agostino-Pearson and Shapiro-Wilk tests were used. For continuous data, parametric tests of means were conducted using t test or one-way ANOVA with Mann-Whitney U or Kruskal-Wallis test as a non-parametric alternative to compare medians. Tukey or Dunn post-hoc tests were performed following a

significant one-way ANOVA or Kruskal-Wallis test, respectively. Differences in proportions of a categorical outcome were evaluated using Chi-square or Fisher's exact test depending on the size of samples. The best-fit mAbs binding kinetics were developed by fitting fluorescence intensity values over a range of serial concentrations of mAbs into a single-site, specific binding model. To further optimise non-linear regression analysis outputs, data points that deviate from the global trend were identified and removed using the ROUT method, also known as robust regression and outlier removal method, on the basis of false discovery rate (FDR). For this analysis, the maximum desired FDR, or Q value, was set to 1% to ensure that less than 1% of the outliers to be falsely or incorrectly identified. A sigmoidal, four-parameter logistic (4PL) regression model was employed to generate dose-response curves from the *in vitro* cytotoxicity experiments. Differences of best-fit values for both binding kinetics and dose-response curves were compared across mAb subsets using extra sum-of-square F test. The strength and direction of linear relationships between two datasets or variables were approximated using either the parametric Pearson or non-parametric Spearman correlation. Fiji software was used throughout for image processing and analysis whereas schematic illustrations were created using BioRender. P values <0.05 were considered statistically significant.

3 Chapter 3: Molecular Characterisation of Immunoglobulin Genes in NMOSD

3.1 Introduction

The immunoglobulin genes have been extensively studied to gain insights into the humoral arm of the adaptive immune response. Knowledge of the organisation of the immunoglobulin genes within the human genome, linked to the current understanding of the B cell developmental stages, enables us to exploit the information embedded in the B cell receptor (BCR) repertoire to study the development of self-reactivity in various autoimmune diseases.

The literature surrounding NMOSD suggest that AQP4-specific B cells exist pre- and post- germinal centre (GC) reactions. *In vitro* cultured naïve (pre-GC) and memory (post-GC) B cells from NMOSD patients were shown to secrete AQP4-specific antibodies⁷⁸. Additionally, intrathecal antibody-secreting cells (ASCs) from affected individuals demonstrated features of antigen-driven clonal expansion⁷⁹. Their BCRs harboured high frequencies of somatic hypermutations, representing a post-GC population. Thus, we asked the following questions: a) how early does specificity to AQP4 emerge along the B cell development in NMOSD?; and b) are there distinct immunoglobulin gene features which characterise the AQP4-specific B cell repertoire in NMOSD?

We approached these questions by isolating single B cells from several developmental lineages from four AQP4-IgG seropositive NMOSD patients. Upon *in vitro* culture and differentiation into antibody-secreting cells, the capacity of these different B cells to produce AQP4-specific antibodies was evaluated to determine the earliest point at which the antigen specificity emerges. Additionally, we performed molecular analyses of the BCRs to explore the positive and negative selection forces that may shape the BCR repertoire and predispose them to specificity against AQP4.

3.2 Specific study aims

The work included in this chapter specifically aimed to:

1. Isolate single B cells from peripheral blood samples from four NMOSD patients;

2. Perform single B cell culture and differentiation into antibody-secreting cells (ASCs);
3. Evaluate single B cell culture supernatants for specificity to AQP4 using live cell-based assay (CBA); and
4. Characterise the immunoglobulin heavy and light V(D)J gene family usage, the frequency of somatic mutations across the immunoglobulin V gene segments, and the physicochemical properties of the complementarity-determining regions 3 (CDR3s) of the AQP4-specific B cell repertoire.

3.3 Methods

3.3.1 Patients

Four patients who met the 2015 criteria for NMOSD with AQP4-IgG from the Highly Specialised Service for NMO in Oxford and had high titres of AQP4-IgG antibodies were selected for this study. Patient demographics and clinical course including disease duration, disease activity, and treatment regime were extracted from medical records. Two age-matched healthy volunteers were selected as controls. Peripheral blood mononuclear cell (PBMC) samples were obtained from all patients and controls (DR11, patient 1; DR14, patient 2; DR26, patient 3, DR20, patient 4; DR34, control 1; DR35, control 2) in addition to a thymic cell sample from one of the patients (DR30, patient 4). Archived patient samples were donated by Prof Nick Willcox. All samples were aliquoted following extraction from patients and stored in a liquid nitrogen tank to prevent multiple freeze-thaw cycles. This study was performed in accordance with research ethics committee approvals (REC16/YH/0013 and 14/SC/0280) after full informed consents were obtained from all participants.

3.3.2 Single B cell isolation and fluorescence-activated cell sorting

These experiments were performed by Dr Sudarshini Ramanathan with the detailed protocols as described in Chapter II, Subsection 2.2.1.1-4. Briefly, PBMC and thymic cell samples were adjusted to a concentration of $1-5 \times 10^6$ cells/mL in ice cold FACS buffer (PBS, 0.1% BSA), blocked with 0.1% normal mouse serum in PBS, and labelled with antibodies against CD3 (clone UCHT1, Pacific Blue), CD10 (clone HI10a, Phycoerythrin 594), CD19 (clone SJ25C1, Allophycocyanin-Cy7), CD20 (clone 2H7, Brilliant Violet 711), CD27 (clone O323, Brilliant Violet 605), IgA (clone IS11-8E10, Phycoerythrin), IgD (clone IA6-2, Fluorescein), IgM (clone MHM-88, Alexa Fluor 647), and IgG (clone G18-145, Alexa Fluor 700) for 30 min protected from light. After three rounds of washing with ice cold FACS buffer, cells were stained with DAPI and analysed with a BD LSRII flow cytometer. Additionally, single CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁺ IgG⁼ CD10⁺ new emigrant (NEm), CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁺ IgG⁼ CD10⁻ mature naïve (MN), CD19⁺ CD20⁺ IgA⁻ CD27⁻ IgD⁻ IgM^{low} double-negative (DNeg), and CD19⁺ CD20⁺ IgA⁻ CD27⁺ IgD⁻ IgM⁻ IgG⁺ memory (Mem) B cells were sorted with FACS Aria III cell sorter and plated on the bed of MS40L feeder cells in 96-well plates in 200 μ L B cell culture media (RPMI 1640 supplemented with 10% FBS, 1% AA, 1% GlutaMAX, 1% HEPES, 1% MEM NEA, 1% sodium pyruvate, and 0.1% β -mercaptoethanol) per well. For each subject, a total of 40 plates with 10 plates per B cell subset were collected.

3.3.3 Single B cell culture and differentiation

Single B cell cultures were maintained and differentiated into ASCs in the presence of the following cytokines: 50 ng/mL interleukin-2 (IL-2), 10 ng/mL interleukin-4 (IL-4), 50 ng/mL interleukin-21 (IL-21), 2.5 μ g/mL R848, and 200 ng/mL BAFF (see Chapter II, Subsection 2.2.1.5). The cytokine cocktail (50 μ L) was added directly to 200 μ L of B cell culture media in each well. Media change was performed every 3 days by replacing half volume (125 μ L) of the old media with 125 μ L of fresh media with 2x cytokine concentrations.

3.3.4 Screening of AQP4-specific antibody production by live cell-based assay (CBA)

After 26 days in vitro, 200 μ L of culture supernatants were collected from all wells and transferred to U-bottom, polypropylene 96-well plates. For every 5 individual plates, 30 μ L supernatant from each corresponding well was taken, pooled together, and transferred to a new plate. For example, supernatant aliquots from wells A1 of individual plates 1-5 were pooled and transferred to well A1 of pooled plate 1. There was a total of 8 pooled supernatant plates made up of aliquots from 40 individual plates for each subject. Initial screening by live CBA was performed by incubating HEK293T cells expressing AQP4-M23-EGFP with 50 μ L of the pooled supernatants using the protocol as described in Chapter II, Subsection 2.2.2.6. Specific binding was detected with an anti-human IgG or IgM, Alexa Fluor 594-conjugated secondary antibody and evaluated visually under a fluorescence inverted microscope. If any of the pooled supernatants showed a positive signal, a second run of live CBA was carried out to investigate which individual supernatant that contributed to the production of AQP4-specific antibodies.

3.3.5 B cell receptor (BCR) sequence analysis

Following amplification of immunoglobulin transcripts (see Chapter II, Section 2.2.3.1-5), the variable, diversity, and joining region sequences (VH-DH-JH and VL-JL for heavy and light chain, respectively) of all BCR repertoires were pairwise aligned against the IMGT human immunoglobulin germline sequence reference directory. The closest V, D, and J genes and alleles were identified based on sequence similarities using a previously standardised algorithm⁸⁰. Silent and non-silent nucleotide mutations across the framework (FR1, FR2, and FR3) and complementarity determining regions (CDR1 and CDR2) of the VH and VL regions were characterised and the total numbers were counted to determine mutation rates.

3.3.6 *Characterisation of CDR3 physicochemical properties*

The hypervariable CDR3 region, encoded by the BCR junction region, was accurately located and identified using the embedded IMGT Junction Analysis tool. The CDR3 amino acid sequence between cysteine at position 104 (C104) and phenylalanine/tryptophan at position 118 (F/W118) was characterised based on different physicochemical properties. The Hopp-Woods⁸¹ and Kyte-Doolittle⁸² scales were used to quantify the degree of hydrophilicity and hydrophobicity of amino acid sequences, respectively.

3.4 Results

3.4.1 *Patient characteristics*

Four patients who fulfilled the 2015 international consensus diagnostic criteria for AQP4-IgG seropositive NMOSD¹⁸ were identified from the patients looked after by the NMOSD specialist service in Oxford. Patient demographic and clinical characteristics at the time of sample collection are summarised in Table 3.1 below.

Patient	Gender	Age (years)	Ethnicity	Disease duration (years)	Disease activity	Treatment	PTPN22 risk allele	Sample collected	Designated sample ID
P1	Female	42	Caucasian	6	Remission	CST, MMF	No	PBMCs	DR11
P2	Female	26	South Asian	14	Remission	CST, MTX	No	PBMCs	DR14
P3	Female	36	South Asian	22	Relapse	CST, MMF	No	PBMCs	DR26
P4	Female	28	Caucasian	N/A	Pre-NMOSD onset	CST, MMF	Yes	PBMCs, TCs	DR20 (PBMCs), DR30 (TCs)

Table 3.1. Patient demographic and clinical characteristics at the time of sample collection.

CST, corticosteroids; MMF, mycophenolate mofetil; MTX, methotrexate; N/A: not applicable; P, patient; PBMCs, peripheral blood mononuclear cells; PTPN22, Protein Tyrosine Phosphatase Non-Receptor Type 22; TCs, thymic cells.

Patients were female with an equal representation of Caucasian and South Asian ethnicity. All of them presented with an aggressive and relapsing episode of NMOSD followed by clinical and imaging evidence of spinal cord (P1) and more extensive CNS involvement including optic nerves, brainstem, and other brain regions (P2, P3, and P4). The relapses predominantly occurred upon corticosteroid weaning. Whilst most patients did not have any history of other autoimmune diseases, a diagnosis of myasthenia gravis 18 years prior to the first clinical episode of NMOSD was reported in P4. Additionally, P4 was later found to carry a PTPN22 risk allele (HD-CT) which has been shown to disrupt the integrity of both central and peripheral B cell tolerance checkpoints and increase the risk of developing autoimmunity⁷⁰. Samples from P1, P2, and P3 were tested and negative for the PTPN22 risk allele.

There was a relatively wide range of age at disease onset (P1, 36; P2, 12; and P3, 14 years old). PBMC samples were taken at 6 (P1), 14 (P2), and 22 years (P3) during the course of their disease at which time the patients were in remission (P1 and P2) or having a relapse (P3) and taking corticosteroids in addition to mycophenolate mofetil (P1 and P3) or methotrexate (P2). In addition to PBMCs, thymic cells were taken from P4, 7 and 15 years prior to NMOSD onset, respectively, while she was on corticosteroids and azathioprine as treatment for her myasthenia gravis (Table 3.1, Figure 3.1). Despite multiple immunotherapy modalities, widely-varying but persistent serum AQP4-IgG levels were still detectable throughout their disease courses as is commonly reported in NMOSD (Figure 3.1).

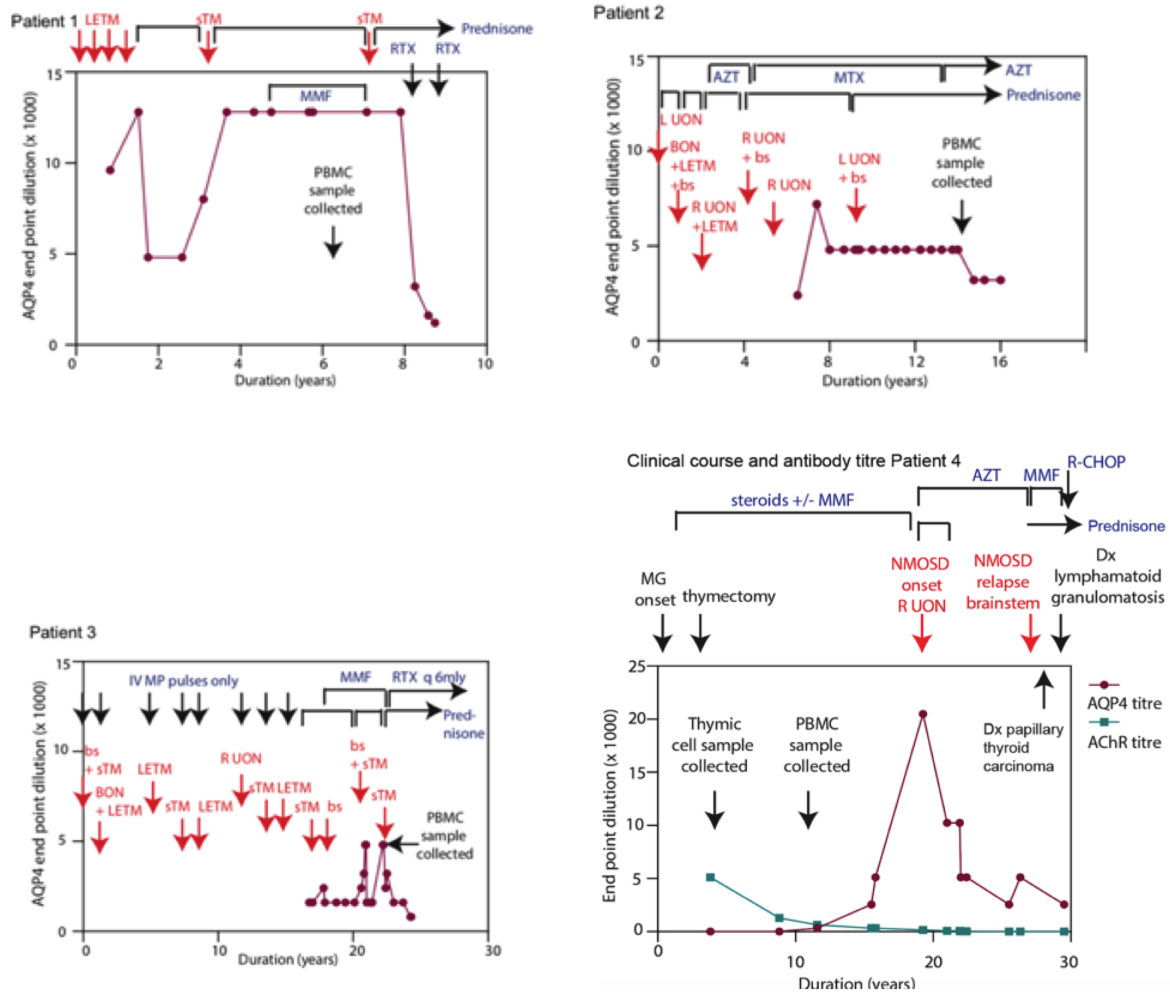


Figure 3.1. Serial AQP4- and AChR-IgG titres, clinical courses, and medications of NMOSD patients.

Clinical and serological profiles of four NMOSD patients are illustrated above. The endpoint titre of serum AQP4-IgG (and AChR-IgG for P4) over time is shown. The times of PBMC sample collection are indicated. Purple dots and lines: AQP4-IgG titres; green dots and lines: AChR-IgG titres; red arrows: NMOSD relapses; black arrows: treatments; AZT: azathioprine; BON: bilateral optic neuritis; bs: brainstem events; IV MP, intravenous methylprednisolone; LETM: longitudinally extensive transverse myelitis; L/R UON: left/right unilateral optic neuritis; MMF: mycophenolate mofetil; MTX: methotrexate; R-CHOP, cyclophosphamide, doxorubicin, prednisone, rituximab, and vincristine; RTX: rituximab; sTM, short transverse myelitis.

3.4.2 Frequencies of peripheral B cell subsets in NMOSD patients and healthy controls

Samples from 4 NMOSD patients and 2 age-matched healthy controls were immunophenotyped and analysed with flow cytometry to identify any expansion of B cell subset(s) in NMOSD compared to controls. An initial basic gating strategy was employed to isolate live and single lymphocytes with further identification of $CD3^- CD19^+ CD20^+$ B cells, shown in Figure 3.2.

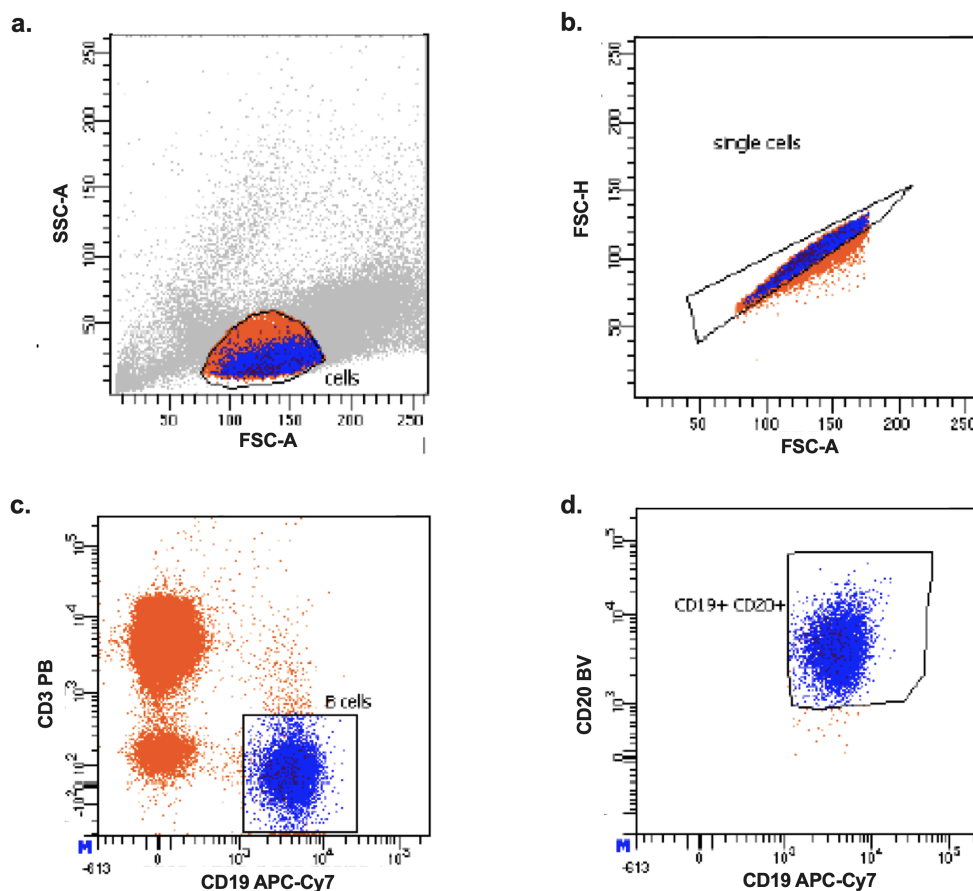


Figure 3.2. Initial basic gating strategy.

a. Identification of live lymphocytes from PBMC populations by forward and side scatter (FSC and SSC); **b.** Exclusion of doublet cells by forward scatter height and area (FSC-H and FSC-A); **c.** and **d.** Identification of $CD3^- CD19^+ CD20^+$ B cells.

An antibody panel of 8 cell surface markers was subsequently used to identify four B cell lineages in the peripheral blood, namely, new emigrant (NEm), mature naïve (MN), double-negative (DNeg), and memory (Mem) B cells (Figures 3.3 and 3.4). Isolation of these

populations allows us to explore the point at which AQP4-reactivity is generated and infer the integrity (or lack thereof) of the central and peripheral B cell tolerance checkpoints.

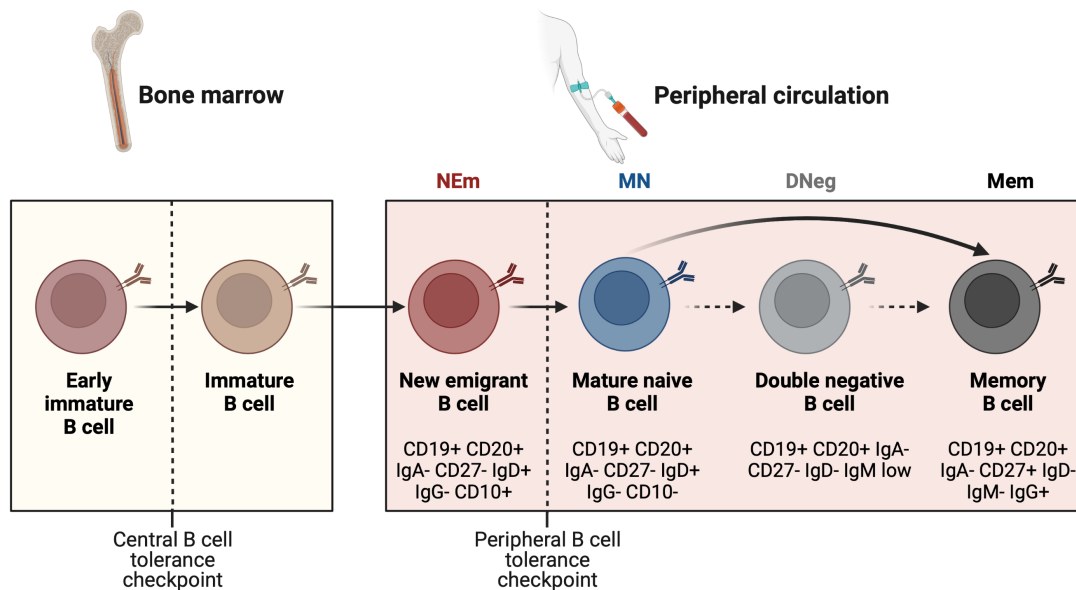


Figure 3.3. B cell populations of interest.

An illustration of the B cell developmental stages and the position of different B cell subsets relative to the well-characterised central and tolerance checkpoints.

Of all B cell subsets studied, the mature naïve B cell population was the largest in both NMOSD patients ($64.00\% \pm 8.165$) and healthy controls ($64.50\% \pm 0.71$), followed by low frequencies of new emigrant (NMOSD vs health, $1.18\% \pm 0.69$ vs $1.69\% \pm 0.35$), double-negative (NMOSD vs health, $0.67\% \pm 0.60$ vs $0.47\% \pm 0.24$), and memory B cells (NMOSD vs health, $3.11\% \pm 3.39$ vs $2.68\% \pm 1.36$) (Figure 3.4). Distributions of these B cell subsets were consistent with the reference values reported in the literature. Additionally, the pooled frequencies of individual B cell subsets were not significantly different between patients and healthy controls (two-way ANOVA fitted to a mixed-effects model, $p > 0.05$), indicating that the state of disease at which the samples were taken and administration of immunosuppressive agents did not significantly alter the frequencies of different B cell populations.

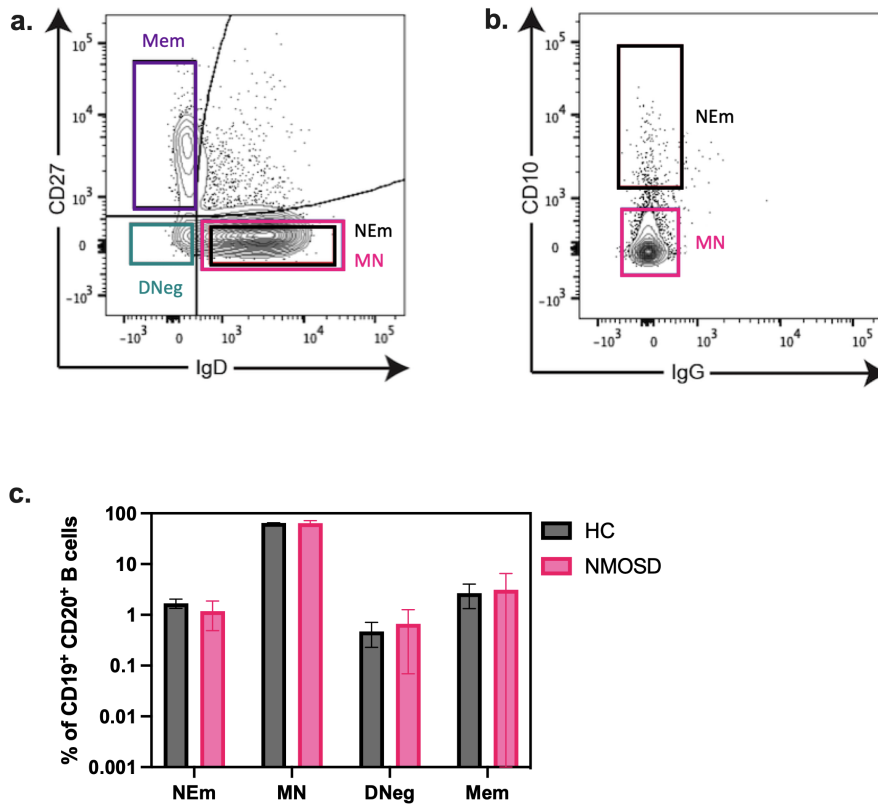


Figure 3.4. A gating strategy to characterise four B cell populations of interest.

PBMC and thymic cell samples were stained for CD19, CD20, IgA, CD27, IgM, IgG, and CD10 surface markers. **a.** IgD and CD27 markers allow separation of IgD⁺ CD27⁺ NEm and MN (Q3), IgD⁺ CD27⁺ DNeg (Q4), and IgD⁺ CD27⁺ mem (Q1) B cells; **b.** NEm and MN B cells were further differentiated based on the expression of CD10 marker; **c.** Pooled frequencies of different B cell subsets from PBMC samples from 4 NMOSD patients and 2 healthy controls. Data are presented as mean $\pm$ SD. NEm: new emigrant; MN: mature naïve; DNeg: double-negative; Mem: memory.

3.4.3 Frequencies of AQP4-specific B cells in NMOSD patients and healthy controls

To investigate how early AQP4 specificities arise along the B cell development stages and to evaluate the fidelity of B cell tolerance checkpoints in NMOSD, approximately 1000 single cells from each B cell subset from each patient and control were sorted, cultured *in vitro*, and differentiated into antibody secreting cells (ASCs) in the presence of a cocktail of soluble cytokines. After 26 days *in vitro*, culture supernatants were collected and screened by live CBA on HEK293T cells expressing AQP4-M23-EGFP. To detect specific binding, new emigrant- and mature naïve-derived supernatants were stained with an anti-human IgM whereas those from memory B cell wells were stained with an anti-human IgG secondary antibody.

Supernatants collected from double-negative single B cells were stained with both secondary antibodies.

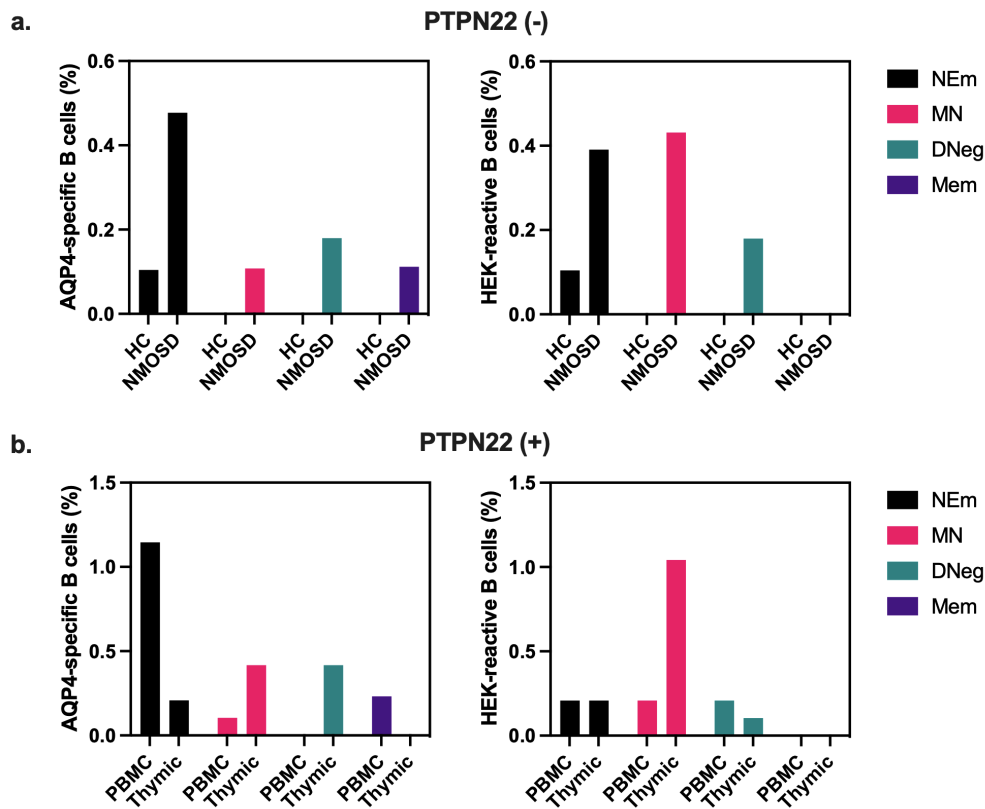


Figure 3.5. Frequencies of AQP4-specific and HEK-reactive B cells in NMOSD patients and healthy controls.

a. Comparative frequencies of AQP4-specific and HEK-reactive B cells across subsets in PTPN22 risk allele-negative NMOSD patients and healthy controls; **b.** Frequencies of PBMC- and thymic cell-derived AQP4-specific and HEK-reactive B cells from an NMOSD patient carrying a PTPN22 risk allele. NEm: new emigrant; MN: mature naïve; DNeg: double-negative; Mem: memory; PTPN22, Protein tyrosine phosphatase non-receptor 22.

As the PTPN22 risk allele(s) are known to cause a global defect in B cell tolerance checkpoints irrespective of disease aetiology, data from patient 4 (P4) was analysed independently of the other patients (P1, P2, P3) (Figure 3.5). The detailed numbers of AQP4-specific B cells across subsets and individuals can be found in Appendix 3. Only 2/7680 (0.03%) B cells from healthy controls were AQP4-specific and they only existed in the earliest B cell stage, which is the new emigrant. In contrast, 22/10,560 (0.21%, Fisher's exact test vs healthy controls, $p < 0.005$) B cells from NMOSD patients who did not carry the PTPN22 risk allele(s) were AQP4-specific with reducing frequencies across lineages (0.48% new emigrant,

0.11% mature naïve, 0.18% double-negative, and 0.11% memory B cells) (Figure 3.5a). This observation suggests a compromise of the central B cell tolerance checkpoint in NMOSD which allows immature AQP4-specific B cells to be released to the periphery. The peripheral tolerance checkpoint, however, may be partially effective in counter-selecting the developing AQP4-specific B cells. PreadSORption experiments could have been considered to further confirm specificity against AQP4-M23-EGFP.

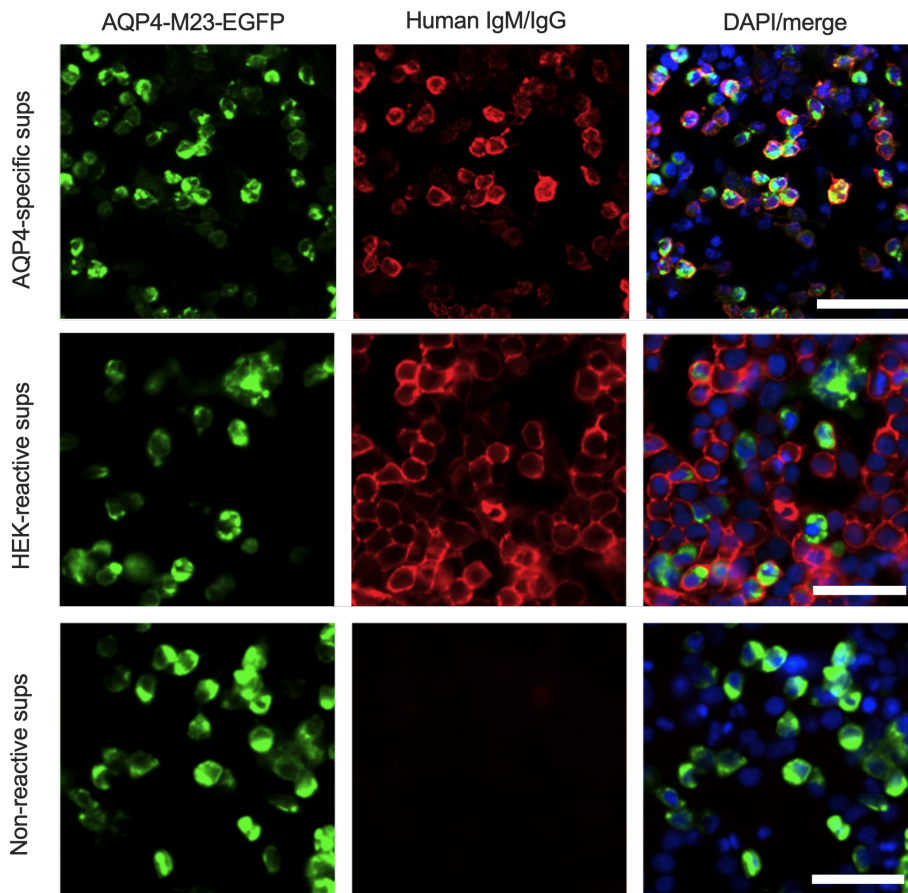


Figure 3.6. Representative images of three different B cell repertoires identified by live CBA using AQP4-M23-EGFP expressing HEK293 cells.

Bound antibodies were detected using an anti-human IgM (new emigrant- and mature naïve B cell-derived supernatants) or IgG secondary antibody (memory B cell-derived supernatants). Supernatants from double-negative single B cell wells were stained using both secondary antibodies. 40x magnification, scale bar=50 μ m.

In addition to B cells with AQP4 specificities, we identified a distinct B cell group, termed HEK-reactive, which showed binding to surface of live HEK293T cells (Figure 3.6, middle panels). The pooled frequency of HEK-reactive B cells from all subsets in both healthy

controls (2/7690, 0.03%) and PTPN22 risk allele-negative NMOSD patients (26/10,560, 0.25%, Fisher's exact test, $p < 0.0001$) largely mirrored of those AQP4-specifics (Figure 3.5a, right panel). However, unlike specificity to AQP4, reactivity to HEK cells were observed at a similarly high level in the new emigrant and mature naïve B cell populations (9/2304 vs 12/2784, Fisher's exact test, $p > 0.05$) in patients. This demonstrates that in NMOSD patients, the central and peripheral B cell tolerance checkpoints do not remove B cells with autoreactivity against generic membrane antigen(s) as robustly as AQP4-specific B cells.

In patient 4, AQP4-specific and HEK-reactive B cells were present in the peripheral blood and thymus years before the first clinical episode of NMOSD (Figure 3.5b). This agrees with what was shown in previous studies whereby carriers of the PTPN22 major risk allele(s) lack the ability to remove self-reactive B cells, even preceding the onset of autoimmunity⁷⁰. Despite the potential confound of the risk allele in the aetiology of NMOSD in this patient, we can still see the same pattern with regard to the distribution of AQP4-specific B cells across lineages in the periphery (Figure 3.5b, left panel). Indeed, the frequency of AQP4-specific new emigrant B cells in this patient alone (1.15%) was more than double than the frequency of the three non-carriers combined (0.48%, Fisher's exact test $p = 0.057$). Interestingly, the thymus harboured a relatively low frequency of AQP4-specific B cells. This may suggest that, after egressing from the bone marrow, intrathymic maturation in this population is rare, or alternatively, AQP4-specific new emigrant B cells may selectively undergo their maturation in other extrathymic sites.

3.4.4 Immunoglobulin heavy VDJ gene family usage in AQP4-specific B cells

To examine whether consistent rearrangements of the immunoglobulin heavy V, D, and J genes predispose to AQP4-specificity in NMOSD patients, B cell receptor (BCR) transcripts from previously identified reactive single wells were isolated, reverse-transcribed, and PCR amplified (see Chapter 2, Subsection 2.2.3.1-5 for methods). The successfully recovered

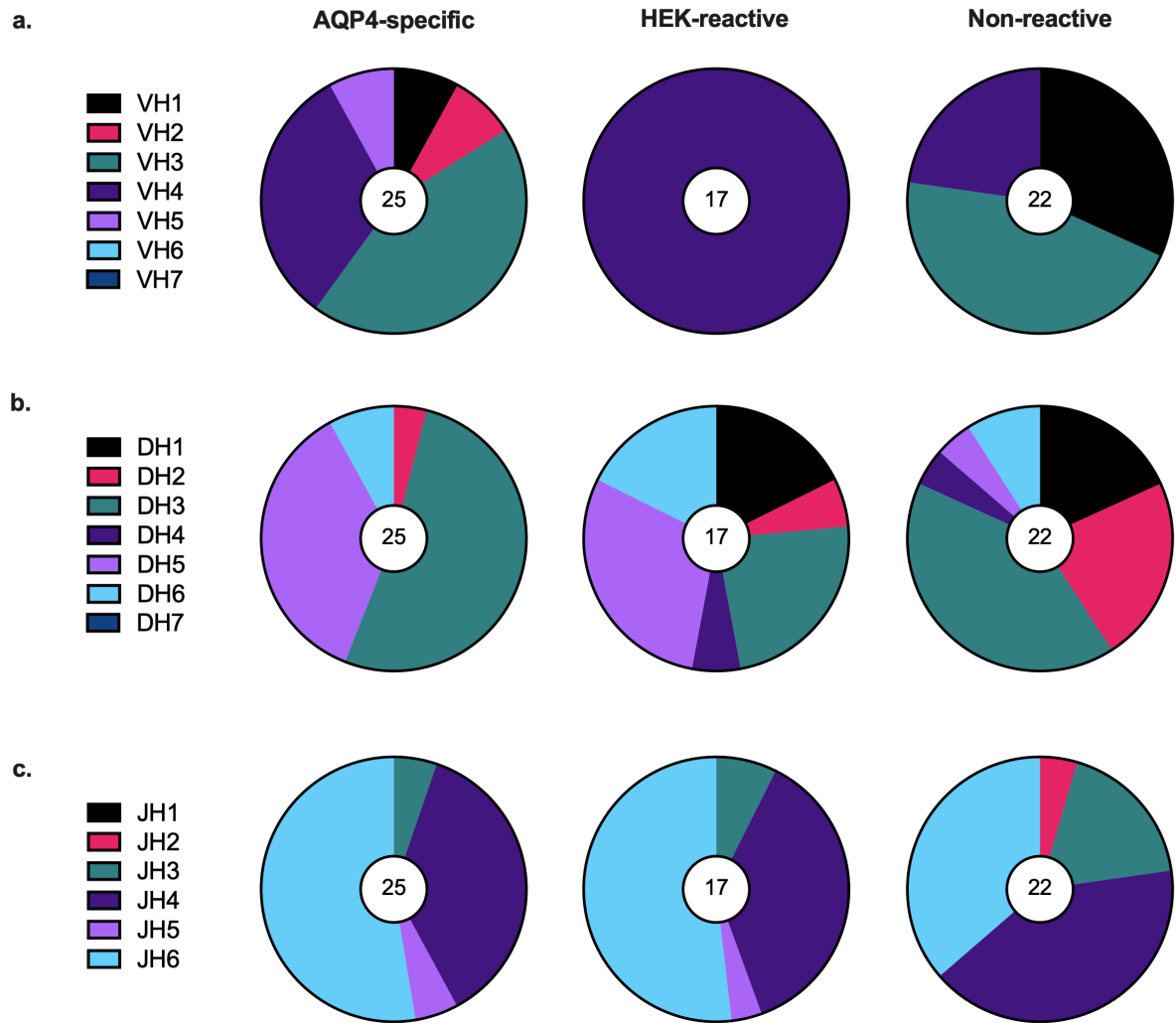
transcripts (Table 3.2) were sequenced and aligned against the IMGT germline reference sequences.

Patient	Reactivity	Screening	B cell subset			
			New emigrant	Mature naïve	Double-negative	Memory
1 (PBMC)	AQP4	Wells identified	3	1	2	1
		Transcripts recovered	3	1	1	1
	HEK	Wells identified	3	7	3	0
		Transcripts recovered	3	5	0	0
2 (PBMC)	AQP4	Wells identified	2	0	3	2
		Transcripts recovered	0	0	2	1
	HEK	Wells identified	5	5	0	0
		Transcripts recovered	0	2	0	0
3 (PBMC)	AQP4	Wells identified	6	2	0	0
		Transcripts recovered	2	1	0	0
	HEK	Wells identified	1	0	2	0
		Transcripts recovered	0	0	0	0
4 (PBMC)	AQP4	Wells identified	11	1	0	2
		Transcripts recovered	5	1	0	1
	HEK	Wells identified	2	2	2	0
		Transcripts recovered	3	2	2	0
4 (Thymic)	AQP4	Wells identified	2	4	4	0
		Transcripts recovered	1	3	2	0
	HEK	Wells identified	2	10	1	0
		Transcripts recovered	0	0	0	0

Table 3.2. Total numbers of recovered AQP4-specific and HEK-reactive BCR transcripts from NMOSD patients.

A total of 25/46 (54.35%) AQP4-specific and 17/45 (37.78%) HEK-reactive transcripts were successfully recovered from single B cell wells from four NMOSD patients. Of 25 AQP4-specific transcripts, 19 were derived from PBMCs and the remaining 6 were from thymic cells. None of the HEK-reactive and non-reactive transcripts were obtained from the thymic cell sample from patient 4. In most cases, the unsuccessful recoveries were due to incomplete

amplification of the heavy and/or light chain, multiple sequences detected with no possible individual reading, and/or poor resolution of the sequencing peaks for accurate determination of base calls. For downstream analyses, 22 non-reactive transcripts from across patients were arbitrarily chosen and recovered to be used as a control. The detailed immunoglobulin gene family usage is shown in Appendix 3.



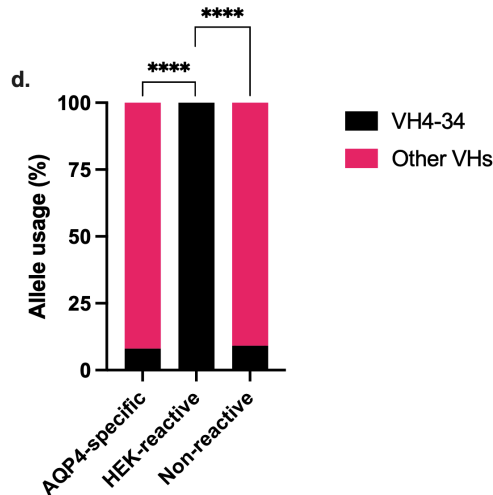


Figure 3.7. Distribution of IgHV, D, and J families in AQP4-specific, HEK-reactive B cell, and non-reactive repertoires.

Pooled BCR transcripts from all NMOSD patients were analysed and compared. The frequencies of VH (a), DH (b), and JH (c) family usage are depicted. Numbers in the centre of pie charts denote the total numbers of BCRs analysed. The HEK-reactive B cell repertoire were exclusively of the IGHV4-34 gene family (d). VH, immunoglobulin heavy V gene; DH, immunoglobulin heavy D gene; JH, immunoglobulin heavy J gene.

As sequences were obtained from BCR transcripts, they represent the productively rearranged immunoglobulin heavy and light chains. Regardless of reactivity, each B cell had a unique heavy V-D-J and light V-J chain gene rearrangements. In pooled PBMC- and thymic cell-derived AQP4-specific BCRs, almost all IgH gene families were represented (5/7 IgHV, 4/7 IgHD, and 4/6 IgHJ). Comparable findings were observed in the non-reactive BCRs (3/7 IgHV, 6/7 IgHD, and 4/6 IgHJ) (Figures 3.7a, b, and c). In contrast, HEK-reactive BCRs exclusively utilised a member of the IgHV4 family (17/17 IgHV4-34, Fisher's exact test vs AQP4-specific and non-reactive, $p < 0.0001$) (Figure 3.7d) in combination with diverse DH (6/7) and JH (4/6) gene families. Although IgHV4-34 was also found in 2/25 AQP4-specific and 2/22 non-reactive BCRs, the same exact VH-DH-JH recombinants were not detected in the HEK-reactive BCRs.

NMOSD patients were further stratified by the presence of the PTPN22 risk allele. The frequencies of the IgHV usage in 12 AQP4-specific BCRs from 3 PTPN22 risk allele-negative patients and 13 from a PTPN22 risk allele-positive patient were analysed and compared to the

expected IgHV gene family distributions obtained from 4,890 unique sequences from 3 healthy volunteers as published somewhere else⁸³.

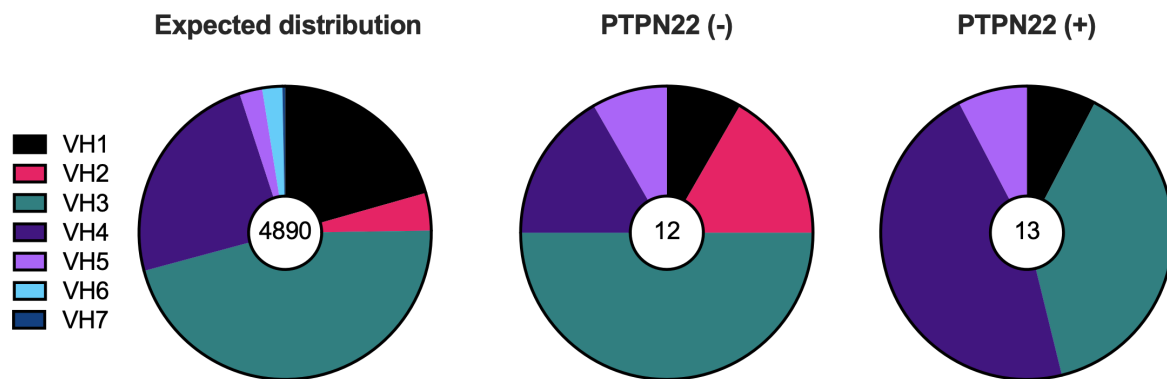


Figure 3.8. Distribution of IgHV families in the PTPN22 carrier and non-carrier AQP4-specific repertoire.

The frequencies of IgHV families in the AQP4-specific BCRs from PTPN22 risk allele-negative and positive NMOSD patients were compared to the expected IgHV gene family distributions obtained from 4,890 unique sequences from 3 healthy young volunteers⁸³. PTPN22, Protein Tyrosine Phosphatase Non-Receptor Type 22; VH, immunoglobulin heavy V gene.

All IgHV families were represented with varying distributions in the reference BCR repertoire. As the largest family, IgHV3 (2,252/4,890, 46.05%) was most often used, followed in decreasing frequencies by IgHV4 (1,180/4,890, 24.13%), IgHV1 (1,008/4,890, 20.61%), IgHV2 (203/4,890, 4.15%), IgHV5 (123/4,890, 2.51%), IgHV6 (109/4,890, 2.22%) and IgHV7 (15/4,890, 0.31%) (Figure 3.8). There were potential trends towards IgHV2 being overrepresented in the AQP4-specific B cells from the PTPN22 risk allele-negative patients (2/12, 16.67% vs 4.15% found in reference BCRs, Fisher's exact test $p=0.09$). There was no detectable usage of this family in the PTPN22 risk allele-positive patient despite the comparable numbers of BCR sequences recovered in both genotypes. On the other hand, carrier of the PTPN22 risk allele demonstrated a trend towards higher representation of the IgHV4 family (6/13, 46.15% vs 24.13% found in reference BCRs, Fisher's exact test $p=0.1$) (Figure 3.8). The frequency of the IgHV4 family was not significantly skewed in the PTPN22 risk allele-negative B cell repertoire from expected (2/12, 16.67% vs 24.13%, Fisher's exact test $p=0.74$). These results suggest that certain IgHV families may predispose towards AQP4-

specificity. Additionally, biases of heavy chain familial usage in the presence or absence of the PTPN22 risk allele may reveal multiple mechanisms (aberrant positive selection and/or inadequate negative selection) that can theoretically contribute to loss of self-tolerance in NMOSD. However, it should be noted that analyses regarding the potential impact of PTPN22 polymorphisms in shaping the AQP4-specific BCR repertoire were purely exploratory and beyond the scope of this study. Recruitment of a larger number of patients carrying the PTPN22 risk allele is needed to confirm these initial findings.

3.4.5 Immunoglobulin kappa/lambda light chain usage in AQP4-specific B cells

The diversity of the immunoglobulin light chain repertoire among B cells with different reactivities were explored to understand the degree of receptor editing in NMOSD. In human, light chain gene rearrangements begin with the kappa locus in one chromosome⁶². If the rearranged kappa V-J gene is unproductive or results in self-reactivity, a second round of rearrangement occurs in the same chromosome until all possible combinations have been exhausted. These serial rearrangements continue in the other kappa locus on the second chromosome should self-reactivity persist. A failure in producing a productive and self-tolerant rearranged light chain gene in association with a readily rearranged heavy chain at this point will be followed by rearrangement of the lambda loci⁸⁴.

Sequences from 25 AQP4-specific, 17 HEK-reactive, and 22 non-reactive BCRs were first aligned against the IMGT germline sequences to identify their light V-J gene families. The relative abundance of kappa vs lambda light chain usage was analysed and the distributions of the kappa J chain (IgJK) families were contrasted with published data⁸⁵ on the expected human IgJK rearrangements.

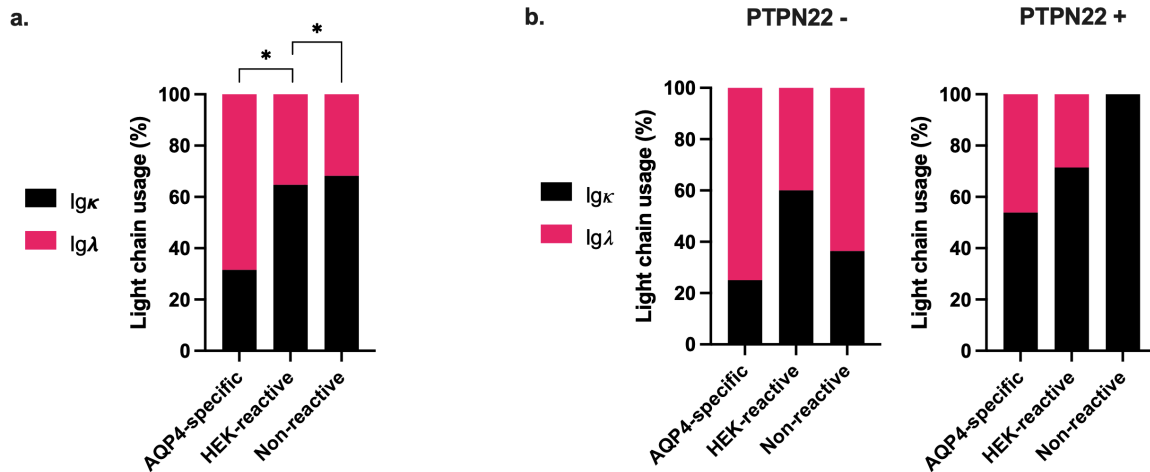


Figure 3.9. Immunoglobulin light chain usage in B cell repertoires with different reactivities.

a. The lambda light chain was predominantly found in the PBMC-derived AQP4-specific B cell repertoire; **b.** The frequency of lambda chain usage was higher in the AQP4-specific and non-reactive repertoire of PTPN22 (-) patients. The percent usage of the kappa light chain increased in all B cell repertoires irrespective of their reactivities in the PTPN22 (+) patient. PTPN22, Protein Tyrosine Phosphatase Non-Receptor Type 22.

Pooled PBMC- and thymic cell-derived AQP4-specific repertoire were dominated by lambda light chain-bearing B cells (60%). In contrast, kappa light chains were more frequently observed in the HEK-reactive (64.71%) and non-reactive repertoires (68.18%). The differential use of the light chain pairing was more pronounced when the observation was focused on the PBMC-derived AQP4-specific BCRs (13/19, 68.42% lambda, Chi-square test vs HEK-reactive and non-reactive BCRs, $p < 0.05$) (Figure 3.9a).

The potential influence of the PTPN22 risk allele in shaping the B cell light chain repertoire was assessed. In PTPN22 risk allele-negative patients, the lambda light was used more frequently by the AQP4-specific BCRs (9/12, 75.00%) (Figure 3.9b). This was also seen in the non-reactive BCRs (7/11, 63.64%). However, there appears to be a shift in the proportion of light chain usage when the PTPN22 risk allele was present. Regardless of their reactivity, the percent usage of the kappa was higher than the lambda light chain in any repertoire (7/13 or 53.85% in AQP4-specific, 6/10 or 71.43% in HEK-reactive, and 1/1 or 100% in non-reactive repertoire).

Thus, the high proportion of lambda chain usage observed in the AQP4-specific BCRs, especially in the PTPN22 risk allele-negative patients, suggests that extensive receptor editing occurred as an attempt to modify self-reactivity. Moreover, the reverse proportion seen in the PTPN22 risk allele-positive patient demonstrates that reduced receptor editing could be one of the mechanisms by which the allele interferes with the establishment of B cell tolerance. A more robust analysis comparing higher numbers of PTPN22 (-) and (+) NMOSD patients is warranted to substantiate these observations.

3.4.6 Immunoglobulin kappa J gene rearrangements in AQP4-specific B cells

Rearrangements of the IgJK gene families have been shown to occur at different frequencies as a result of the unique position of the proximal kappa J transcript promoter to the J gene segments which facilitates receptor editing^{85,86}. Observation from a published review⁸⁵ with a compilation of 26 human BCR sequences revealed a marked difference in the frequency of the IgJK genes relative usage during V-J rearrangements: the IgJK1 (11/26, 42%) and IgJK2 (10/26, 38%) were the most commonly used followed by the IgJK3 (2/26, 8%), IgJK4 (1/26, 4%), and IgJK5 (2/26, 8%) at similarly low frequencies (Figure 3.10a). Interestingly, in our study, the IgJK1 only contributed 20% to the total distribution of IgJK families in the AQP4-specific BCRs whereas in the HEK-reactive BCRs, there appears to be an extensive use of the IgJK1 (6/11, 54.55%) compared to the other IgJK gene families. It should also be noted that there was an expansion of the IgJK4 family (7/15, 46.67%) relative to the expected distribution in the non-reactive BCRs (Fisher's exact test, $p < 0.005$) (Figure 3.10a).

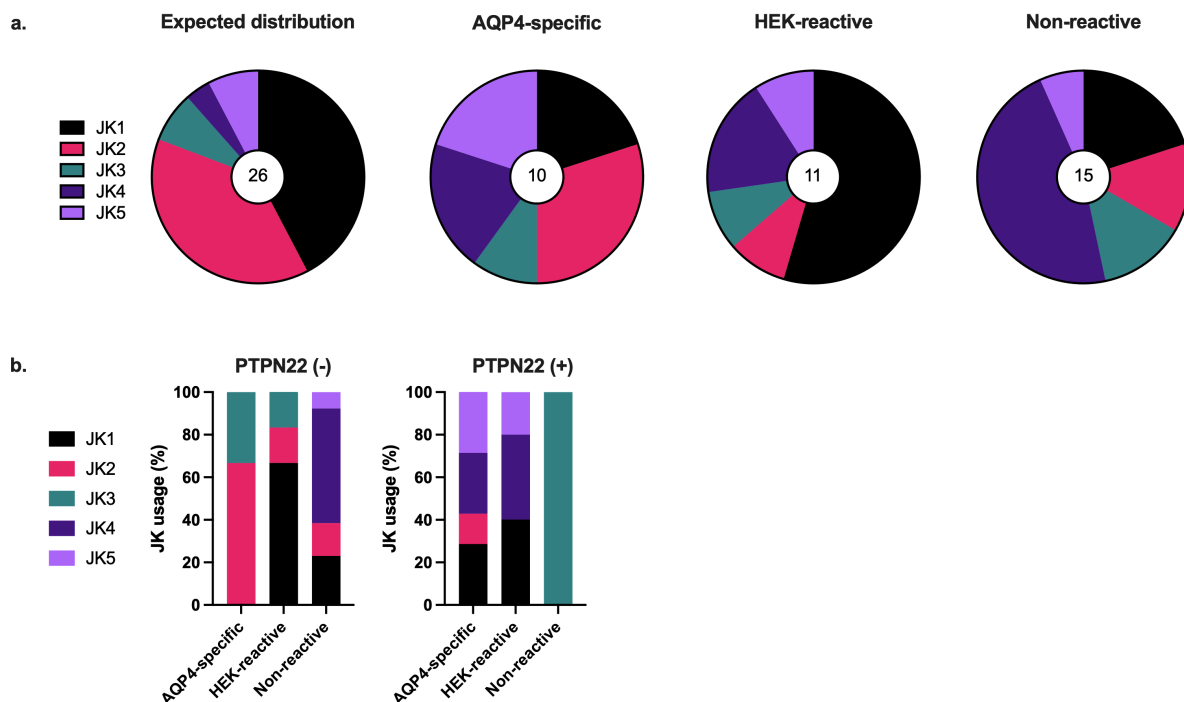


Figure 3.10. Relative frequencies of IgJK family usage in different B cell repertoires.

a. The observed distributions of IgJK families in the AQP4-specific, HEK-reactive, and non-reactive B cell BCRs were compared to the expected distributions obtained from published data from 26 human BCR sequences⁸⁵. The number in the centre each pie chart denotes the total number of BCR sequence analysed; **b.** The frequencies of IgJK family usage among B cells with different reactivities in patients with and without the PTPN22 risk allele are depicted. PTPN22, Protein Tyrosine Phosphatase Non-Receptor Type 22.

When B cells were grouped based on the presence of the PTPN22 risk allele, differential patterns of IgJK family usage were observed (Figure 3.10b). The IgJK1 family was completely absent in the AQP4-specific BCRs from the PTPN22 risk allele-negative patients whilst it was detected in 2/7 (28.57%) BCRs with AQP4 specificity in the PTPN22 risk allele-positive patient. Additionally, the HEK-reactive BCRs from any patients had an expected if not slightly expanded frequency of IgJK1 family usage (4/6 or 66.67% in the PTPN22-negative patients and 2/5 or 40% in the PTPN22-positive patient). From these observations, we could infer that receptor editing mediated by kappa J gene rearrangement may serve as a mechanism to minimise specificity against AQP4 in NMOSD and this key mechanism could be impacted by PTPN22 polymorphisms.

3.4.7 IgHV-IgHJ gene combinations in AQP4-specific B cells

As shown in the previous sections, AQP4-specific BCRs in our study were polyclonal whereby each individual B cell had a unique rearranged germline heavy and light chain V-(D)-J gene segments. Only two AQP4-specific mature naïve B cells from two different patients shared the same heavy chain V and J gene segment combinations (IgHV2-5 and IgHJ4) but had a different D gene segment usage (IgHD3 vs IgHD5). However, two studies in the literature demonstrated that several clonally expanded CD138⁺ plasma cell populations were identified in the CSF and peripheral blood of NMOSD patients, including up to 10 members belonging to a single B cell clone^{79,87}. This led us to speculate whether certain heavy chain gene rearrangements may confer a survival advantage and allow AQP4-specific B cells to undergo clonal expansion and continue their maturation process to become professional antibody secreting cells (ASCs) upon encountering their cognate antigen.

We approached this question by contrasting the heavy chain gene families used by the AQP4-specific B cells with those annotated in either of the two studies. The number of BCRs sharing the same exact IgHV gene or the whole IgH-IgHJ gene combinations was counted. Matching of the IgHD gene usage was not possible as data were not available from the published studies.

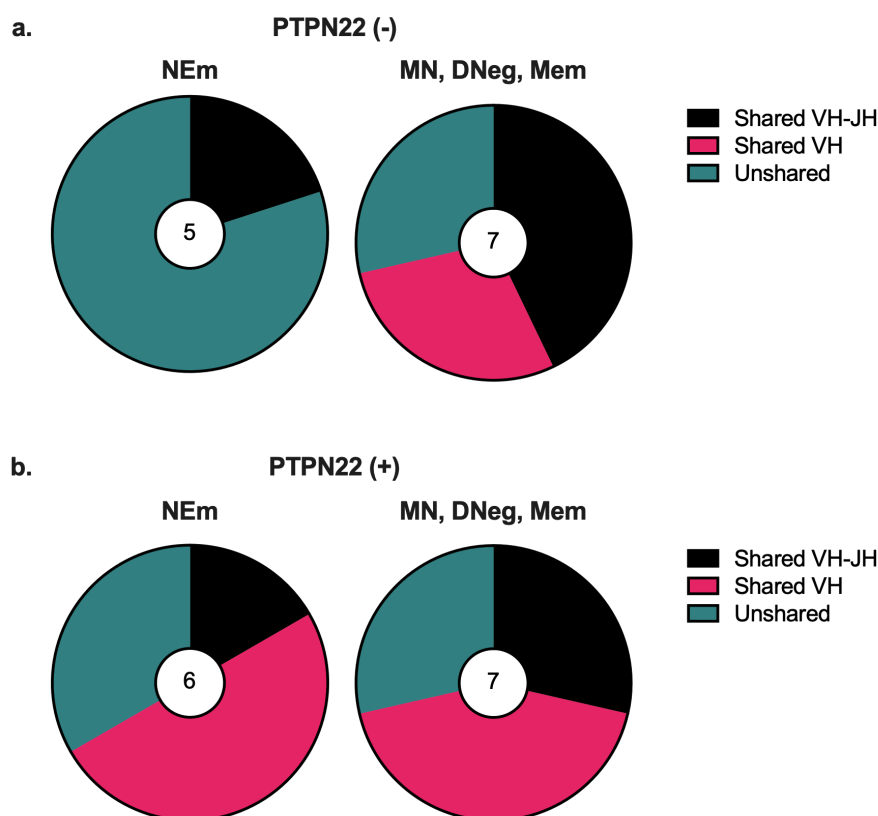


Figure 3.11. Clonotype analysis of the AQP4-specific B cell repertoire.

The IgHV-IgHJ gene segment usage in the PTPN22 risk allele negative (a) and positive (b)-derived AQP4-specific repertoire were mapped to the previously published^{79,87}, clonally expanded CD138⁺ plasma cell populations to explore the potential effect of heavy chain gene rearrangements on the survival of AQP4-specific B cells. B cells were segregated by subsets to reflect the relative positions of those subsets to the well-known central and peripheral tolerance checkpoints.

In the PTPN22 risk allele-negative patients, 1/5 (20%) of the AQP4-specific new emigrant and 5/7 (71.43%) of the pooled mature naïve, double-negative, and memory B cells shared IgHV-IgHJ gene combinations or IgHV gene only with the clonally expanded ASC populations (Figure 3.11a). Meanwhile, more than half of the AQP4-specific B cells in both the new emigrant (4/6, 66.7%) and the more mature B cell subsets (5/7, 71.43%) from the PTPN22 risk allele-positive patient showed matched heavy chain gene rearrangements with the ASC populations (Figure 3.11b). Regardless of B cells subsets, a higher frequency of shared clonotypes were seen in the PTPN22 risk allele carrier (9/13, 69.23%) than non-carriers (6/12, 50%). These data suggest that in NMOSD patients, AQP4-specific B cell clones expressing certain rearranged heavy chain gene segments may be favoured to survive and mature.

Alternatively, these potentially pathogenic B cell clones may not be adequately removed from the immune repertoire as a result of compromised central and peripheral tolerance checkpoints.

3.4.8 Frequencies of somatic mutations across B cell development stages

The frequencies of mutations in the immunoglobulin heavy and light V gene segments across B cell reactivities and subsets were examined. In general, the mutation rates in the IgHV and IgLV were similar to one another. Irrespective of their reactivities, a few new emigrant and mature naïve B cells harboured minimum mutations (0-3 nucleotides) in both the heavy and light V gene segments (Figure 3.12a). The AQP4-specific double-negative subset comprised 2 unmutated and 3 heavily mutated B cells (7-30 nucleotide mutations) (Figure 3.12a, top left panel). Interestingly, during the initial live CBA screening following single cell sorting and culture, the unmutated and mutated double-negative B cells were found to secrete IgM and IgG antibodies, respectively. As predicted, the memory B cell subsets had considerably high mutation loads (10-27 nucleotide mutations) (Figure 3.12a, top left panel).

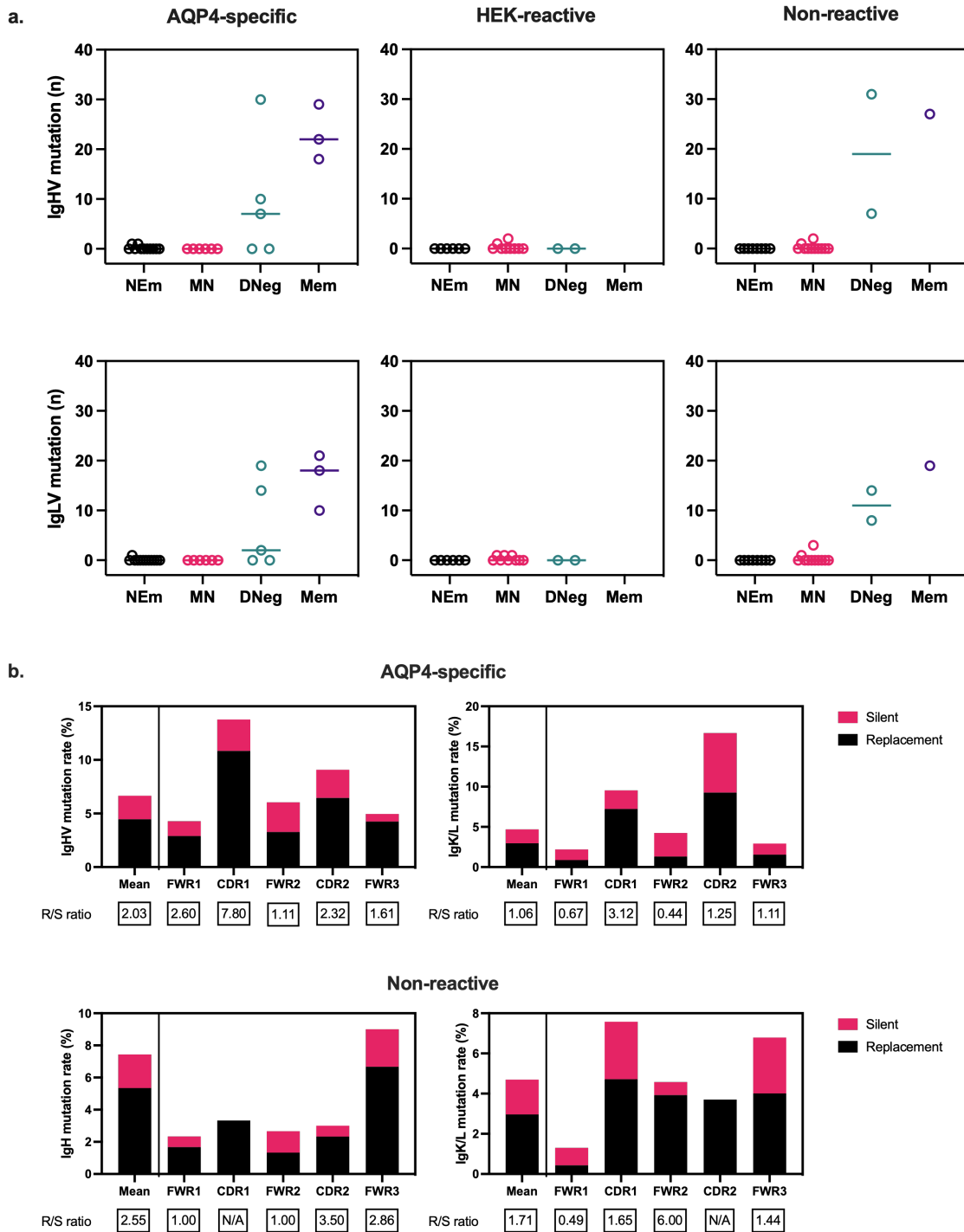


Figure 3.12. Mutation rates in the IgHV and IgLV gene segments across antigen reactivities and B cell subsets.

a. The number of nucleotide mutations in the heavy (top panels) and light (bottom panels) V gene segments across B cell subsets and reactivities; **b.** The frequency of replacement and silent mutations across framework (FWRs 1-3) and complementarity-determining regions (CDRs 1-2) in the somatically hypermutated BCRs. Numbers at the bottom of bar charts represent the R/S ratios of the corresponding regions. R, replacement; S, silent. Horizontal bars represent median values. NEm: new emigrant; MN: mature naïve; DNeg: double-negative; Mem: memory.

Nucleotide mutations in the heavy and light chain V gene segments can alter the resulting amino acid sequences. The rates of replacement (R) and silent (S) mutations across 3 framework (FWRs 1-3) and 2 complementarity-determining regions (CDRs 1-2) of the somatically hypermutated double-negative and memory B cells are depicted in Figure 3.12b. The mean mutation rates and the R/S ratios in the IgHV and IgLV gene segments between the AQP4-specific and non-reactive repertoires were comparable. The FWRs are generally longer than the CDRs and, thus, mutations were more frequently observed in the FWRs. However, replacement mutations were enriched in the CDRs compared to the FWRs.

All the above results suggest that mutation rates reflect B cell development stages rather than reactivity. Additionally, this provides evidence that, for the most part, our in vitro culture and BCR transcript amplification conditions introduced minimal artificial mutations and were able to maintain the integrity of the native BCR sequences.

3.4.9 Heavy chain CDR3 physicochemical properties

The heavy chain CDR3 (HCDR3) has been thought to be the major determinant of an antibody's binding specificity⁸⁸. Therefore, we sought to characterise the physicochemical properties of HCDR3 across B cell reactivities and subsets based on length, net charge, hydrophilicity, and hydrophobicity. The HCDR3 amino acid sequences are summarised in Appendix 3.

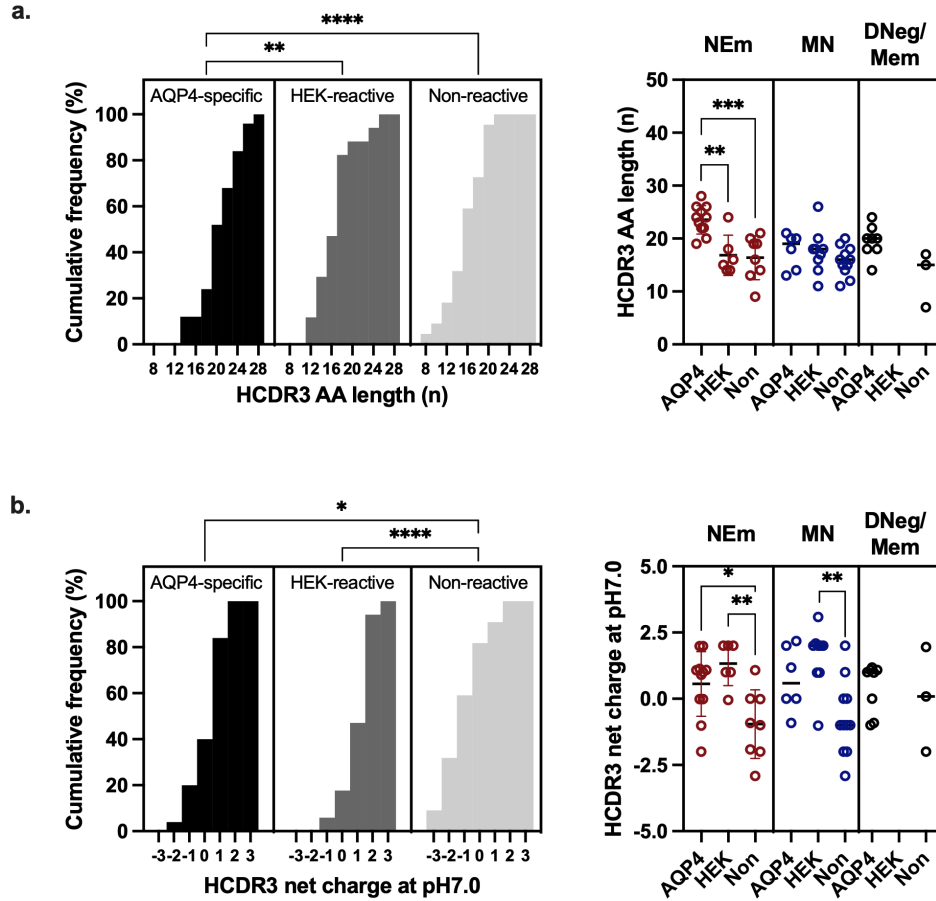


Figure 3.13 Heavy chain CDR3 length and net charge across different antigen reactivities and B cell subsets.

Cumulative frequency histograms and plots of HCDR3 amino acid length (**a**) and net charge at pH7.0 (**b**) are shown. Data are presented as mean $\pm$ SEM. NEm: new emigrant; MN: mature naïve; DNeg: double-negative; Mem: memory. One-way ANOVA, *= $p<0.05$, **= $p<0.005$, ***= $p<0.0005$, ****= $p<0.0001$.

Pooled AQP4-specific HCDR3s were significantly longer than HEK-reactive and non-reactive CDR3s with an average length of 20.84 ± 3.80 amino acids (one-way ANOVA with Tukey post-hoc tests, vs HEK-reactive $p<0.005$, vs non-reactive $p<0.0001$) (Figure 3.13a, left panel). The average length of the HEK-reactive and non-reactive HCDR3s were 16.94 ± 3.85 and 15.59 ± 3.63 amino acids, respectively. These differences were largely contributed by the new emigrant subset (Figure 3.13a, right panel). Furthermore, amino acids that make up the AQP4-specific (0.56 ± 1.10) and HEK-reactive HCDR3s (1.36 ± 1.01) were more positively charged at pH7.0 compared to the ones that constitute the non-reactive HCDR3s (-0.71 ± 1.41 , Kruskal-Wallis test with Dunn's post-hoc test, vs AQP4-specific $p<0.05$, vs HEK-reactive

$p < 0.0001$) (Figure 3.13b, left panel). Upon grouping based on B cell subsets, these findings were mostly observed in the new emigrant but not in the later B cell populations. (Figure 3.13b, right panel).

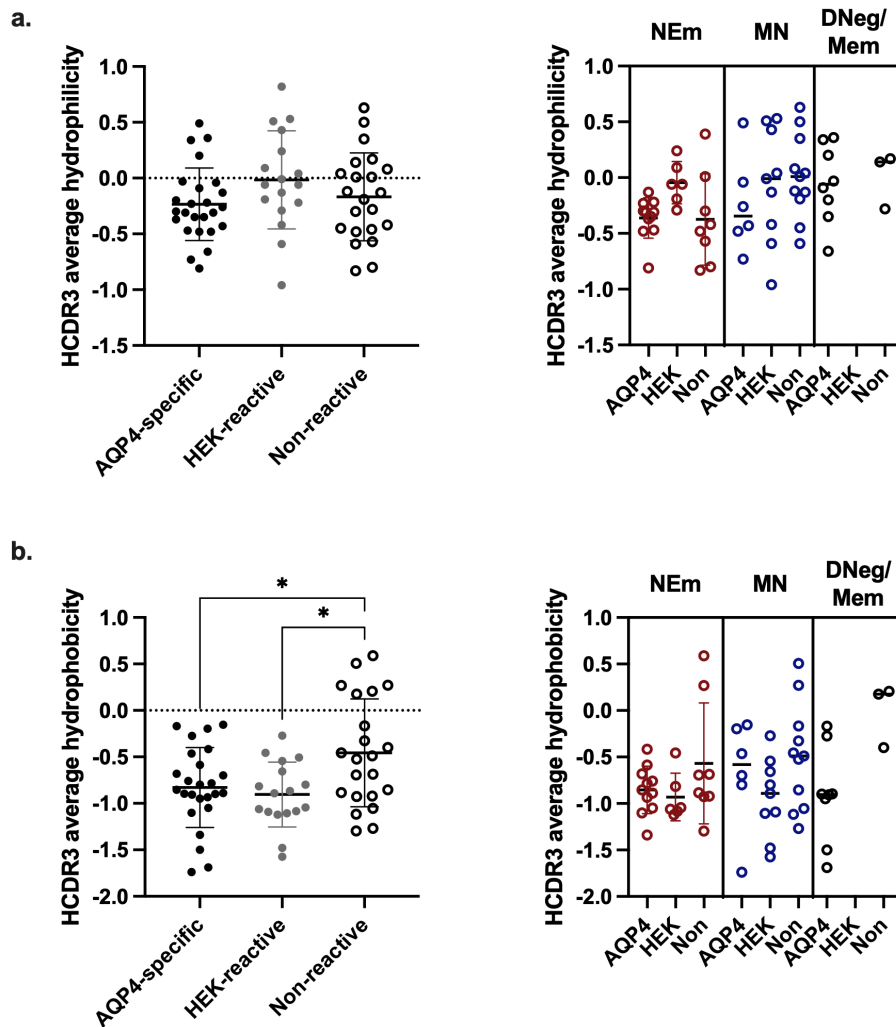


Figure 3.14. Heavy chain CDR3 average hydrophilicity and hydrophobicity values across antigen reactivities and B cell subsets.

Plots show HCDR3 hydrophilicity (a) and hydrophobicity (b) of individual BCRs stratified by antigen reactivities and B cell subsets. Data are presented as mean $\pm$ SEM. NEm: new emigrant; MN: mature naive; DNeg: double-negative; Mem: memory. One-way ANOVA, *= $p < 0.05$, **= $p < 0.005$, ***= $p < 0.0005$, ****= $p < 0.0001$.

To measure the water solubility and hydropathy values of the HCDR3 amino acid residues, the Hopp-Woods⁸¹ and the Kyte-Doolittle⁸² scale (also known as GRAVY index) were used, respectively. The HCDR3 average hydrophilicity values were comparable among B cell groups with differing reactivities (mean, AQP4-specific = -0.23 ± 0.33 , HEK-reactive =

-0.02 ± 0.44 , non-reactive = -0.17 ± 0.39) (Figure 3.14a). However, HCDR3s with reactivity against AQP4 (mean, -0.83 ± 0.43) and HEK (mean, -0.90 ± 0.35) were found to be more hydrophobic than the non-reactive HCDR3s (mean, -0.46 ± 0.58 , one-way ANOVA with Tukey post-hoc test, $p < 0.05$) (Figure 3.14b, left panel). This unique feature seems to be independent of the B cell subsets. (Figure 3.14b, right panel).

3.4.10 Light chain CDR3 physicochemical properties

As shown in one of the preceding sections, there was a bias in the kappa vs lambda light chain usage among the different B cell repertoires. We asked a question whether the physicochemical properties of the light chain CDR3 (LCDR3) may characterise different BCR reactivities independent of the light chain isotype. We employed the same analyses as what we did with the HCDR3.

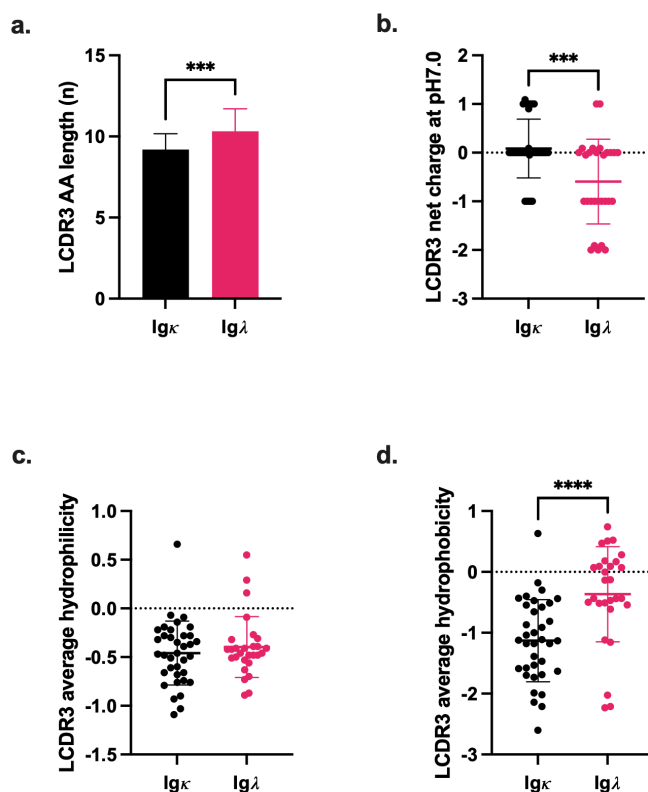


Figure 3.15. CDR3 physicochemical properties of the two light chain isotypes.

LCDR3s from pooled BCRs with differing reactivities were grouped by kappa/lambda light chain usage and their amino acid length (a), net charge (b), hydrophilicity (c) and hydrophobicity (d) were compared. Data are presented as mean $\pm$ SEM. AA, amino acid; LCDR3, light-chain CDR3. Unpaired *t*-test, *= $p<0.05$, **= $p<0.005$, ***= $p<0.0005$, ****= $p<0.0001$.

Regardless of reactivity, LCDR3s in the lambda chain-bearing BCRs were longer (mean lambda vs kappa, 10.32 ± 1.39 vs 9.19 ± 0.98 , *t*-test $p<0.0005$), more negatively charged (mean lambda vs kappa, -0.59 ± 0.87 vs 0.08 ± 0.60 , *t*-test $p<0.0005$), and more hydrophobic (mean lambda vs kappa, -0.37 ± 0.78 vs -1.13 vs 0.68 , *t*-test $p<0.0001$) compared to the kappa counterparts (Figure 3.15). The hydrophilicity of the LCDR3 amino acid residues in both light chain isotypes was not significantly different. Furthermore, when the LCDR3s were grouped by reactivities and B cell subsets, no unique signatures with regard to any of the physicochemical properties were observed (Figure 3.16). These findings demonstrate that the kappa and lambda light chains possess intrinsic physicochemical properties irrespective of antigen specificity and B cell developmental stages.

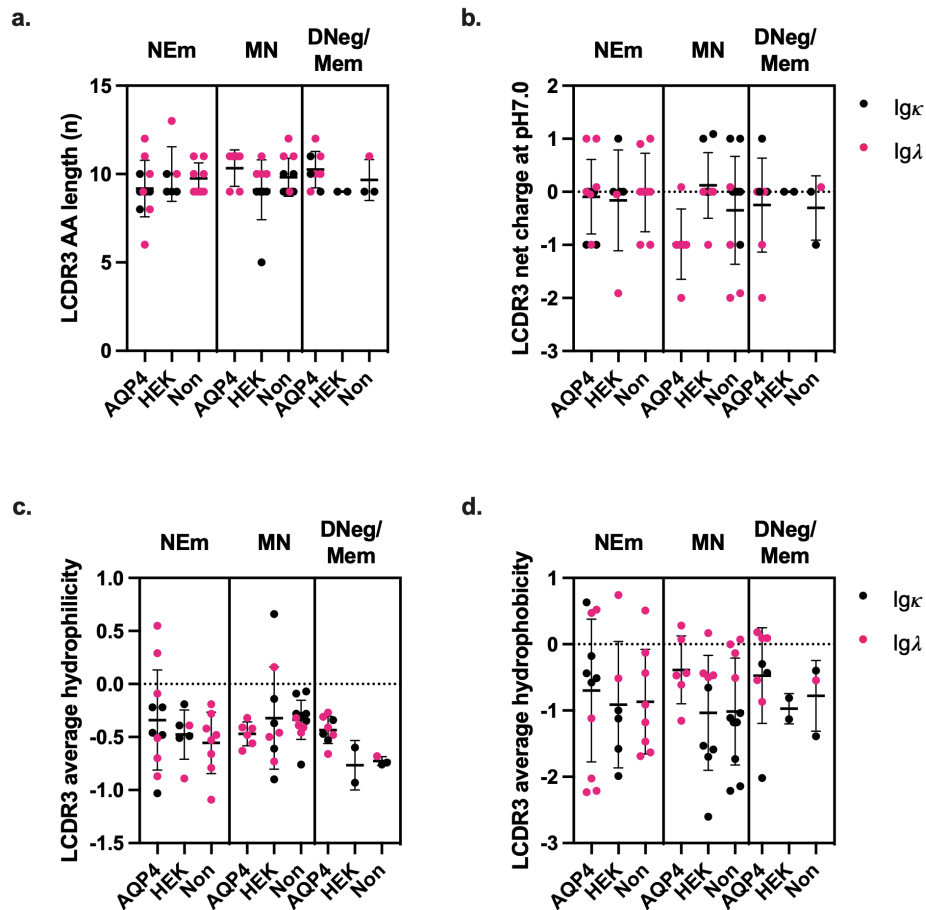


Figure 3.16. LCDR3 physicochemical characteristics across antigen reactivities and B cell subsets. B cells were grouped by antigen reactivities and subsets. The amino acid length (a), net charge (b), hydrophilicity (c), and hydrophobicity (d) of the LCDR3s from individual BCRs are shown. Data are presented as mean $\pm$ SEM. NEm: new emigrant; MN: mature naïve; DNeg: double-negative; Mem: memory.

3.5 Summary

Molecular characterisation of the immunoglobulin genes of AQP4-specific B cells isolated from NMOSD patients revealed very relevant findings with implications for understanding the aetiology and propagation of the disease. Immunophenotyping of B cell subsets did not distinguish patients from healthy controls. However, using a single-cell approach, we found that a small number of B cells at the earliest stage of development from healthy controls were AQP4-specific. In contrast, significantly higher frequencies of AQP4-specific B cells were observed in patients with reducing magnitude across developmental lineages, suggesting a defective central with partially functioning peripheral tolerance

checkpoint in NMOSD. Furthermore, we described a distinct population of B cells with reactivity against HEK membrane antigen(s). The distribution of these HEK-reactive B cells across subsets mirrored those of AQP4-specific. Upon characterisation of the B cell receptors (BCRs), there were trends towards IgHV2 being overrepresented in the AQP4-specific BCRs whereas the HEK-reactive BCRs exclusively utilised the IgHV4-34 gene family. An attempt to control AQP4 specificity through receptor editing was shown by a bias in favour of lambda chain and distal IgJK gene segments usage. Inferring from the shared heavy V and J genes with the previously published, clonally expanded, professional antibody-secreting cells (ASCs), certain gene combinations may confer a survival advantage to early AQP4-specific B cells to continue their maturation process. Additionally, the rate of somatic hypermutations across the immunoglobulin heavy and light V genes was shown to reflect B cell developmental stages rather than reactivity. Meanwhile, physicochemical properties of the heavy, but not light chain CDR3 characterised B cells with reactivity to self-antigens. Taken together, AQP4-specific B cells demonstrated unique immunoglobulin gene features as a consequence of aberrant positive and/or negative selection forces involved in B cell tolerance.

4 Chapter IV: Characterisation of Binding Affinity of NMOSD Patient-derived AQP4-specific Monoclonal Antibodies

4.1 Introduction

V(D)J recombination during early B cell development diversifies the BCR repertoire within an individual. As this happens randomly, non-functional BCRs as well as those with reactivity against autoantigens are also frequently generated. To minimise autoreactivity, at least two tolerance checkpoints are present along the B cell developmental pathway. The central tolerance checkpoint removes a substantial proportion (35 to 90%) of autoreactive early immature B cells before egressing to the peripheral blood as new emigrant B cells⁶⁷. If autoreactive clones persist, they are further eliminated by the peripheral checkpoint prior to transitioning into mature naïve B cells⁶⁷. Various neurological and non-neurological autoimmune conditions are characterised by the high frequency of circulating autoreactive new emigrant and mature naïve B cells, suggesting an impairment in these tolerance checkpoints^{68,69,71,72}.

The overarching theme of impaired tolerance mechanisms is also observed in NMOSD. A line of evidence showed that the peripheral humoral immune repertoire of NMOSD patients were enriched with autoreactive new emigrant and mature naïve B cells⁷². Additionally, it was proposed that pathogenic AQP4-specific antibodies of the IgG class might originate from these unmutated, autoreactive precursor B cells which subsequently gained specificity to AQP4 through somatic hypermutations⁷². In contrast, we have demonstrated that AQP4-specific B cells emerged as early as the new emigrant stage and were maintained throughout lineages (see Chapter III, section 3.4, subsection 3.4.3). Indeed, serum IgM antibodies to AQP4 exist in 10-44% of NMOSD patients and were detectable in active NMOSD lesions. This led us to speculate as to whether the precise maturation of AQP4-specific B cells in the presence of tolerance defects confers higher binding affinity to AQP4 which may then contribute to autoimmunity in NMOSD. To prove this hypothesis, we generated monoclonal antibodies (mAbs) from the BCR transcripts of individually cultured B cells from different B cell subsets

and characterised their antigen specificity and binding affinity using multiple in vitro assay platforms.

4.2 Specific study aims

In this chapter, we specifically aimed to:

1. Generate and produce mAbs from previously cultured single B cells from NMOSD patients;
2. Develop a microplate-based method for quantitative measurement of antibody binding affinity;
3. Characterise binding affinity of AQP4-specific mAbs from different B cell subsets by live CBA and microplate-based immunofluorescence assay;
4. Generate unmutated common ancestors (UCAs) from somatically hypermutated AQP4-specific mAbs and characterise their binding affinity by live CBA and microplate-based immunofluorescence assay; and
5. Evaluate the poly and autoreactivity properties of AQP4-specific mAbs and UCAs.

4.3 Methods

4.3.1 Generation of monoclonal antibodies (mAbs)

Following single B cell culture and differentiation into ASCs, RNA from single wells was isolated by adding 30 μ L RNA lysis solution containing 10 mM DTT and 40 units RNasin ribonuclease inhibitor. RNA was reverse-transcribed to synthesise cDNA using 2.5 μ L random primer, 0.25 μ L CH5 universal immunoglobulin primer, 2.5 μ L 10 mM dNTP, 0.5 μ L 25% IGEPAL CA-630, 1 μ L 0.1 mM DTT, 0.5 μ L RNasin ribonuclease inhibitor, 1 μ L SuperScript IV reverse transcriptase, 10 μ L 5x SuperScript IV reaction buffer in a total reaction volume of 50 μ L. Reverse transcription was performed at 25°C for 10 min, 50°C for 60 min, and 85°C for 10 min in a thermocycler.

cDNA (2 μ L) was used to amplify immunoglobulin heavy (Ig γ and Ig μ) and light (Ig κ , Ig λ) chain transcripts by two rounds of PCR (PCR-1 and 2) in 20 μ L reactions containing 2 μ L primers and 10 μ L GoTaq G2 Green Master Mix. PCR-1 was performed for 45 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec whereas PCR-2 was run performed for 35 cycles at 95°C for 30 sec, 55-60°C for 30 sec, and 72°C for 60 sec. PCR-2 products were purified and sequenced.

Expression vectors containing immunoglobulin heavy (Ig γ and Ig μ) and light (Ig κ , Ig λ) chain constant regions were linearised by restriction digest and gel-purified. PCR-2 inserts were then cloned into the linearised vectors, transformed into 5-alpha competent *E. coli*, and plated on ampicillin agar plates for overnight culture. For each transformation, 10-15 colonies were screened by colony PCR in 10 μ L reactions containing 1 μ L insert-specific primer and 5 μ L 2x GoTaq G2 Green Master Mix at 98°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec for 34 cycles. The PCR products were then loaded into a 1% agarose gel for electrophoresis with the backbone vector without an insert as a negative control. Positive colonies were minipreped and submitted for Sanger sequencing to confirm the insert sequences (see Chapter II, Subsection 2.2.3.1-13 for the detailed methods).

4.3.2 Generation of unmutated common ancestors (UCAs)

During V(D)J recombination, extra nucleotides (N) were randomly added by terminal deoxynucleotidyl transferase (TdT) between the VH-DH, DH-JH and VL-JL segments in the heavy and light chain genes, respectively, to increase CDR3 diversity. As these insertions are not encoded by the germline genes, the N regions were preserved. The resulting unmutated common ancestors (UCAs), also referred to as revertant mAbs, were named after their parent mAbs with a letter R added at the end, e.g., a revertant of parent mAb DR11 D4 would be assigned as DR11 D4R.

4.3.3 *Production and purification of monoclonal IgG*

HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% AA and co-transfected with 0.75 µg of each heavy (Ig γ) and light (Ig κ/λ) chain plasmid DNA and 4.2 µg of PEI per mL of culture media. After 6 hours, culture media was aspirated and replaced with the same volume of fresh, phenol red-free DMEM supplemented with 10% Nutridoma-SP. Supernatants were collected after 3-5 days of culture. IgG in culture supernatants was affinity-purified using protein G agarose beads. Bound IgG was eluted in 3-4 fractions, spin-concentrated using a 10 kD molecular weight cut-off centrifugal filter device, and buffer-exchanged into PBS. Concentrated IgG samples were stored at 4°C until use (Chapter II, Subsection 2.2.4.1 and 2.2.4.3).

4.3.4 *Production and precipitation of monoclonal IgM*

To promote the formation of pentameric IgM antibodies (instead of hexameric), HEK293T cells grown in DMEM supplemented with 10% FBS and 1% AA were co-transfected with 0.5 µg of each heavy (Ig μ), light (Ig κ/λ), and joining (IgJ) plasmid DNA, and 4.2 µg of PEI per mL of culture media. Culture media was replaced with fresh, phenol red-free DMEM supplemented with 10% Nutridoma-SP 6 hours post-transfection and supernatants were collected 3-5 days afterwards. IgM was precipitated from supernatants by slowly adding an equal volume of saturated ammonium sulphate solution (4.32 M) and incubating the mixture overnight at 4°C. Precipitates were reconstituted in 1 mL PBS, desalted, spin-concentrated using a 100 kD molecular weight cut-off centrifugal filter device. Recovered IgM samples were stored at 4°C until use (Chapter II, Subsection 2.2.4.2 and 2.2.4.4).

4.3.5 *Polyacrylamide gel electrophoresis (PAGE) and western blot*

To verify antibody expression, IgG and IgM samples were analysed by PAGE under reducing and non-reducing conditions as described in Chapter II, Subsection 2.2.4.7

Commercially available, high performance liquid chromatography (HPLC)-purified human IgG and IgM were used as controls. Protein bands were visualised by Coomassie Blue staining and the molecular sizes were estimated based on the migration pattern of pre-stained protein standards. The identities of the protein bands were further confirmed by transferring the bands to a polyvinylidene fluoride (PVDF) membrane using a dry transfer method and staining the membrane with HRP-conjugated, goat anti-human IgG, Fc γ and IgM, Fc γ antibodies (Chapter II, Subsection 2.2.4.9).

4.3.6 *Microplate-based immunofluorescence assay*

The general live CBA protocol is described in Chapter II, Subsection 2.2.2.1-8. HEK293T cells were seeded in a PLL-coated, flat bottom, 96-well plate at 40,000 cells/well in 100 μ L DMEM supplemented with 10% FBS and 1% AA. The following day, a transfection mixture was prepared by adding 1.5 μ g untagged AQP4-M23 plasmid DNA and 4.2 μ g PEI into 25 μ L of serum-free DMEM per mL of culture media. The mixture was incubated for 10 min at RT and added into fresh culture media from which 100 μ L was dispensed into each well after removing the old media. At 6 hours post-transfection, culture media containing the transfection mixture was removed and replaced with fresh media. Cells were allowed to express the protein for 24 hours prior to performing a live CBA.

Quantitative measurement of antibody binding affinity was performed by incubating AQP4 M23-expressing HEK293T cells with 1:2 dilutions of AQP4-specific mAbs (0.02-20 μ g/mL) in a total volume of 50 μ L. for 1 hour at RT. Cells were then fixed with 4% PFA and specific binding was detected with an anti-human IgG or IgM, Alexa Fluor 594-conjugated secondary antibody. Fluorescence intensities at 560/600 nm excitation/emission wavelengths were read using a microplate reader, normalised against values obtained from wells incubated with an isotype control antibody, and plotted as a function of mAb concentration. Binding

kinetics parameters including equilibrium dissociation constant (K_D) and maximum binding level (Bmax) were determined by fitting data to the one-site specific binding model.

4.3.7 Poly and autoreactivity ELISA

To evaluate antibody polyreactivity, flat bottom, 96-well plates were coated with specific antigens including 20 µg/ml dsDNA, 10 µg/mL LPS, or 15 µg/mL recombinant human insulin. Meanwhile, semi quantitative detection of antibody autoreactivity was performed using commercially available QUANTA Lite ANA ELISA plates pre-coated with human epithelial type 2 (Hep-2) cell lysate. For all ELISAs, highly reactive ED38, a monoclonal IgG generated from VpreB⁺ λ5⁺ peripheral B cells, was used as a positive control. Monoclonal IgG samples and control were tested at 1 µg/mL, 0.25 µg/mL, 0.063 µg/mL, and 0.016 µg/mL. Signals were developed with an HRP-conjugated, goat anti-human IgG, Fcγ and ABTS substrate. The minimum optical density (OD) values at 405 nm at which antibodies were considered reactive was 0.5 at 1 µg/mL (Chapter II, Subsection 2.2.5.1-2). Polyreactivity was defined as simultaneous reactivity to all three antigens (dsDNA, LPS, and insulin) whereas autoreactivity was defined as reactivity to Hep-2 cell lysate.

4.4 Results

4.4.1 Generation of patient-derived monoclonal antibodies (mAbs)

To study antigen specificity and binding affinity, we generated monoclonal antibodies (mAbs) from BCR transcripts from individually cultured B cells. Regardless of reactivity (AQP4-specific or HEK-reactive) and B cell subsets, all transcripts were cloned into both human IgG1 and IgM expression vectors. Antibody cloning was performed with assistance from Dr Deniz Akdeniz.

In total, 25 AQP4-specific and 17 HEK-reactive mAbs were cloned from 4 patients with serum AQP4-IgG positive NMOSD. The detailed numbers of successfully cloned mAbs

from each patient are summarised in Table 4.1 below. For purposes of this study, mAbs were named based on the initials of a researcher (DR) and an ID number given to a patient from which they were generated (patient 1, DR11; patient 2, DR14; patient 3, DR26; patient 4's PBMCs, DR20; and patient 4's thymic cells, DR30) followed by the alphanumeric designation of an individual well in which the cDNA was PCR amplified. Hereafter, a fully designated identity of a particular mAb would appear as, for example, DR11 C6 which indicates that it was derived from patient 1 and well C6 of the PCR amplification plate.

Patient	Designated ID	Reactivity	B cell subset			
			New emigrant	Mature naïve	Double-negative	Memory
1 (PBMC)	DR11	AQP4	3	1	1	1
		HEK	3	5	0	0
2 (PBMC)	DR14	AQP4	0	0	2	1
		HEK	0	2	0	0
3 (PBMC)	DR26	AQP4	2	1	0	0
		HEK	0	0	0	0
4 (PBMC)	DR20	AQP4	5	1	0	1
		HEK	3	2	2	0
4 (Thymic)	DR30	AQP4	1	3	2	0
		HEK	0	0	0	0
Total		AQP4	25			
		HEK	17			

Table 4.1. The total numbers of successfully cloned monoclonal antibodies from reactive BCR transcripts.

4.4.2 Expression of patient-derived monoclonal antibodies (mAbs)

As production of mAbs of IgM isotype was not regularly conducted in our laboratory, we sought to determine the optimum culture vessels and timepoints of supernatant collection. A pilot expression of 6 unique IgMs using HEK293T cells grown in 3 different culture vessels (6-well plates, T25 flasks, and T75 flasks) was performed. The volume of culture media added in each culture vessel was 2 mL/well for 6-well plates, 5 mL for T25 flasks, and 15 mL for T175 flasks. Culture supernatants were serially harvested from days 3 to 5 post-transfection and the total amounts of IgM produced were quantified by human IgM-specific ELISA (see Chapter II, Subsection 2.2.4.6 for methods) and compared.

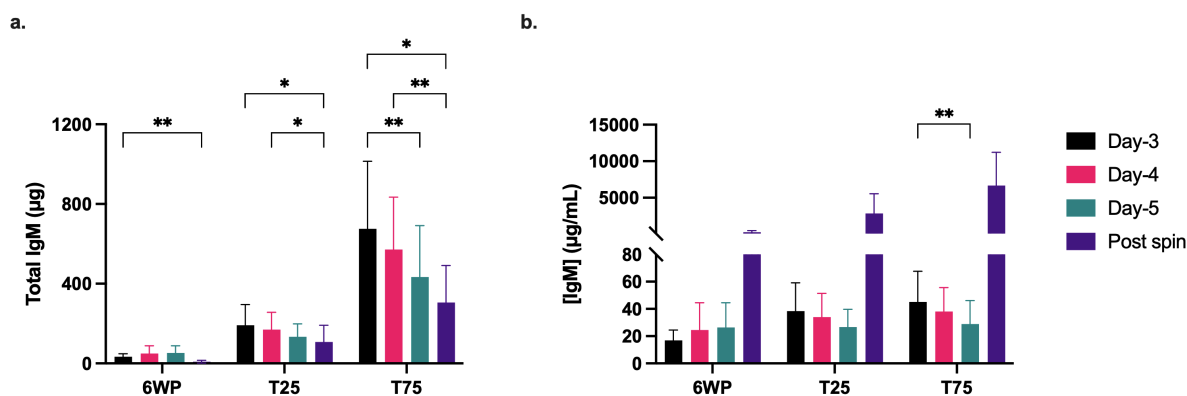


Figure 4.1. Optimisation of IgM expression.

HEK293T cells grown in different culture vessels were transfected with 6 unique IgM plasmids. The total amounts (a) and concentrations (b) of IgM were quantified and compared among different expression scales and timepoints of supernatant collection. At the end of culture (day 5), supernatants were spin concentrated and their amounts and concentrations are shown (post-spin). Two-way ANOVA, Mean±SEM, *= $p<0.05$, **= $p<0.005$, ***= $p<0.0005$, ****= $p<0.0001$. 6WP, 6-well plate.

Culture vessel (volume, mL)	Amount of secreted IgM (mean, µg)				Concentration of secreted IgM (mean, µg/mL)			
	Day 3	Day 4	Day 5	Post spin	Day 3	Day 4	Day 5	Post spin
6WP (2 mL)	33.88	49.01	52.94	8.53	16.94	24.50	26.47	291.0
T25 flask (5 mL)	191.60	169.90	133.20	107.20	38.33	33.99	26.64	2838.00
T75 flask (15 mL)	676.00	571.10	433.30	305.8	45.07	38.07	28.89	6672.00

Table 4.2. The total amounts and concentrations of secreted IgM across different expression scales and timepoints.

As expected, there was more secreted IgM in the supernatant in larger culture vessels at any timepoints (mean across timepoints, 6-well plate vs T25 vs T75, 34-53 µg vs 133-192 µg vs 433-676 µg, two-way ANOVA with Tukey's post-hoc tests, $p<0.05$) (Table 4.2). These differences were not observable with respect to IgM concentration (Figure 4.2b, Table 4.2). Additionally, about 100 times higher concentrations of IgM were generated by spin-concentrating solutions with ultracentrifugal filters in exchange for reduced amounts of total IgM in the final product due to precipitation of IgM during the process.

The yields of IgM expression using 6-well plates and T25 flasks did not significantly improve over time (Figure 4.2a, Table 4.2). Interestingly, in a larger expression scale using T75 flasks, there was a trend of reducing yield at later timepoints which could be due to the

release of proteases from dying cells. Whilst different expression scales can be employed to suit different amounts of antibody needed for downstream experiments, day 3 post-transfection seems to be the most optimum timepoint to harvest culture supernatants for IgM expression.

4.4.3 Identification of mAbs oligomers by SDS-PAGE and western blot

In vivo, immunoglobulin molecules are produced as oligomers, with IgG as a monomer and IgM as a pentamer, and to a lesser extent, a hexamer⁸⁹. Pentameric IgM antibodies are formed by multimerization of five IgM monomers associated with a joining (J) chain, or IgJ, via disulphide bonds involving the cysteine residue at the end of the I μ heavy chain tailpiece. When IgJ is not available for incorporation, the secreted IgM is dominated by hexameric species whereas increasing levels of IgJ expression promote the secretion of more abundant IgM pentamers⁸⁹.

To confirm the expression of antibody oligomers, mAbs were run on SDS gels under reducing and non-reducing conditions and the molecular sizes were compared to HPLC-purified IgG and IgM standards from human serum. Samples and standards were loaded into a 4-12% Bis-Tris gradient gel and electrophoresis were carried out at a constant 200V for 50 min. Coomassie Blue staining was employed to visualise protein bands.

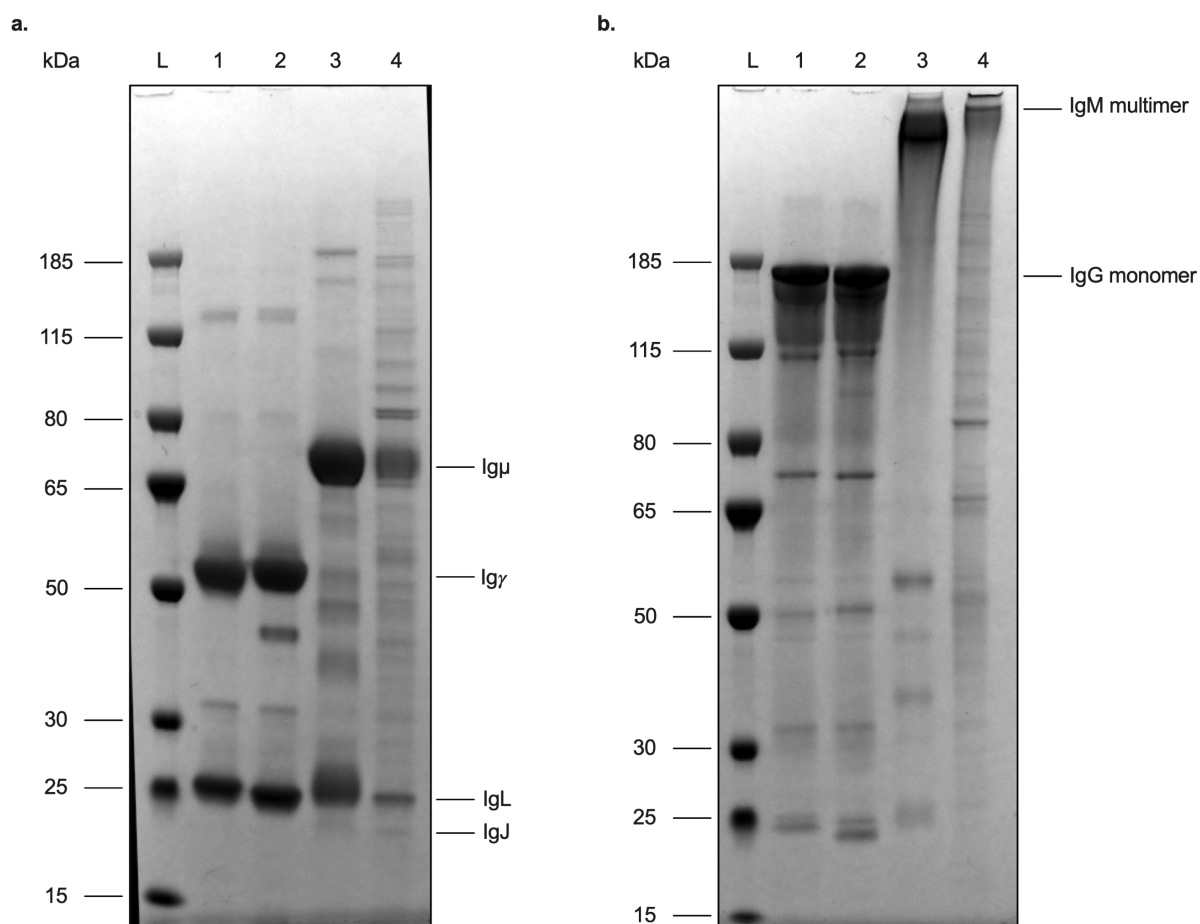


Figure 4.2. Representative images of Coomassie Blue staining of SDS-PAGE gels following the separation of IgG and IgM molecules.

IgG and IgM samples and standards were run on 4-12% Bis-Tris gradient gels under reducing (a) and non-reducing (b) conditions to confirm the production of monomeric IgG and multimeric IgM. L, protein ladder; lane 1, IgG standard; lane 2, IgG sample; lane 3, IgM standard; lane 4, IgM sample.

Under denaturing-reducing conditions (Figure 4.2a), separation of the immunoglobulin heavy chain (Ig γ and Ig μ) and light chain (IgL) was observed. In lane 2, our in-house produced IgG showed two thick bands which correspond to the Ig γ and IgL with the theoretical masses of ~50 and ~25 kD respectively. Two dominant bands, Ig μ (~70 kD) and IgL (~25 kD), were also revealed in the IgM standard (lane 3) and sample (lane 4). A lighter IgJ band was detected in both IgM standard and sample, but not in the IgG preparations, at ~17 kD. Identification of this immunoglobulin chain is sometimes challenging as it only represents ~1.5% of the total mass of an IgM molecule.

Formation of monomeric IgG and multimeric IgM species was resolved by non-reducing SDS gel electrophoresis (Figure 2b). Two heavy-light chain pairs of an IgG monomer make up a total mass of ~150 kD. Bands corresponding to this size were seen in both the IgG standard (lane 1) and sample (lane 2). Meanwhile, multimeric IgM with an approximate size of 900 kDa was detected as a thick band at the upper end of lanes 3 (IgM standard) and 4 (IgM sample).

Next, protein bands were transferred to a PVDF membrane and co-stained with HRP-conjugated anti-human Ig γ and Ig μ antibodies. The detected bands on the protein gels were specifically identified as the heavy chain of each immunoglobulin isotype (Figure 4.3). We conclude that our method is optimised to express monoclonal IgG and IgM antibodies.

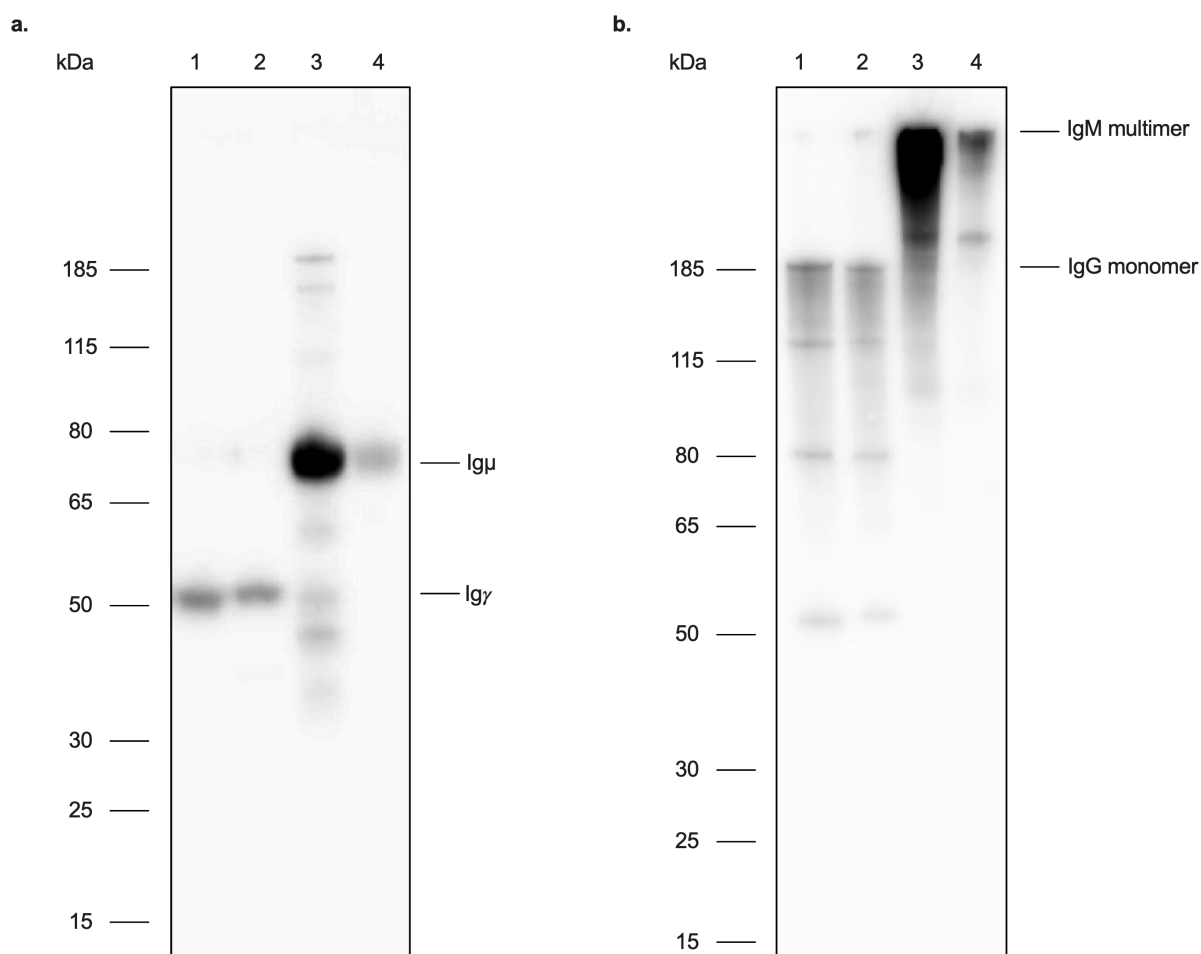


Figure 4.3. Western blot analysis post separation of IgG and IgM using heavy-chain specific antibodies after SDS-PAGE.

Proteins bands post separation under reducing (**a**) and non-reducing (**b**) conditions were transferred to PVDF membranes and probed with anti-human heavy chain-specific secondary antibodies. $Ig\gamma$ and $Ig\mu$ were identified and shown. Lane 1, IgG standard; lane 2, IgG sample; lane 3, IgM standard; lane 4, IgM sample.

4.4.4 Confirmation of AQP4-specificity by live CBA

To confirm AQP4-specificity post-cloning, AQP4-IgM and IgG1 were tested on live CBA using AQP4 M23-expressing HEK293T cells as a substrate. Cells were incubated with 20 μ g/mL of mAbs and stained with fluorophore-conjugated, isotype-specific secondary antibodies. A non-reactive IgM from one of the patients (DR11 E9) and an anti-hen egg lysozyme (HEL) IgG1 were used as isotype controls (Figure 4.4a).

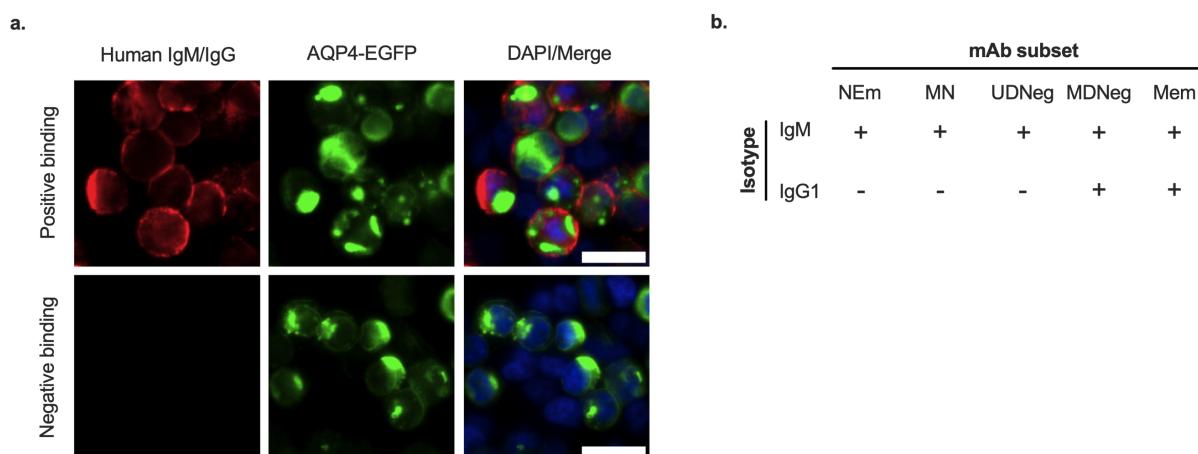


Figure 4.4. Differential binding of AQP4-specific mAbs as IgM and IgG1 species across subsets.

a. Representative images of specific binding to AQP4 evaluated by live CBA using AQP4 M23-expressing HEK293T cells as a substrate. A non-reactive IgM generated from one of the patients and an anti-hen egg lysozyme (HEL) IgG1 were used as controls. **b.** AQP4-specific new emigrant (NEm, $n=11$), mature naïve (MN, $n=6$), and unmutated double-negative (UDneg, $n=2$) showed detectable binding as IgM multimers but not IgG1 monomers. In contrast, somatically hypermutated AQP4-specific double-negative (MDNeg, $n=3$) and memory (Mem, $n=3$) mAbs were able to recognise their antigen across two antibody isotypes. 40x magnification, scale bar=20 μm . AQP4, Aquaporin-4; DAPI, 4'6-diamidino-2-phenylindole.

New emigrant- ($n=11$) and mature naïve-derived ($n=6$) mAbs showed detectable binding to AQP4 as IgM, but not IgG1 (Figure 4.4b). The double-negative subset comprised 2 populations, those with ($n=3$) and without ($n=2$) somatic mutations across their rearranged immunoglobulin genes. The unmutated double-negative exclusively bound as IgM whereas the mutated counterparts bound as both IgM and IgG1. All memory mAbs ($n=3$) demonstrated AQP4 specificity across both antibody isotypes.

From these observations, we reason that the ability of the unmutated, early B cell subsets-derived mAbs to bind AQP4 is mostly conferred by the multivalent properties of IgM multimers which compensate for the low affinity of the antigen-binding site in its individual monomers. Furthermore, incorporation of somatic mutations refines their affinity and allows them to bind as IgG1 monomers.

4.4.5 Confirmation of HEK-reactivity by live CBA

An additional exploratory analysis with regard to differential binding of HEK-reactive mAbs as IgM and IgG1 antibodies was performed. Non-transfected HEK293T cells were used as a substrate for live CBA. None of the HEK-reactive mAbs were derived from the mutated B cell subsets, i.e., double-negative and memory. HEK-reactive new emigrant, mature naïve, and unmutated double-negative mAbs demonstrated binding to surface membrane as IgM multimers, but not IgG1 monomers (Figure 4.5). Similar to AQP4 specificity in early B cells, this result indicates that HEK reactivity is mediated by low-affinity, multivalent antibody-antigen interactions.

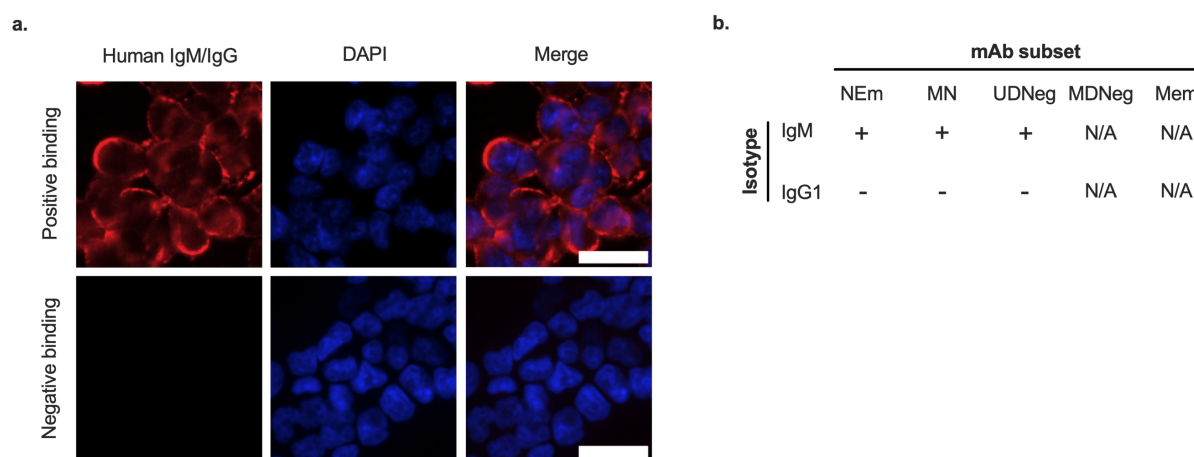


Figure 4.5. Differential binding of HEK-reactive mAbs as IgM and IgG1 species across subsets.

a. Representative images of surface membrane binding of HEK-reactive mAbs on non-transfected HEK cells; **b.** HEK reactivity was only observed when mAbs were expressed as IgM, but not IgG1 antibodies. 40x magnification, scale bar=20 μ m. N/A, not applicable; NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory. DAPI, 4'-diamidino-2-phenylindole.

4.4.6 Characterisation of AQP4-specific mAbs binding affinity by CBA endpoint titre

To study the evolution of binding affinity to AQP4 across B cell subsets, we determined the endpoint titres of mAbs by live CBA. AQP4 M23-transfected HEK293T cells were incubated with serial dilutions of mAbs starting at a concentration of 20 μ g/mL. The endpoint titre was defined as the highest dilution which gave a positive signal evaluated visually by fluorescence microscopy. As different antibody isotypes, i.e., IgM vs IgG1, may influence the

nature of antibody-antigen interactions, all mAbs were tested as IgM multimers. Comparisons across subsets as IgG1 were not possible as the new emigrant, mature naïve, and unmutated double negative mAbs did not show binding to AQP4 as IgG1 monomers. There was a trend of increasing binding affinity as represented by endpoint titres across mAbs subsets (mean $\mu\text{g/mL}$, NEm=3.010, MN=0.5534, UDNeg=0.8205, MDNeg=0.1840, Mem=0.1140) (Figure 4.6). This suggests that, in the presence of defective B cell tolerance, the precise development of early AQP4-specific B cells may give rise to affinity-matured AQP4-specific antibodies.

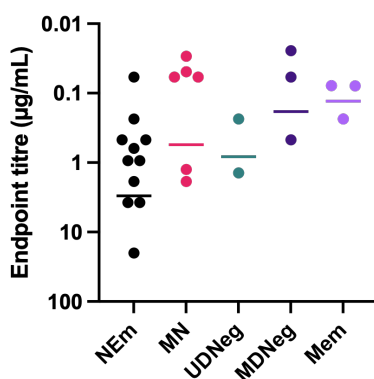


Figure 4.6. CBA endpoint titres of AQP4-IgMs across subsets.

All mAbs were tested as IgM multimers to eliminate the potential influence of different isotypes in the nature of mAb binding to AQP4. There was a trend of increasing endpoint titre across mAbs subsets (n , NEm=11, MN=6, UDNeg=2, MDNeg=3, and Mem=3). Horizontal bars indicate median values. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

4.4.7 Development of a quantitative measurement of antibody binding affinity

Up to this point, antibody binding to AQP4 has been characterised by a single parameter, i.e., CBA endpoint titre. To gain a deeper understanding of the binding kinetics, we developed a quantitative assay whereby fluorescence signals from specific binding were read using a microplate reader and plotted against a range of mAb concentrations.

In microplate readers, incoming signals are amplified by the photomultiplier tubes (PMTs) based on the voltage, also termed gain, set by the user. To identify the setting which provides the best signal resolution, fluorescence signals of a serially diluted AQP4-IgM over a range of gain values (500-4000) were recorded and plotted (Figure 4.7). DR14 F2 IgM was

chosen for this purpose as it was shown on live CBA to be one of the strongest AQP4 binders and, thus, was expected to have the highest signal output.

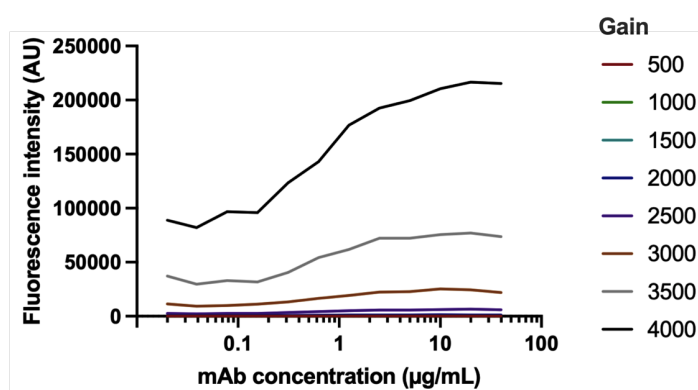


Figure 4.7. Optimisation of fluorescence gain on a microplate reader.

A positive control AQP4-IgM mAb (DR14 F2) was tested across a range of concentrations and gain values to determine the setting which provides the best signal resolution. AU, arbitrary unit.

There was an upward shift of the fluorescence intensity (FI) curves with higher gain values. The clearest discrimination between the highest and lowest mAb concentrations was achieved at gain 4000, which was the maximum capacity of the microplate reader. Additionally, the dynamic range (0.02-20 µg/mL) was deemed adequate for binding kinetics analyses as the data defined a complete sigmoidal curve within the range of tested concentrations. Therefore, subsequent binding assays were performed using this optimised setting.

4.4.8 Quantitative measurement of AQP4-specific mAbs binding affinity

Quantitative measurements of antibody binding to AQP4 were performed using the aforementioned microplate-based method. All mAbs were serially diluted from 20 µg/mL starting concentration and tested as IgM multimers. Isotype control-subtracted fluorescence intensity (FI) values of four biological replicates for each mAb were recorded and plotted as a function of mAb concentration. Equilibrium dissociation constant (K_D) values and maximum binding (B_{max}) levels were estimated by fitting data to the single-site specific binding model under the assumption that one antibody arm binds to one antigenic target. To further optimise

non-linear regression analysis outputs, data points that deviate from the global trend were identified and removed using the ROUT method on the basis of false discovery rate (FDR). For this analysis, the maximum desired FDR, or Q value, was set to 1% as we aimed for less than 1% of the outliers to be falsely or incorrectly identified.

Binding curves and estimated kinetics parameters of individual AQP4-IgM mAbs are shown in Figure 4.8 and Table 4.3, respectively. Whilst a complete curve was obtained from most of the mAbs, a few of them did not reach a saturation point even at the highest concentration tested. Performing the assay at a higher concentration range might solve this issue but it would also impose a significant technical challenge: an industrial scale expression platform would be needed to produce immense amounts of antibodies.

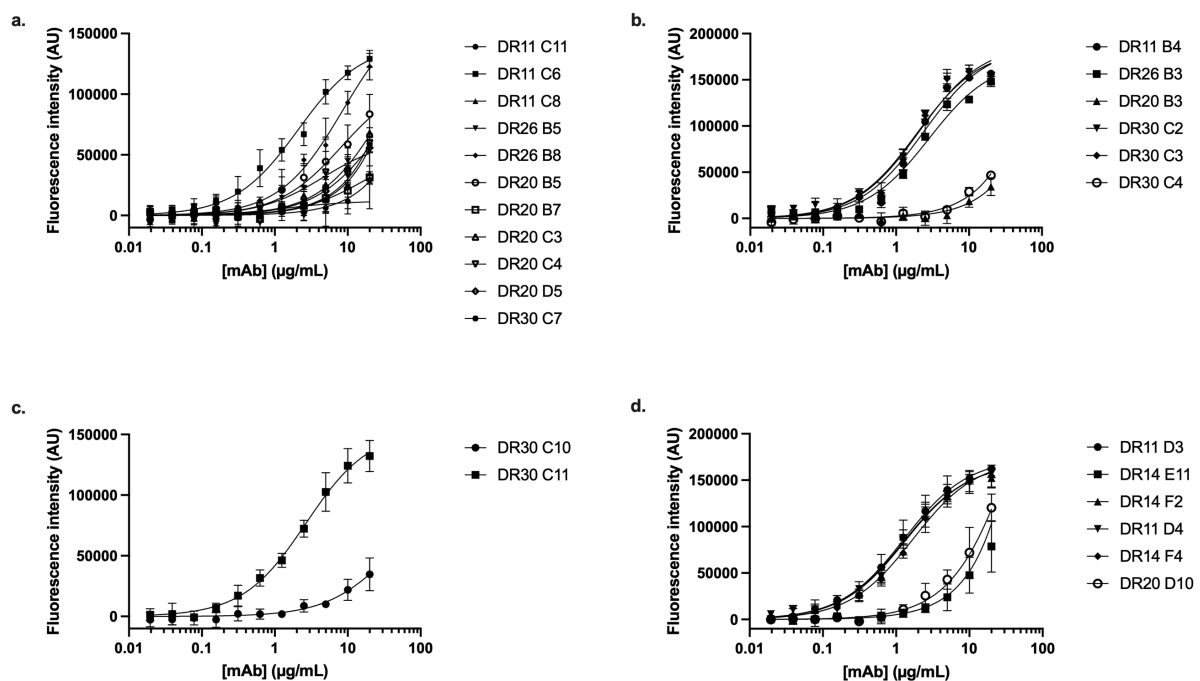


Figure 4.8. Binding curves of AQP4-IgMs.

Isotype control-subtracted fluorescence intensity values of serially diluted new emigrant (a), mature naïve (b), unmutated double-negative (c), and mutated double-negative and memory (d) IgMs were read using a microplate reader and curve-fitted to a single-site specific binding model. Data are presented as mean $\pm$ SEM.

Subset	mAb ID	K _D (µg/mL)	SEM (µg/mL)	Bmax (AU)	SEM (AU)
New emigrant	DR11 C6	2.099	0.2256	141598	4681
	DR11 C8	1.392	1.211	11982	3371
	DR11 C11	1.036E+15	1.811E+28	Unstable	Unstable
	DR26 B5	4.623	1.129	62969	6080
	DR26 B8	8.625	1.038	173998	9631
	DR20 B5	8.235	2.271	113501	13956
	DR20 B7	16.84	7.5	57450	14603
	DR20 C3	31.68	14.93	171097	57901
	DR20 C4	1.8702E+11	2.75E+20	Unstable	Unstable
	DR20 D5	27.25	10.73	133753	34709
	DR30 C7	7.928E+15	6.864E+29	Unstable	Unstable
Mature naïve	DR11 B4	2.38	0.2571	187002	6405
	DR26 B3	2.819	0.2833	172449	5736
	DR20 B3	8.775E+15	8.613E+29	Unstable	Unstable
	DR30 C2	1.93	0.2243	184024	6450
	DR30 C3	2.082	0.2293	188507	6374
	DR30 C4	579.5	3391	1448474	8259161
Unmutated double-negative	DR30 C10	36.72	29.72	98928	57048
	DR30 C11	2.667	0.2813	153359	5278
Mutated double-negative	DR11 D3	1.287	0.1189	173587	4424
	DR14 E11	4.449E+15	1.018E+29	Unstable	Unstable
	DR14 F2	1.276	0.08883	168111	3224
Memory	DR11 D4	1.321	0.08934	168655	3166
	DR14 F4	1.692	0.1472	172695	4398
	DR20 D10	45.7	19.39	389802	123265

Table 4.3. Estimated binding kinetics parameters of AQP4-IgMs.

Kinetics parameters were derived from the single-site specific binding model. K_D indicates the equilibrium dissociation constant of antibody binding to AQP4 as IgM multimers. Bmax denotes the maximum binding level at a saturating antibody concentration. Some mAbs had an incomplete curve using the selected model and, thus, their parameters were defined as unstable and should be interpreted with caution.

As kinetic parameters for some of the mAbs could not be confidently estimated using the selected model, they were excluded from analysis. Nevertheless, a reducing trend of K_D values, or increasing binding affinity to AQP4, was observed across subsets, consistent with the CBA endpoint titre results (median K_D, µg/mL, NEm=8.43, MN=2.38, UDNeg=19.69, MDNeg=1.282, Mem=1.692) (Figure 4.9a). Comparative analysis of the Bmax values, however, did not distinguish mAbs generated from different B cell lineages (Figure 4.9b).

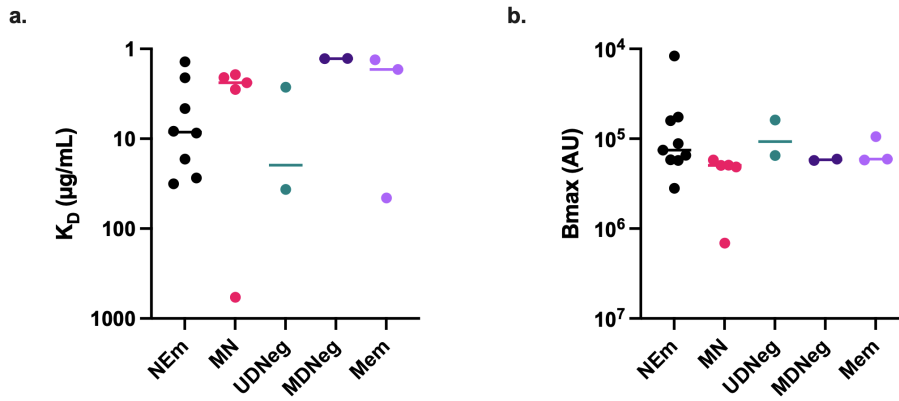


Figure 4.9. Binding kinetics of AQP4-IgMs across subsets.

There was a trend of reducing K_D (a), but not B_{max} (b) values of mAbs generated from B cells at different developmental stages. Horizontal bars represent median values. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

To gain statistical power, we pursued a more robust analysis whereby the average fluorescence intensity values of individual mAbs within a subset were treated as replicates to generate a global binding curve for that particular subset. The best-fit K_D values were compared among all groups using the extra sum-of-squares F test. If the compared parameter was identified to be different for at least one group, multiple comparisons between each group with every other group were performed.

The global binding curves of individual AQP4-IgM subsets are presented in Figure 4.10a. Analyses involving the unmutated double-negative subset did not yield any statistical significance due to the wide variability of the two member-mAbs (DR30 C10 and DR30 C11). The new emigrant was consistently demonstrated to have high K_D , followed in reducing value by the mature naïve and the somatically mutated mAb subsets (mutated double-negative and memory) (Figure 4.10b).

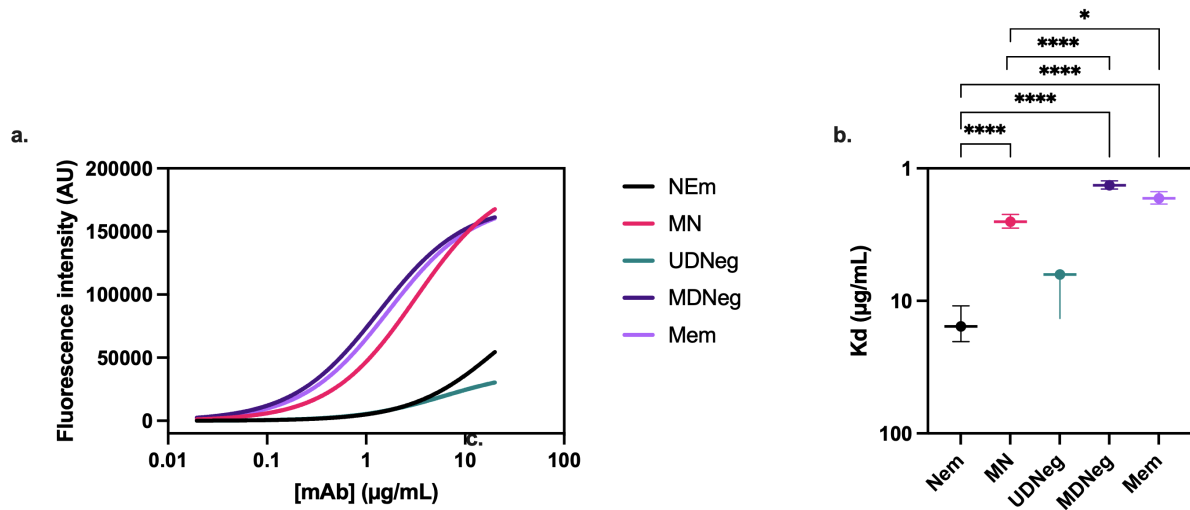


Figure 4.10. Global binding curves of individual AQP4-IgM subsets.

a. Average fluorescence intensity values of member mAbs within individual subsets were treated as replicates to generate global binding curves. **b.** There was a significant and gradual reduction in K_D values, or increment in binding affinity toward AQP4, across mAb subsets. Comparison of Fits, Mean $\pm$ SEM, $\ast=p<0.05$, $\ast\ast=p<0.005$, $\ast\ast\ast=p<0.0005$, $\ast\ast\ast\ast=p<0.0001$. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

4.4.9 Correlation between CBA endpoint titre and K_D value

The strength of interactions between AQP4-specific mAbs and their cognate antigen has been characterised using two approaches: visual determination of CBA endpoint titres and estimation of K_D values. Visual CBA is a relatively straightforward method if performed by trained observers, but it may only capture a certain aspect of antibody-antigen interactions. In contrast, kinetics measurements provide a more comprehensive view of antibody binding although it requires extensive analyses to reliably calculate related parameters. Nevertheless, a significant correlation was observed between CBA endpoint titres and K_D values of the AQP4-IgMs (Spearman $r=0.6604$, $p=0.0015$) (Figure 11). These two approaches can therefore be employed in parallel to evaluate and rank order antibody binding affinity.

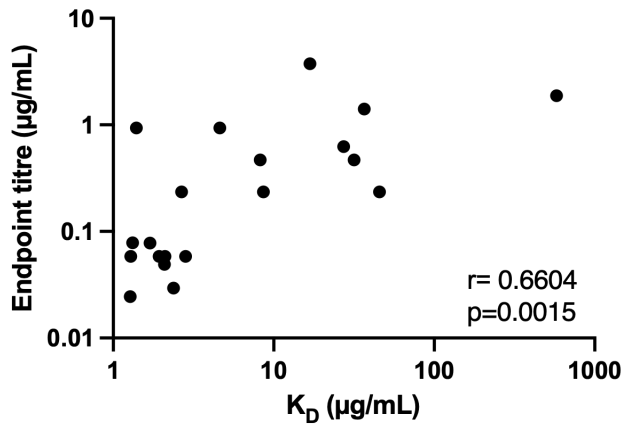


Figure 4.11. CBA endpoint titres and K_D values of AQP4-IgMs.

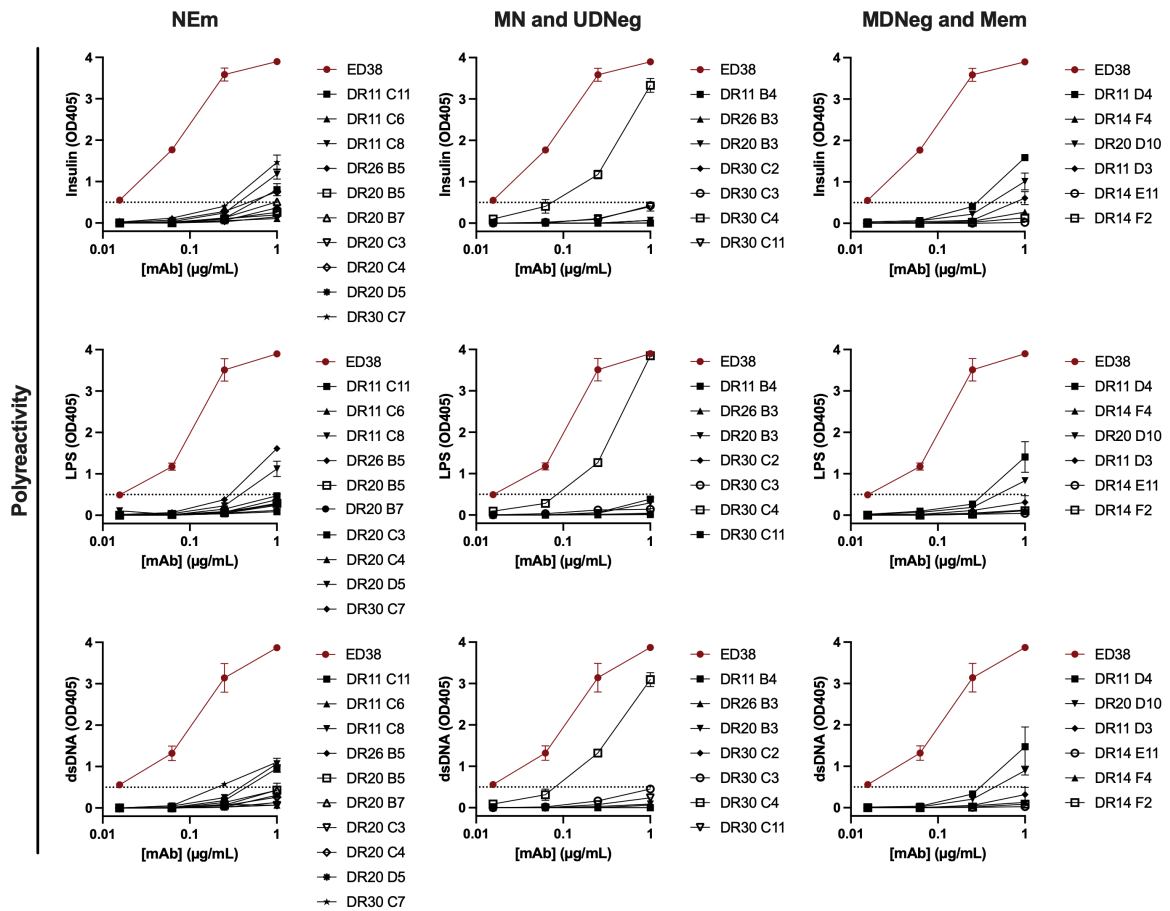
The two parameters are shown to correlate with one another. Spearman $r=0.6604$, $p<0.005$.

4.4.10 Evaluation of poly and autoreactive properties of AQP4-specific mAbs

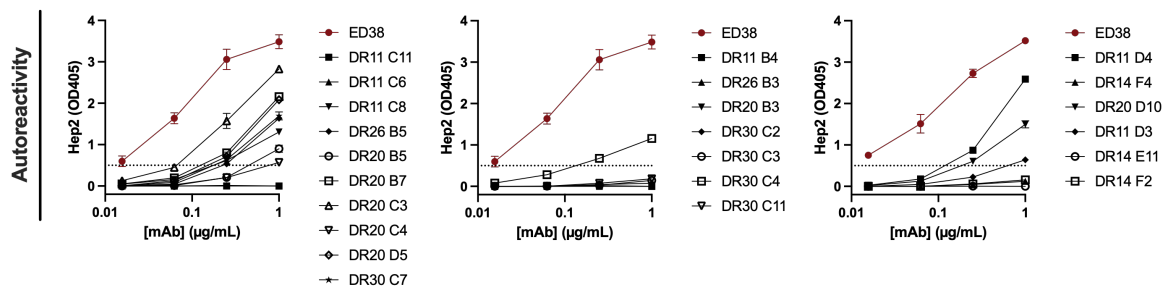
In an earlier study, mature AQP4-specific B cells were suggested to develop from poly and/or autoreactive precursor B cells which subsequently gain their AQP4 specificity through somatic hypermutations. On the other hand, we have clearly demonstrated that AQP4 specificities were detected as early as the new emigrant stage and maintained throughout lineages. To investigate whether AQP4-specific B cells in our study exhibit reactivities beyond AQP4, they were tested for poly and/or autoreactivity.

Poly and autoreactive antibodies were detected by ELISA using a panel of antigens comprising insulin, LPS, dsDNA, and human epithelial type-2 (Hep2) whole cell lysates. All mAbs were tested as IgG1 monomers as these assays were not optimised for testing of pentameric IgM molecules. Optical density (OD) values of serial dilutions of mAbs were read using a microplate reader at 405 nm wavelength and recorded. Highly poly- and autoreactive antibody ED38 was used as a positive control. A positive reading was defined as an OD value of >0.5 at 1 µg/mL antibody concentration.

a.



b.



b.

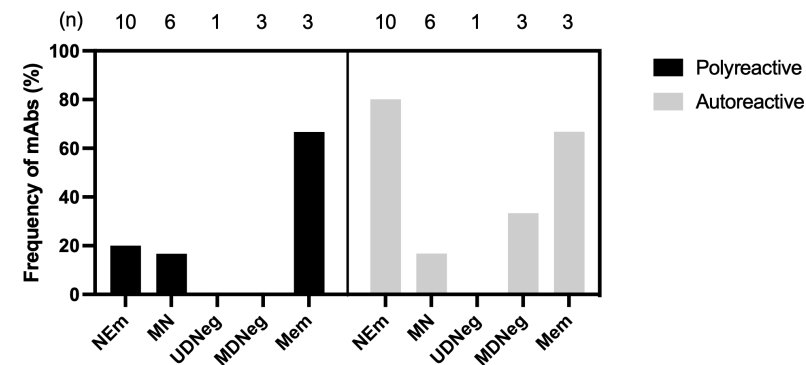


Figure 4.12. Poly and autoreactivity assays of AQP4-specific mAbs.

a. AQP4-specific mAbs were tested for polyreactivity against insulin, LPS, and ds DNA and autoreactivity against Hep2 cell lysates by ELISA. A highly poly and autoreactive mAb cloned from a VpreB+ $\lambda 5^+$ peripheral B cells (ED38) was used as a positive control. All mAbs were tested as IgG1 monomers. Each data point represents the mean value of two replicates. Dotted horizontal lines mark the cut-off of positive reactivity value; **b.** Frequencies of poly and autoreactive antibodies across subsets. Numbers at the top of bar charts denote the total numbers of mAbs tested. Data are presented as mean $\pm$ SEM. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

Graphs of OD values and a summary of results of the poly and autoreactivity assays are shown in Figure 4.12a and Table 4.4. Interestingly, some AQP4-specific mAbs exhibited reactivities to one or more test antigens. Polyreactive antibodies, defined as simultaneous reactivities to insulin, LPS, and dsDNA, comprised 2/10 (20.00%) and 1/6 (16.67%) of the NEm and MN subsets, respectively (Figure 4.12b). None of the double-negative mAbs were polyreactive whereas 2/3 (66.67%) of the memory mAbs were polyreactive. With respect to reactivity against autoantigens from Hep2 cell lysates, 8/10 (80.00%) of the NEm and 1/6 (16.67%) of the MN mAbs were considered to be autoreactive. Additionally, autoreactive antibodies constituted 1/3 (33.33%) of the MDNeg and (2/3) 66.67% of the Mem subsets. These data suggest that a proportion of AQP4-specific B cells also display broader reactivities against common antigens.

Subset	mAb ID	Insulin	LPS	dsDNA	Hep2	Polyreactive	Autoreactive
New emigrant	DR11 C6	-	-	-	-	No	No
	DR11 C8	++	++	++	++	Yes	Yes
	DR11 C11	-	+	+	-	No	No
	DR26 B5	-	-	-	+	No	Yes
	DR26 B8	NT	NT	NT	NT	NT	NT
	DR20 B5	-	-	-	+	No	Yes
	DR20 B7	-	+	-	+++	No	Yes
	DR20 C3	-	-	-	+++	No	Yes
	DR20 C4	-	+	-	+	No	Yes
	DR20 D5	-	-	-	+++	No	Yes
	DR30 C7	++	++	++	++	Yes	Yes
Mature naïve	DR11 B4	-	-	-	-	No	No
	DR26 B3	-	-	-	-	No	No
	DR20 B3	-	-	-	-	No	No
	DR30 C2	-	-	-	-	No	No
	DR30 C3	-	-	-	-	No	No
	DR30 C4	+++	+++	+++	++	Yes	Yes
Unmutated double-negative	DR30 C10	NT	NT	NT	NT	NT	NT
	DR30 C11	-	-	-	-	No	No
Mutated double-negative	DR11 D3	-	+	-	+	No	Yes
	DR14 E11	-	-	-	-	No	No
	DR14 F2	-	-	-	-	No	No
Memory	DR11 D4	++	++	++	+++	Yes	Yes
	DR14 F4	-	-	-	-	No	No
	DR20 D10	+	++	+	++	Yes	Yes

Table 4.4. Summary of poly and autoreactivity assay results.

Some mAbs were not tested (NT) due to inadequate sample volumes. Binding to test antigens was indicated as: (-) negative; (+) OD value 0.5-1; (++) OD value 1-2; (+++) OD value 2-4 as measured at 405 nm wavelength.

4.4.11 Frequencies of poly and autoreactive early B cells in NMOSD patients and healthy individuals

Most newly generated antibodies are poly and autoreactive, but a huge proportion of these are removed from the repertoire by rigorous controls of self-reactivity in the central and peripheral B cell tolerance checkpoints. Data from 15 healthy donors provided by Dr Kevin O'Connor showed that, on average, 7.38% of new emigrant and 8.84% of mature naïve B cells were polyreactive (Figure 4.13). In contrast, polyreactive B cells constituted 2/10 (20.00%) of the new emigrant 1/6 (16.67%) of the mature naïve populations obtained from the 4 NMOSD patients in our study. However, one should note that we only screened for poly and

autoreactivity among the AQP4-specific B cell populations, not the whole B cell repertoire of the patients. Nonetheless, this observation may suggest that there is a compromised in the central and, to a lesser degree, the peripheral tolerance checkpoints in NMOSD.

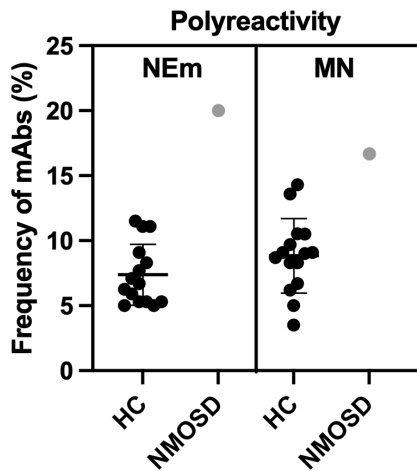


Figure 4.13. The frequency of polyreactive mAbs generated from B cells from healthy donors and NMOSD patients.

Each dot in the healthy donor group represents an average frequency per individual donor whereas the dot in the NMOSD group is a pooled frequency from 4 patients in this study. Data are presented as mean $\pm$ SEM. HC, Healthy control; MN, Mature Naïve; NEm, New Emigrant; NMOSD, Neuromyelitis Optica Spectrum Disorder.

4.4.12 Generation of unmutated common ancestors (UCAs)

In total, 25 AQP4-specific antibodies were identified from 4 individuals with AQP4-IgG positive NMOSD: 11 from new emigrant, 6 from mature naïve, 5 from double-negative, and 3 from memory B cells. Of these, 3 double-negative, and 3 memory mAbs were shown to have high mutation loads across their immunoglobulin heavy and light genes. To study the precursors of these mutated mAbs, we reverted the mutated BCR sequences to their germline counterparts by replacing any point mutations with nucleotides as annotated in the IMGT database. This was carried out with an assumption that the mutated BCRs once had germline conformations and they underwent somatic hypermutations in active germinal centres. The reversion strategy is illustrated in Figure 4.14.

During V(D)J recombination, extra nucleotides (N) were randomly added by terminal deoxynucleotidyl transferase (TdT) between the VH-DH, DH-JH and VL-JL segments in the

heavy and light chain genes, respectively, to increase CDR3 diversity. As these insertions are not encoded by the germline genes, the N regions were preserved. The resulting unmutated common ancestors (UCAs), also referred to as revertant mAbs, were named after their parent mAbs with a letter R added at the end, e.g., a revertant of parent mAb DR11 D4 would be assigned as DR11 D4R (Chapter IV, Subsection 4.3.2).

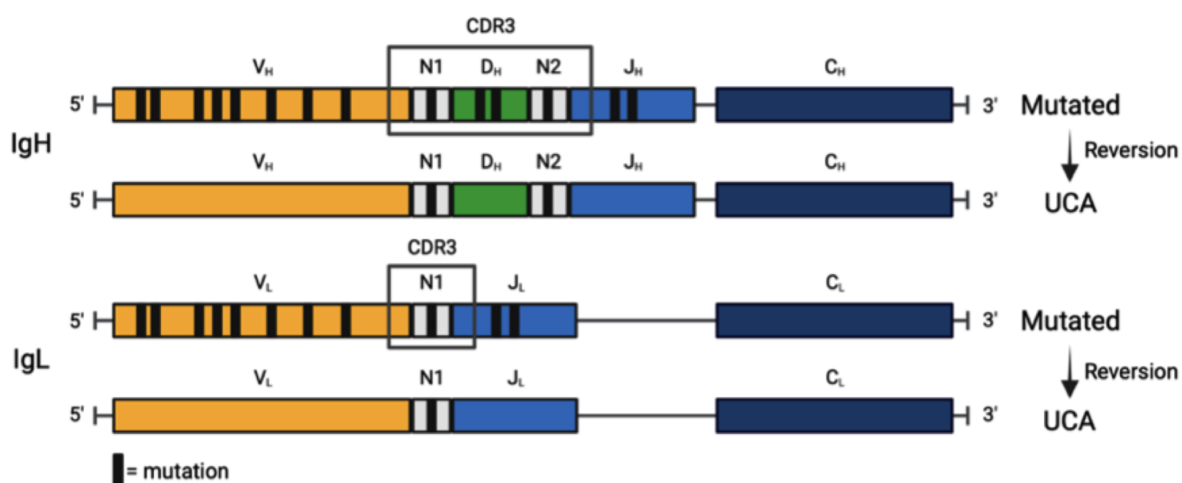


Figure 4.14. Generation of unmutated common ancestors (UCAs).

Closely aligned germline precursors were reconstructed by reverting mutations across the immunoglobulin genes of the parent mAbs. During V(D)J recombination, N nucleotides are randomly inserted at the junctions between the V_H-D_H and D_H-J_H segments in the rearranged heavy chain gene and the V_L-J_L segment in the rearranged light chain gene. These N regions were not reverted as they are not encoded by the germline immunoglobulin genes.

As the UCAs were generated experimentally and may or may not naturally exist in vivo, we assessed whether the nucleotide reversions would yield successful expression of antibodies. A total of 6 UCAs from 3 double-negative (DR11 D3R, DR14 E11R, DR14 F2R) and 3 memory (DR11 D4R, DR14 F4R, DR20 D10R) parent mAbs were cloned into both IgM and IgG1 expression vectors. These UCAs were loaded into 4-12% Bis-Tris SDS gels under denaturing-reducing conditions and stained with Coomassie Blue (Figure 4.15).

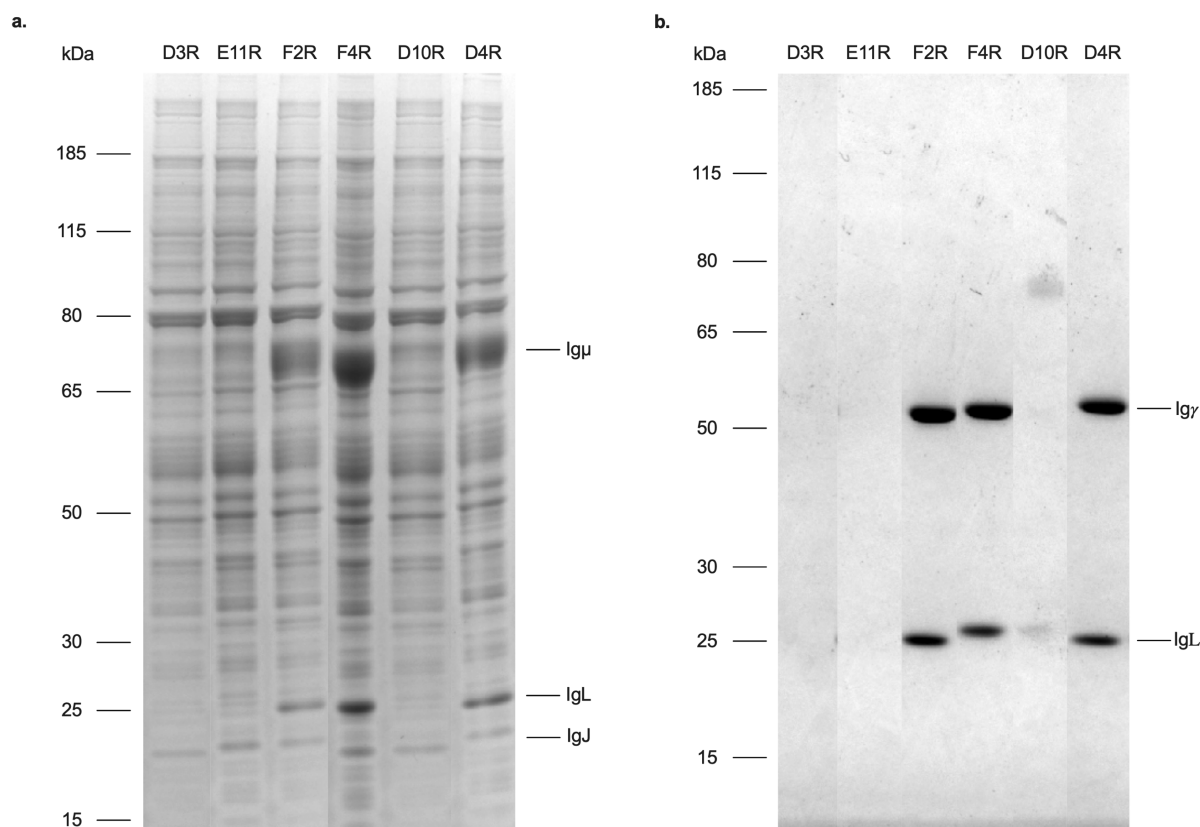


Figure 4.15. Expression of experimentally generated UCAs.

A total of 6 UCAs comprising 3 double-negative and 3 memory mAbs were reconstructed from their mutated parent mAbs. Expression of UCAs as IgM (**a**) and IgG1 (**b**) was evaluated by SDS-PAGE followed by Coomassie Blue staining. Reversion of mutations in some UCAs had an impact on the secretion of functional antibodies (lanes DR11 D3R, DR14 E11R, and DR20 D10R).

Only 3/6 UCAs expressed as IgM (Figure 4.15a) and IgG1 (Figure 4.15b) antibodies. No Igμ, Igγ, and IgL were detected in those which failed to express (Figure 4.15a and b; lanes DR11 D3R, DR14 E11R, and DR20 D10R). As the production of the IgJ chain was unaffected, this suggests that changes in the heavy and light chain domains of some UCAs alter their structural conformations to a particular degree that it interferes with the ability of cells to secrete functional antibody molecules.

4.4.13 Evaluation of AQP4 specificity of UCAs by live CBA

To explore whether the naïve precursors of mutated AQP4-specific B cells display reactivity to AQP4, the CBA endpoint titre and K_D value of the UCAs as multimeric IgM molecules were characterised. All UCA IgMs were shown to retain binding to AQP4 with

varying degrees of affinity (Figure 16a-c). By comparison to their parent IgMs, DR11 D4R and DR14 F2R had lower endpoint titre and higher K_D values. The binding parameters of DR14 F4R were relatively unaffected by somatic hypermutations. Collectively, somatic hypermutations are not necessary for AQP4 binding, and in most cases, it enhances the affinity of BCRs toward AQP4.

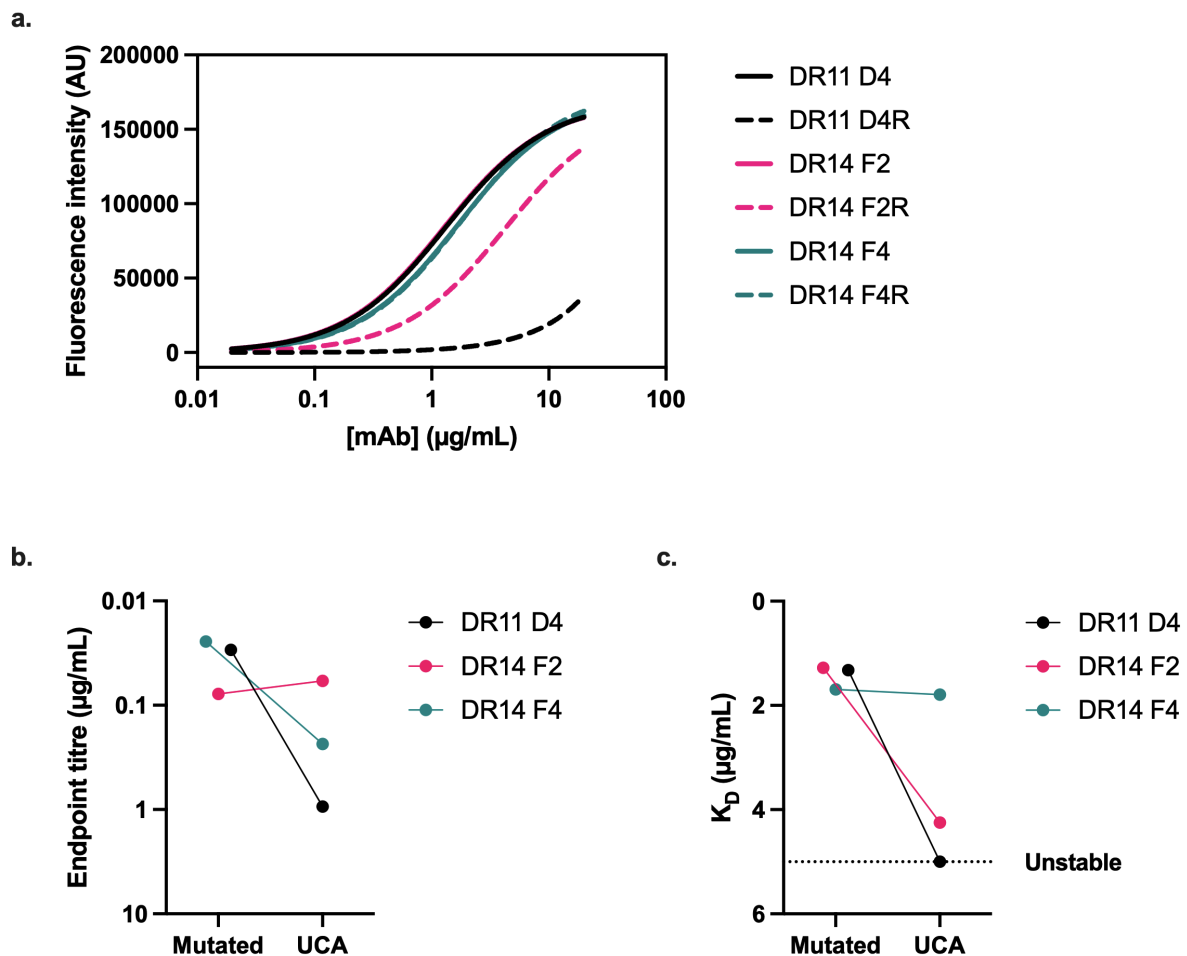


Figure 4.16. Unmutated common ancestors of mature AQP4-specific B cells retain AQP4 reactivities. UCAs were tested for AQP4 binding as IgM multimers. Binding curves (a), CBA endpoint titres (b), and estimated K_D values (c) are shown.

4.4.14 Evaluation of poly and autoreactive properties of UCAs

We have shown that a proportion of mutated AQP4-specific B cells exhibited broader reactivity against multiple common antigens, i.e., insulin, LPS, dsDNA, and Hep2 cell lysate. To explore whether these reactivities are modulated by somatic mutations, we tested the

generated UCAs for poly and autoreactivity assays. By comparison to their parent mAbs, the UCAs displayed higher levels of poly and autoreactivity (Figure 4.17). These properties were abrogated by somatic hypermutations as exemplified by DR14 F2R and DR14 F4R. In contrast, acquisition of mutations along the immunoglobulin genes had less impact, if any, on the poly and autoreactivity of the precursor mAb DR11 D4R. We conclude that somatic hypermutations have heterogenous effects on the innate poly and autoreactivity profiles of AQP4-specific B cell precursors.

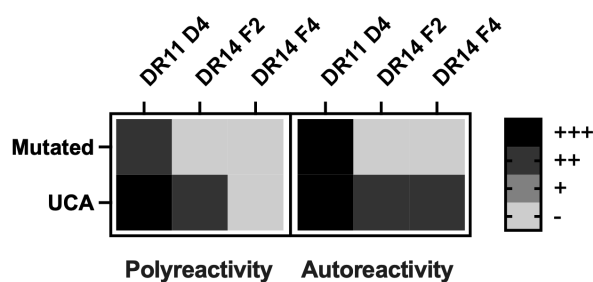


Figure 4.17. Poly and autoreactivity characteristic of mutated AQP4-specific mAbs and their precursors.

Degrees of poly and autoreactivity were evaluated by ELISA and scored as previously described: (-) negative; (+) OD value 0.5-1; (++) OD value 1-2; (+++) OD value 2-4. UCA, Unmutated Common Ancestor.

4.5 Summary

Characterisation of binding affinity of AQP4-specific monoclonal antibodies generated from distinct B cell lineages has provided a new perspective on the developmental profiles of AQP4 specificity in NMOSD. Unlike monomeric IgG1, production of multimeric IgM molecules poses a challenge due to their complex protein structure. Transient co-expression of IgM heavy, light, and joining chains in HEK293T cells followed by precipitation of secreted antibodies with saturated ammonium sulphate was shown to be a rapid and efficient method for the enrichment of IgM multimers. Interestingly, the native, unmutated mAbs only showed detectable binding to AQP4 on live CBA as IgM, but not IgG1 antibodies, whereas the hypermutated mAbs bound AQP4 as both antibody isotypes. Detailed characterisation of

AQP4-specific mAbs through determination of CBA endpoint titre and equilibrium dissociation constant (K_D) value further confirmed a gradual evolution of binding affinity across subsets, consistent with B cell maturation stages. Additionally, a proportion of mAbs exhibited autoantigen reactivities beyond AQP4, termed poly and/or autoreactivity. Higher frequencies of polyreactive new emigrant and mature naïve B cells were found in NMOSD patients than healthy individuals, supporting the overarching theme of defective B cell tolerance checkpoints as a contributing factor to NMOSD pathogenesis. To study the origin of mutated AQP4-specific mAbs, we reconstructed unmutated common ancestors (UCAs) from the most closely aligned germline immunoglobulin genes of the respective parent mAbs. It should be noted that the reversion process may have an impact on the secretion of functional antibodies. Upon reversion to UCAs, specificity to AQP4 was retained, albeit in reducing affinity. This substantiates our earlier findings that somatic hypermutations enhance, but is not necessary for, AQP4 binding. Furthermore, somatic hypermutation was also demonstrated to modulate the poly and/or autoreactivity of naïve AQP4-specific B cell precursors. Taken together, in NMOSD, the precise maturation of AQP4-specific B cells in the presence of impaired B cell tolerance checkpoints gives rise to high-affinity AQP4 specificities which may then contribute to autoimmunity in NMOSD.

5 Chapter V: *In Vitro* Functional Characterisation of NMOSD Patient-derived AQP4-specific Monoclonal Antibodies

5.1 Introduction

CNS inflammation and astrocytopathy in NMOSD is thought to be mediated by AQP4-specific antibody of the IgG class. The direct pathogenicity of AQP4-specific IgG has been consistently shown through cellular^{41,43} and rodent studies^{48,49,52,90}. Binding of the IgG to AQP4 expressed on the membrane of target cells initiates several molecular cascades, including complement-dependent cytotoxicity (CDC)^{41,43} and antibody-dependent cell-mediated cytotoxicity (ADCC)^{52,90}. Deposition of complement activation products in NMOSD lesions^{39,40} and the apparent effects of eculizumab⁹¹, a therapeutic humanised monoclonal IgG against the complement protein C5, in improving clinical profiles of affected patients suggests the pivotal role of CDC as a disease mechanism.

In NMOSD, the pathogenicity of AQP4-IgM remains elusive. Serum AQP4-specific IgM antibodies are often detected during clinical relapse⁷⁷, indicating ongoing germinal centre activity and potential disease relevance. In general, despite similarities through which IgM and IgG interact with C1q, the first component of the classical complement pathway, they do not activate complement to the same extent. IgM has been consistently shown to be a more potent complement activator than IgG^{74,75,92,93}.

Given the above evidence, we hypothesised that AQP4-IgM antibodies can activate CDC and induce lysis of cells bearing the target antigen. Additionally, in parallel with higher binding affinity, their maturation endows them with greater pathogenic potential. To prove this, we measured the levels of cytotoxic activity of the generated AQP4-IgM mAbs and characterised their evolution across B cell lineages.

5.2 Specific study aims

The work discussed in this chapter aimed to:

1. Develop an in vitro complement-dependent cytotoxicity (CDC) assay;
2. Characterise in vitro CDC of AQP4-IgMs from different B cell subsets; and

3. Compare in vitro CDC of somatically hypermutated AQP4-IgMs and their respective UCAs.

5.3 Methods

5.3.1 *Immunostaining of C5b9 deposition on cell membrane*

HEK293T cells were seeded on PLL-coated coverslips in a 6-well plate at 900,000 cells/well in 2 mL DMEM supplemented with 10% FBS and 1% AA and grown overnight at 37°C and 5% CO₂. The following day, cells were transfected with AQP4-M23-EGFP plasmid DNA and PEI (see Chapter II, Subsection 2.2.2.1 and 2.2.2.5 for HEK293T cell seeding and transfection methods, respectively). After 24 hours, coverslips were transferred to a 24-well plate and incubated with 20 µg/mL AQP4- or isotype-control IgM in 250 µL blocking buffer (DMEM, 20 mM HEPES, 1% BSA) for 30 min at 37°C. Cells were washed twice with PBS, exposed to 10% human complement in 250 µL blocking buffer for 30 min at RT to decelerate the lytic process, immediately washed with PBS twice, and fixed with 4% PFA for 10 min at RT. To label C5b9 complex deposition on cell membrane, cells were sequentially washed twice with PBS, incubated with a rabbit polyclonal anti-human C5b9 diluted 1:500 in blocking buffer for 45 min at RT, washed twice with PBS, and labelled with a goat anti-rabbit, Alexa Fluor 594 secondary antibody (1:750) for 45 min at RT. Cells were washed twice with PBS before mounting on a glass slide in fluorescence mounting medium supplemented with DAPI (1:1000) and examined under a fluorescence microscope.

5.3.2 *In vitro complement-dependent cytotoxicity (CDC) assay*

Adherent HEK293S cells stably expressing AQP4-M23 were seeded at 20,000 cells/well in a PLL-coated, flat bottom, 96-well plates in 100 µL DMEM supplemented with 10% FBS and 200 µg/mL geneticin. The following day, cells were washed twice with PBS and incubated with 1:2 dilutions of heat-inactivated patient sera starting from 1:20 or AQP4-

specific mAbs (0.04-20 µg/mL) in the presence of 10% human complement (v/v) in 100 µL phenol red-free DMEM for 24 hours. After two rounds of washing with PBS, cells were incubated with 20% Alamar Blue in 100 µL phenol red-free DMEM for 1 hour at 37°C and 5% CO₂. Fluorescence intensities (FI) of the active metabolite, or resorufin, were recorded with a microplate reader at 560/590 nm excitation/emission wavelengths as a measure of cell viability which is inversely proportional to antibody cytotoxicity. Fi values were plotted as a function of serum or mAb concentration and fitted to a sigmoidal, four-parameter logistic (4PL) regression model to determine half maximal effective concentration (EC₅₀) and maximum cytotoxicity level (B_{max}) (see Chapter II, Subsection 2.2.6.1-3 for the detailed methods).

5.4 Results

5.4.1 *Evaluation of C5b9 deposition on AQP4 M23-expressing HEK293T cells*

The capacity of AQP4-IgG in initiating the classical complement pathway upon binding to AQP4 on the surface membrane has been extensively studied. To investigate the pathogenicity of the IgM counterpart, we first evaluated the deposition of membrane attack complexes (MACs) or C5b9 on cell membranes of HEK293T cells expressing AQP4-M23-EGFP by indirect immunofluorescence. In the presence of 20 µg/mL of AQP4-IgMs and 10% human complement, C5b9 was clearly visualised on the membrane of target cells and absent in cells incubated with the same concentration of an isotype control antibody and human complement. These results suggest that AQP4-IgMs are able to induce complement deposition through activation of the classical complement pathway.

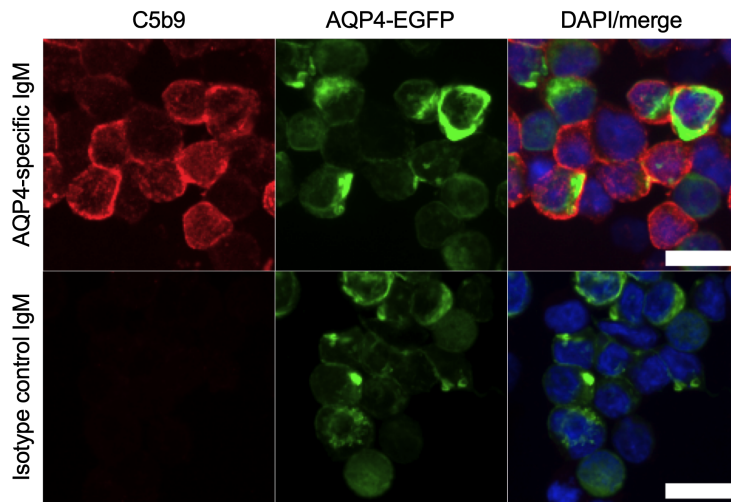


Figure 5.1. Representative images of complement deposition induced by AQP4-IgM antibodies. AQP4 M23-expressing HEK293T cells were incubated with 20 $\mu\text{g}/\text{mL}$ of either an AQP4-specific or an isotype control IgM in the presence of 10% human complement. Deposition of terminal complement complexes was visualised with an anti-human C5b9 antibody. 40x magnification, scale bar=20 μm . AQP4, Aquaporin-4; DAPI, 4'6-diamidino-2-phenylindole.

5.4.2 Generation of AQP4 M23-stable cell line

HEK293S cells stably expressing AQP4-M23 were used as a substrate for subsequent CDC assays. Cells were transfected with an untagged AQP4-M23 plasmid containing a geneticin resistance gene cassette. Stable expression over generations was achieved by growing cells under a selective pressure with 200 $\mu\text{g}/\text{mL}$ geneticin and sorting x2 by flow cytometry the brightest 10% of cells. Upon revival from a cryopreserved state, the cell line was evaluated for their surface AQP4 expression by live CBA using AQP4+ serum samples from NMOSD patients. Transiently transfected HEK293T cells that were used as a substrate for routine diagnostics assays were used as a positive control. We confirmed the expression of AQP4 on the surface membrane of stably-transfected HEK293S cells (Figure 5.2).

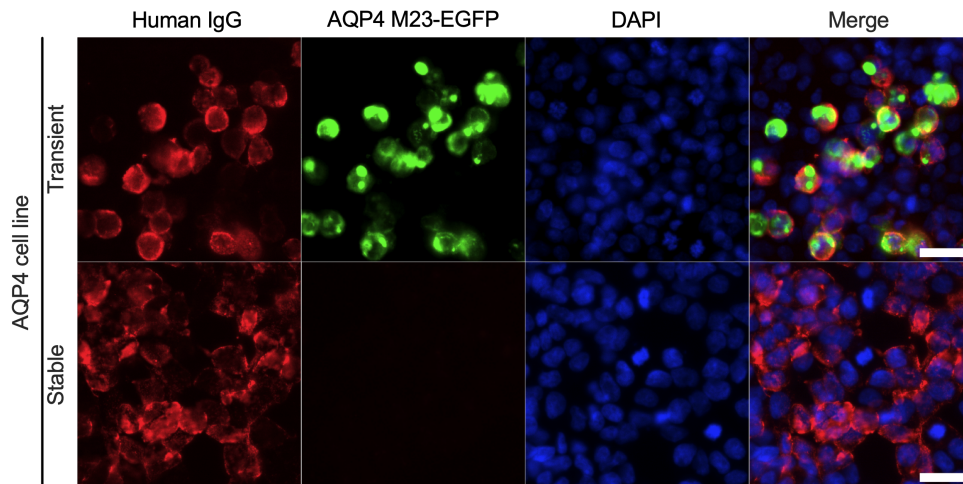


Figure 5.2. Representative images of AQP4 M23-expressing cell lines.

The AQP4 surface membrane expression of the revived HEK293S stable line was evaluated by live CBA. Transiently transfected HEK293T cells from routine diagnostics assays were used as a positive control. Cells were incubated with the same AQP4+ serum samples from NMOSD patients and stained with an anti-human IgG secondary antibody. 40x magnification, scale bar=20 μ m. AQP4, Aquaporin-4.

5.4.3 Validation of AQP4 M23-stable cell line

To further assess the AQP4 expression of the stable cell line, we performed live CBA and determined the endpoint titres of 40 AQP4+ serum samples. The results were then compared to those obtained using the transient cell line as a substrate. There was no detectable difference in serum CBA endpoint titres tested on the two cell lines ($p > 0.05$, Wilcoxon test) (Figure 5.3). Furthermore, the observed titres correlated strongly with one another (Spearman $r = 0.9535$, $p < 0.0001$). We validated the AQP4 expression of the stable AQP4-M23 cell line and concluded that they were suitable for downstream CDC assays.

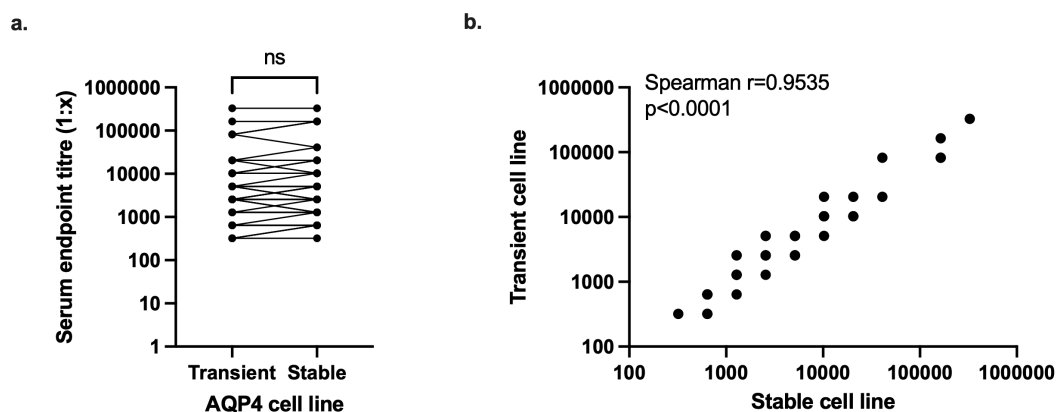


Figure 5.3. Serum CBA endpoint titres on transient and stable AQP4 cell lines.

a. Endpoint titres of 40 AQP4+ serum samples as determined by live CBA using the transient and stable AQP4 cell lines as a substrate. Dots and lines represent paired serum samples. Some samples are not visible due to overlaps (Wilcoxon tets, $p=0.1521$); **b.** The observed endpoint titres on the transient and stable cell line correlated strongly with one another (Spearman $r=0.9535$, $p<0.0001$). AQP4, Aquaporin-4; ns, not significant.

5.4.4 Optimisation of in vitro complement-dependent cytotoxicity assay (CDC)

To optimise the experimental conditions of in vitro CDC assays, AQP4 M23-stable cells were incubated with AQP4+ serum samples ($n=2$) diluted 1:20. These samples were known to have high endpoint titres (1:163,840) as evaluated by live CBA. Cells treated with the same dilution of healthy control serum samples ($n=2$) were used as negative controls. Incubation with sera was performed for 24 hours in the presence of varying complement concentrations (0, 2.5, 5, 10, 20, 40%). Cell viability post-treatment was assessed with Alamar Blue. The fluorescence intensities of resorufin, the product of the active ingredient resazurin were recorded using a microplate reader to calculate degrees of cytotoxicity.

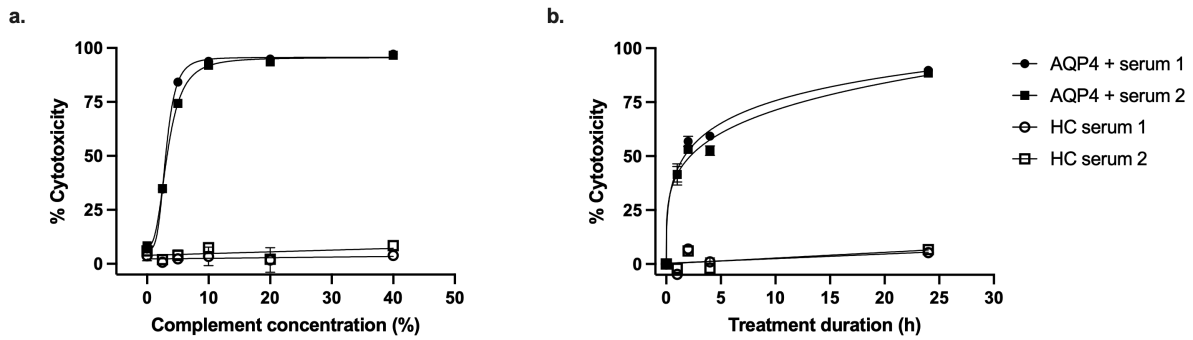


Figure 5.4. Complement-dependent cytotoxicity of AQP4 serum samples as a function of complement concentration and treatment duration.

a. AQP4 M23-stable cells were incubated for 24 hours with a fixed concentration (5%) of either AQP4+ or healthy control serum sample in the presence of varying concentrations of complement (0, 2.5, 5, 10, 20, 40%); **b.** At a fixed concentration of serum sample (5%) and complement (10%), CDC assay was performed at a range of treatment durations. Sigmoidal, four-parameter logistic (4PL) regression model, Mean $\pm$ SEM.

The degree of cytotoxicity of AQP4+ serum samples was shown to be dependent on the abundance of complement in the solution and reached a maximum effect starting from 10% complement concentration (Figure 5.4a). At a saturating condition (10% complement), the percentage of cytotoxicity was reduced with shorter treatment durations (0, 1, 2, 4, 12 hours) (Figure 5.4b).

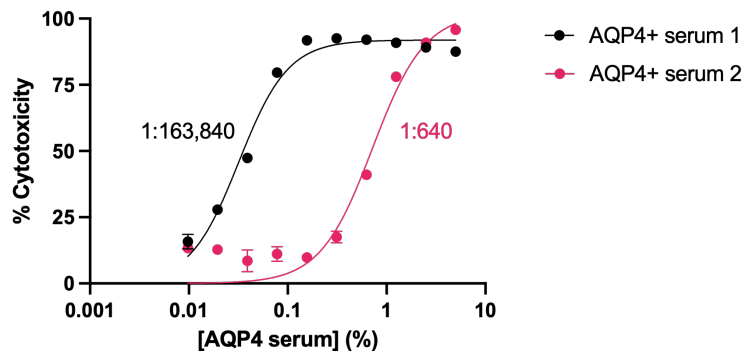


Figure 5.5. Cytotoxicity curves of AQP4+ serum samples.

AQP4 M23-stable cells were incubated with serial dilutions of two AQP4+ serum samples with contrasting CBA endpoint titres as indicated. Using the optimum experimental conditions, the *in vitro* CDC assay was able to discriminate the degree of cytotoxicity between the two samples. Sigmoidal, four-parameter logistic (4PL) regression model, Mean $\pm$ SEM.

Supplementation with 10% complement and incubation for 24 hours were found to be the optimum conditions for CDC assays. To evaluate the assay sensitivity and dynamic range, cells were incubated with serial dilutions of two AQP4+ serum samples with contrasting CBA

endpoint titres (serum 1 = 1:1,163,840, serum 2 = 1:640). Each sample was tested in duplicates at a starting concentration of 5%. The assay conditions were shown to be able to discriminate cytotoxic activity of the two samples. Serum 1 was demonstrated to activate CDC at a lower concentration and hence, was a more potent complement activator than serum 2 (Figure 5-5).

5.4.5 Characterisation of AQP4-specific mAbs cytotoxic capacity

Using the optimised in vitro CDC assay, we sought to characterise the cytotoxicity profiles of the AQP4-IgMs. A non-reactive mAb DR11 E9 was used as an isotype control. The percentage of cytotoxicity for each mAb was calculated and curve-fitted to the sigmoidal, four-parameter logistic (4PL) regression model. Individual dose-response curves and a summary of the estimated parameters are shown in Figure 5.7 and Table 5.1. EC50 indicates the concentration of mAb needed to cause 50% lysis in the presence of 10% complement whereas Bmax denotes the maximum cytotoxicity observed at a saturating mAb concentration.

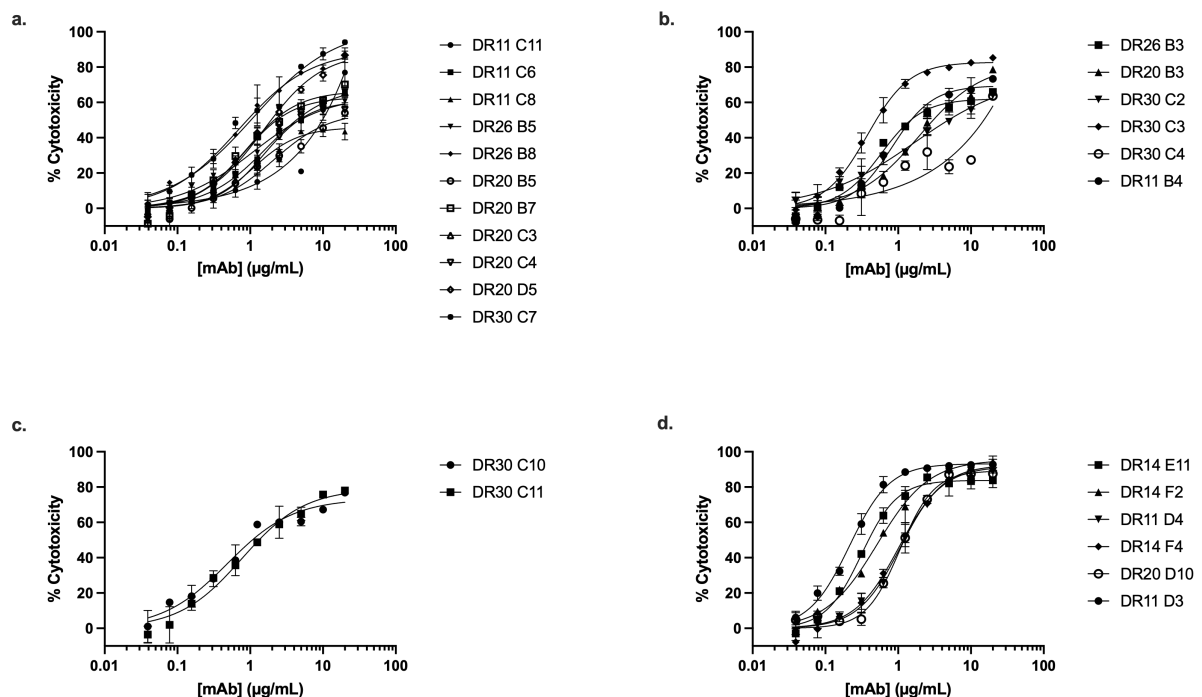


Figure 5.6. Dose-response curves of AQP4-IgMs.

Percentage cytotoxicity values of the new emigrant (a), mature naïve (b), unmutated double-negative (c), mutated double-negative and memory (d) mAbs were plotted and curve-fitted to the sigmoidal, four-parameter logistic (4PL) regression model. Data are presented as mean±SEM.

Subset	mAb ID	EC50 ($\mu\text{g/mL}$)	SEM ($\mu\text{g/mL}$)	Bmax (% cytotoxicity)	SEM (% cytotoxicity)
New emigrant	DR11 C6	1.706	0.3639	68.98	5.004
	DR11 C8	1.105	0.1741	45.91	2.54
	DR11 C11	Unstable	Unstable	Unstable	Unstable
	DR26 B5	1.1	0.2165	64.06	3.64
	DR26 B8	0.6998	0.1041	89.61	3.677
	DR20 B5	2.094	0.9425	54.71	9.028
	DR20 B7	0.8791	0.1516	66.49	3.713
	DR20 C3	0.8211	0.09935	63.05	2.466
	DR20 C4	1.534	0.2538	60.92	3.767
	DR20 D5	1.515	0.2284	88.41	4.502
Mature naïve	DR30 C7	1.101	0.2945	102.8	7.474
	DR11 B4	0.826	0.1051	69.57	3.03
	DR26 B3	0.5847	0.09415	62.07	3.177
	DR20 B3	1.943	0.4226	82.01	6.08
	DR30 C2	1.695	0.6406	74.88	7.314
	DR30 C3	0.3763	0.02706	82.8	1.747
Unmutated double- negative	DR30 C4	Unstable	Unstable	Unstable	Unstable
	DR30 C10	0.4861	0.09925	73.34	4.069
Mutated double- negative	DR30 C11	0.7698	0.1298	79.06	3.925
	DR11 D3	0.2138	0.009437	93.21	1.126
	DR14 E11	0.3142	0.01898	83.84	1.518
Memory	DR14 F2	0.5167	0.03762	95.44	2.034
	DR11 D4	1.085	0.0747	92.51	2.275
	DR14 F4	1.023	0.09184	91.77	2.876
	DR20 D10	1.06	0.05712	89.2	1.82

Table 5.1. A summary of dose-response parameters of AQP4-IgMs.

EC50 indicates the effective concentration of mAb required to cause 50% lysis of AQP4-expressing cells in the presence of 10% complement. Bmax denotes the maximum percentage of cytotoxicity at a saturating mAb concentration. The cytotoxicity parameters of some mAbs could not be adequately estimated using the selected model and were defined as unstable as the data did not define a complete dose-response curve.

The cytotoxicity parameters of some IgM mAbs could not be adequately estimated as the data did not define a complete dose-response curve. For those demonstrating a complete curve, the best-fit values were compared among subsets and analysed. There was a gradual increase in cytotoxicity levels from the new emigrant to the mutated double-negative subsets, followed by a slight reduction in the memory subset (median EC50, $\mu\text{g/mL}$, NEm=1.10, MN=0.83, UDNeg=0.63, MDNeg=0.31, Mem=1.06) (Figure 5.8a). At a saturating IgM concentration, most NEm, MN, and UDNeg IgMs induced an incomplete cell lysis whereas

the MDNeg and Mem mAbs were able to achieve a maximum response of approximately 100% lysis (median Bmax, % cytotoxicity, NEm=65.28, MN=74.88, UDNeg=76.20, MDNeg=93.21, Mem=91.77) (Figure 5.8b).

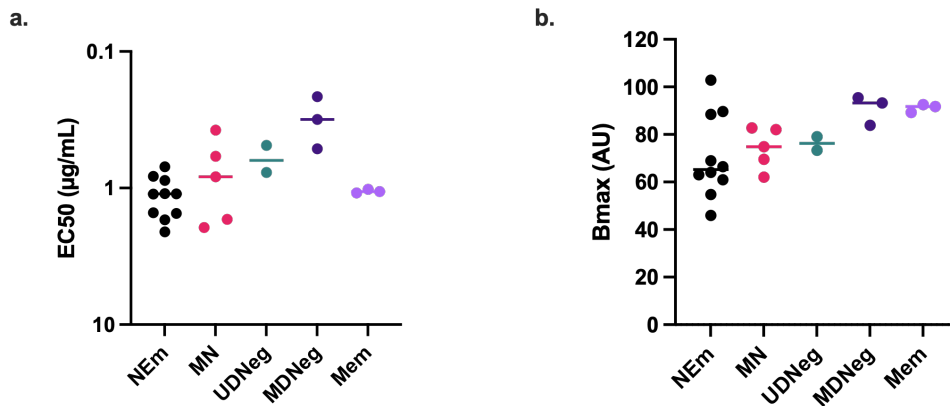


Figure 5.7. Cytotoxicity parameters of AQP4-IgMs across subsets

a. There was a trend of increasing degrees of cytotoxicity between the new emigrant and mutated double-negative subsets, followed by a sudden drop in the memory mAb subset; **b.** Most of the unmutated IgM mAbs (NEm, MN, and UDNeg) induced an incomplete cell lysis whereas the mutated IgM mAbs demonstrated a total lysis at a saturating IgM concentration. Horizontal bars indicate median values. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

To increase statistical power, average fluorescence intensities of member mAbs within individual subsets were pooled to generate global dose-response curves. Comparisons among subsets were performed using the extra sum-of-squares F test. Consistent with the above observation, the MDNeg had the lowest CDC EC50 value and therefore, was the most potent complement activator of all subsets (EC50, µg/mL, NEm=1.43, MN=0.83, UDNeg=0.61, MDNeg=0.32, Mem=1.06) (Figure 5.9a and b). The cytotoxicity levels of the NEm, MN, and Mem subsets were not significantly different from one another.

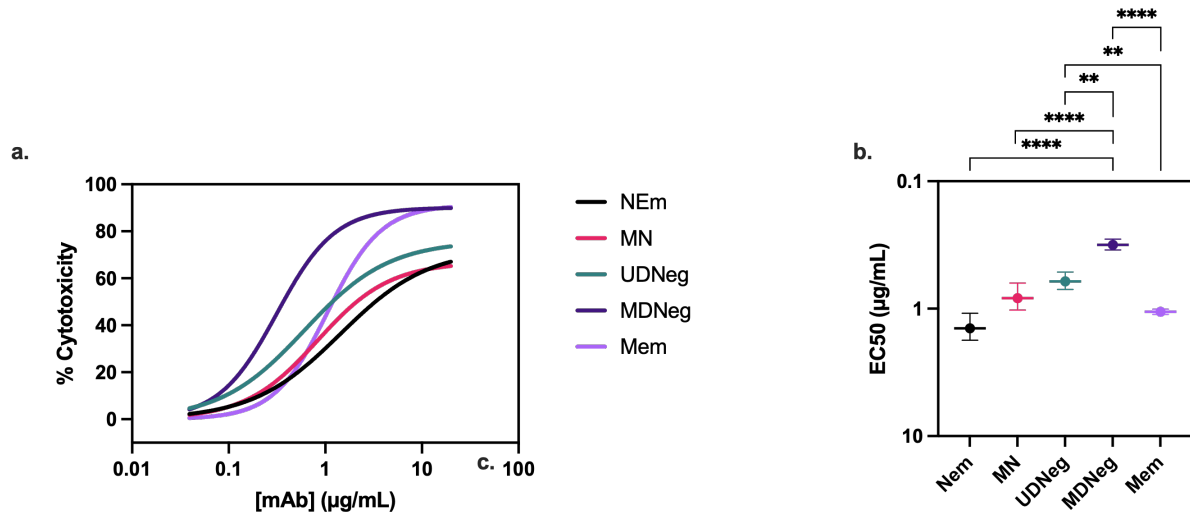


Figure 5.8. Global dose-response curves of AQP4-IgMs across subsets.

a. Average fluorescence intensities of member mAbs within individual subsets were treated as replicates to generate global dose-response curves. Data were fitted to the sigmoidal, four-parameter logistic (4PL) regression model; b. The estimated CDC EC50 values of mAbs subsets are shown. The MDNeg subset was demonstrated to have the most potent cytotoxic activity. Comparison of Fits, Mean±SEM, $*=p<0.05$, $**=p<0.005$, $***=p<0.0005$, $****=p<0.0001$. NEm, new emigrant; MN, mature naïve; UDNeg, unmutated double-negative; MDNeg, mutated double-negative; Mem, memory.

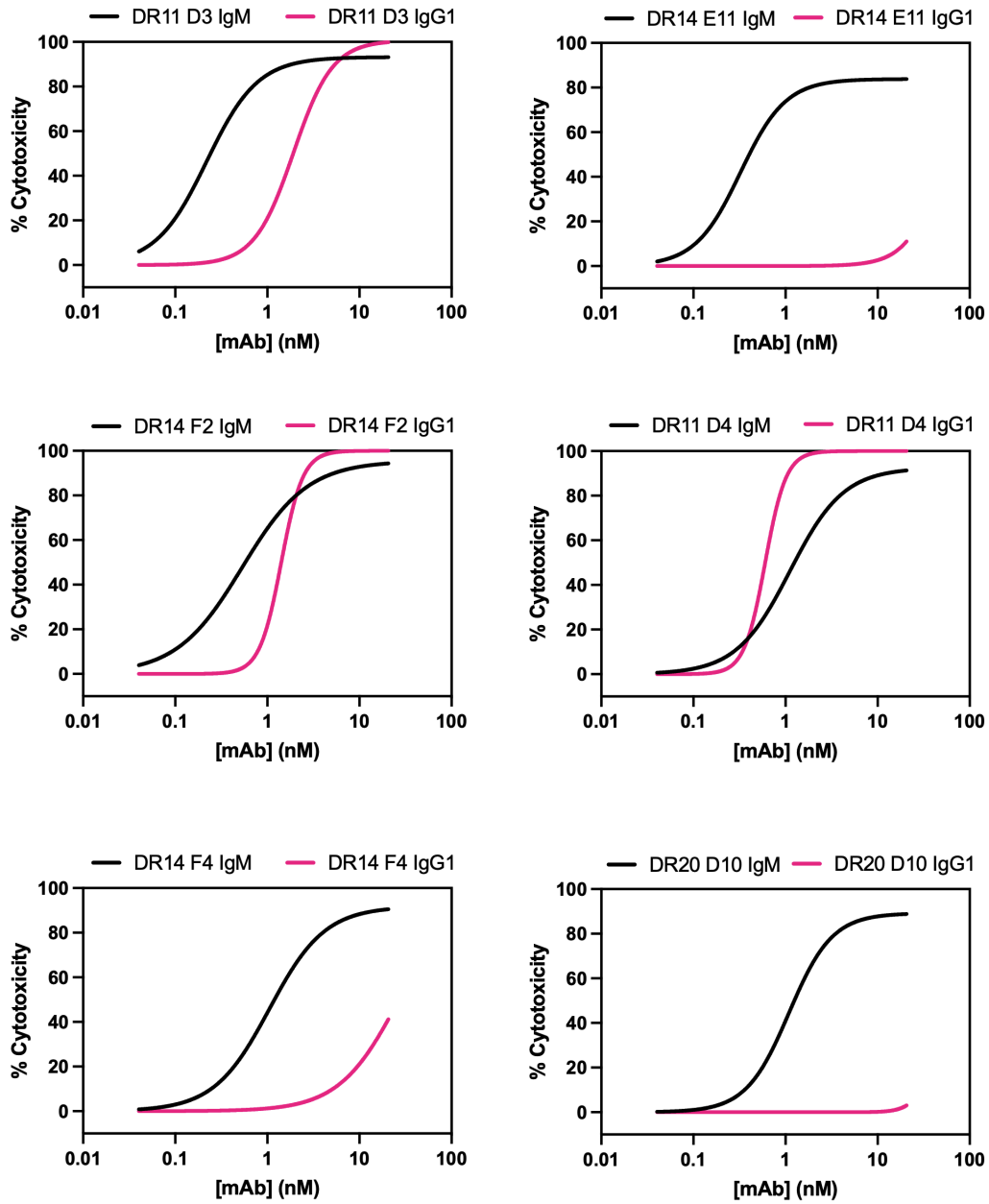
Taken together, AQP4-specific mAbs exhibited discrete levels of CDC activity. Unlike binding affinity, the relative potencies of complement activation across mAbs subsets were more variable and less predictable in relation with B cell development stages. The relatively comparable CDC activity of the Mem with the NEm and MN mAbs was surprising, considering that the former was derived from the most mature B cell subset of all and their immunoglobulin heavy and light chain genes were somatically hypermutated (Chapter III, Subsection 3.4.8). One would expect that the Mem mAbs would be the most potent complement activator. However, it should be noted that we adopted an experimental design whereby all mAbs were tested as a single antibody isotype, i.e., IgM, for comparison purposes. This was due to the exclusive binding of the unmutated mAbs (NEm, MN, and UDNeg) to AQP4-M23 as IgM (Chapter IV, Subsection 4.4.4). Consequently, having an IgG molecule in an IgM backbone may impede the mutated (MDNeg and Mem) mAbs' natural cytotoxicity and possibly explain these findings. Additionally, an interesting observation with regard to maximum response (Bmax) between the unmutated and mutated mAbs was noted whereby the former elicited an

incomplete cell lysis even at a saturating mAb concentration (Figure 5.9a). This phenomenon, however, should be interpreted with caution as measurement of Bmax values is dependent on the experimental setup.

5.4.6 Evaluation of cytotoxic capacity of AQP4-specific mAbs as IgG1 versus IgM

We found that the memory mAbs displayed relatively low CDC activities (see Subsection 5.4.5 of this chapter) which could be due to their artificial expression as an IgM rather than IgG molecule. Motivated by these findings, we sought to specifically explore the differential CDC activity of AQP4-IgM and IgG using a set of matched, mutated mAbs comprising 3 MDNeg (DR11 D3, DR14 E11, DR14 F2) and 3 Mem (DR11 D4, DR14 F4, DR20 D10) mAbs. As we recall from our previous observations (Chapter IV, Subsection 4.4.4), these mAbs demonstrated binding to AQP4 as both antibody isotypes. The dose-response curves of each mAb as IgG and IgM are presented in Figure 5.9a.

a.



b.

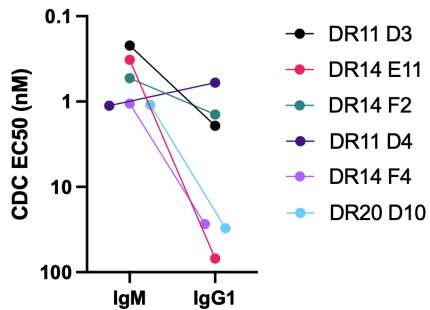


Figure 5.9. Differential CDC activity of AQP4-specific mAbs as IgM and IgG1.

a. A set of matched, mutated mAbs were evaluated for their efficiencies in activating the classical complement cascade as IgM and IgG1. Dose-response curves of individual mAbs are shown; **b.** A summary of CDC EC50 values of AQP4-specific mAbs as IgM and IgG1. CDC, Complement-dependent Cytotoxicity; UCA, Unmutated Common Ancestor.

On a per molecule basis, the effector functions of 5/6 mutated AQP4-specific mAbs were reduced when tested as monomeric IgG1 compared to multimeric IgM antibodies. The inverse effect was noted for memory mAb DR11 D4 whereby its efficiency in activating the complement cascade was improved on an IgG1 versus IgM backbone (EC50, nM, 0.60 vs 1.11) (Figure 5.9a and b). Whilst these results are in line with current literature, the artificial expression of the memory mAbs as IgM rather than IgG1 may not fully explain our previous findings with regard to the relatively comparable CDC activities of the memory to the unmutated AQP4-specific mAb subsets.

5.4.7 Evaluation of cytotoxic capacity of UCAs

In the preceding chapter (Chapter IV, Subsection 4.4.13), we reported that somatic hypermutations enhance the binding affinity of AQP4-specific B cell precursors. We wondered whether greater cytotoxicity profiles can be gained through the same manner. The unmutated common ancestor (UCA)-mutated parent IgM mAb pairs were tested on the CDC assay. Upon reversion of mutations, all UCAs had reduced complement activation compared to the parent mAbs (Figure 5.10a and b). This suggests that, in individual B cells, somatic hypermutations confer improved ability to fix complement.

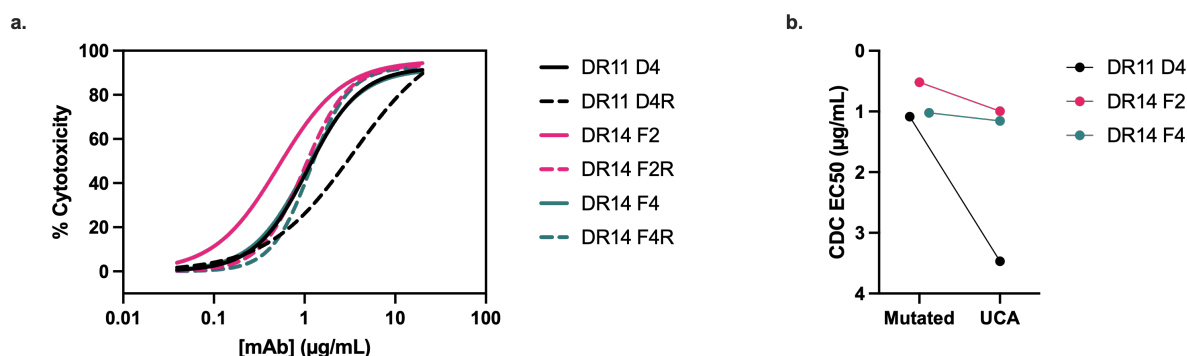


Figure 5.10. Cytotoxicity profiles of mutated AQP4-IgMs and their unmutated precursors.

a. Dose-response curves of individual UCA and parent IgM mAbs; **b.** The UCAs exhibited lower cytotoxic activity compared to the parent mAbs. UCA, unmutated common ancestor. CDC, Complement-dependent Cytotoxicity; UCA, Unmutated Common Ancestor.

5.4.8 Evaluation of cytotoxic capacity of HEK-reactive mAbs

AQP4-IgM mAbs were shown to induce lysis of cells expressing the target antigen in the presence of human complement. We wondered whether IgM with reactivity to HEK membrane antigen(s) also display classical complement activity. Non-transfected HEK cells were incubated with serially diluted HEK-reactive mAbs starting from 20 $\mu\text{g/mL}$ supplemented with 10% human complement. The CDC assays were performed under the same conditions as described in the preceding sections. A non-AQP4 and non-HEK reactive IgM (DR11 E9) was used as an isotype control. Representative dose-response curves of 3 HEK-reactive IgM mAbs are depicted in Figure 5.12. There was no observable difference in cell viability between those treated with HEK-reactive IgM and isotype control. We conclude that binding of IgM antibodies to HEK membrane antigen(s) do not activate the classical complement pathway. The CDC activity of IgM binding to AQP4 is therefore, unique to the nature of interactions between the two molecules which facilitates optimum engagement with C1q and activation of the remaining complement cascade.

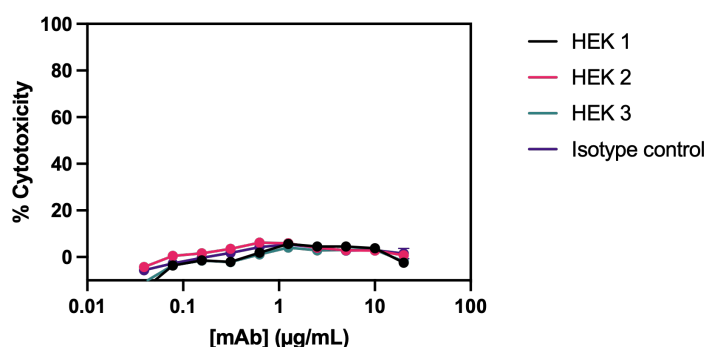


Figure 5.11. Representative dose-response curves of HEK-reactive IgMs.

Non-transfected HEK293T cells were incubated with serial dilutions of either a HEK-reactive or control IgM in the presence of 10% human complement. CDC assays were performed under the optimum conditions as described in the preceding sections. Each sample was tested in duplicates. There was no observable difference in cell viability between the treatment and control group. HEK, Human Embryonic Kidney.

5.5 Summary

Characterisation of *in vitro* effector functions of NMOSD patient-derived monoclonal antibodies highlights the potential contribution of AQP4-IgM to disease pathogenesis. We demonstrated that, in the presence of human complement, AQP4-IgM had the capacity to induce terminal complement complex (C5b9) deposition on the membranes of AQP4 M23-expressing HEK293T cells. We subsequently developed an *in vitro* complement-dependent cytotoxicity (CDC) assay using HEK293S stably expressing AQP4-M23 as a substrate. The AQP4 expression on the stable cells was first evaluated and validated through live CBA using AQP4+ serum samples. The observed endpoint titres of 40 AQP4+ serum samples on the stable cells were identical to the transiently-transfected cells which are used for routine diagnostics assays, suggesting similar surface AQP4-M23 expression between the two substrates. Next, the time allowed for complement fixation at 37°C and the amount of human complement added were examined. CDC was found to be time- and complement concentration-dependent. Sample incubation for 24 hours with 10% human complement supplementation were shown to give the best signal on the CDC assay. Following identification of the optimum conditions, the cytotoxicity profiles of AQP4-specific mAbs were characterised. To compare among different

subsets, all mAbs were tested as IgM with the caveat that the IgM backbone may impact the natural CDC ability of the mutated mAbs. There was a significant increase in the degree of cytotoxicity between the unmutated (NEm, MN, and UDNeg) and MDNeg mAb subsets. Interestingly, the complement activating potencies of the mutated memory mAbs were relatively comparable to the unmutated ones. The artificial expression of the former as a multimeric IgM molecule may not solely explain these findings as 5/6 of the mutated mAbs displayed superior CDC abilities as IgM than IgG1. Nonetheless, when we looked at individual B cell lineages as represented by the UCA and their respective mutated parent mAbs, it was consistently shown that somatic hypermutations augment AQP4-IgM cytotoxic capacity.

6 Chapter VI: *In Vivo* Functional Characterisation of NMOSD Patient-derived AQP4-specific Monoclonal Antibodies

6.1 Introduction

Active NMOSD lesions are characterised by loss of astrocytes in the lesion centre demonstrated by loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity, IgG and IgM deposition co-localising with complement products in a vasulocentric pattern, immune cell infiltration, and a loss of myelin staining demonstrating demyelination^{39,40}. Over the past decade, acute lesional models have demonstrated that AQP4-IgG with⁴⁸ or without added human complement⁴⁹, depending on the specific model and species used, is sufficient to replicate these histopathological hallmarks of NMOSD.

Earlier work involving intraperitoneal administration of IgG isolated from AQP4-seropositive NMOSD patients produced lesions with astrocyte depletion and complement deposition in rats with experimental autoimmune encephalomyelitis (EAE)⁴⁶ or in rats pre-treated with complete Freund's adjuvants⁴⁷. However, the pre-existing neuroinflammatory state in these models confounds the interpretation of lesion pathology in relation to disease mechanisms. A more robust model employing a single intracerebral infusion of patient-derived IgG to naïve rats subsequently demonstrated the direct pathogenicity of IgG with reactivity to AQP4 in NMOSD⁴⁹.

As opposed to rats, injections of AQP4-IgG alone did not generate NMOSD-like lesions in naïve mice⁴⁸. Pathology similar to that of human NMOSD was reproduced only when IgG antibodies were co-administered with human complement directly into mouse brains⁴⁸. It is believed that, in comparison to other species (human, rat, and guinea pig), mouse complement has a low intrinsic complement activity^{50,51}. Additionally, mouse serum was reported to contain complement inhibitor(s)⁵¹, further limiting the utility of mouse models to study diseases which involve the classical complement pathway including NMOSD.

In the preceding chapter, we have reported that AQP4-IgM antibodies had varying efficiencies in activating the classical complement pathway *in vitro*. As IgM deposits are

observed in autopsy and biopsy samples from NMOSD patient brain samples, this led us to consider the pathogenic potential of AQP4-IgM *in vivo*. To examine the capability of AQP4-IgM to fix complement and cause lesions in rodent brain, we developed an acute model of NMOSD employing a single intracerebral co-injection of AQP4-IgM and human complement in naïve rats, followed by histopathological characterisation of the produced lesions.

6.2 Specific study aims

The work presented in this chapter aimed to:

1. Evaluate the rat AQP4-specificity of human-derived AQP4-IgM by three *in vitro* binding assay platforms: live CBA, astrocyte indirect immunofluorescence assay (IIFA), and fresh tissue IIFA;
2. Examine the ability of human AQP4-IgM to activate the classical complement pathway and reproduce the hallmark of human NMOSD lesions *in vivo*; and
3. Develop an acute rat model of NMOSD employing a single intracerebral co-injection of human AQP4-IgM and complement.

6.3 Methods

6.3.1 *In vitro* rat AQP4 binding assays

The specificity of human AQP4-IgM to rat AQP4 was evaluated by three *in vitro* binding assay platforms, namely, live CBA, astrocyte IIFA, and brain section IIFA. The general protocols for these different immunostainings have been described in Chapter II, Subsections 2.2.2.1-7 (live CBA), Subsections 2.2.7.1-2 (astrocyte IIFA), and Subsections 2.2.8.1-2 (rat brain IIFA). For live CBA, HEK293T cells transiently transfected with rat AQP4-M23-EGFP plasmid were used as a substrate. AQP4-IgM antibodies were serially diluted from 20 µg/mL starting concentration and their endpoint titres were visually determined using a fluorescence microscope. Astrocyte and rat brain IIFA were performed using enriched primary rat astrocyte

cultures and lightly-fixed rat brain section, respectively. AQP4-IgM antibodies were tested at a single dilution (20 µg/mL) and specific binding was visualised with a fluorescence or confocal microscope. For all assays, a non-reactive DR11 E9 IgM was used as an isotype control.

6.3.2 Intracerebral injection

The procedure for intracerebral co-injection of AQP4-IgM and human complement has been described in Chapter II, Subsections 2.2.9.1-2. In summary, male, wild-type Wistar rats were anaesthetised with 2% isoflurane/oxygen at 2 L/min and mounted onto a stereotaxic frame. A mixture of 10 µg AQP4-IgM and 10% human complement (v/v) in 2 µL PBS was injected into the left striatum at 1 mm anterior, 3 mm lateral, and 4 mm ventral to bregma using a finely drawn glass microcapillary. Rats injected with the same concentration of DR11 D4R and non-reactive DR11 E9 IgM were used as controls.

6.3.3 Immunohistochemistry

At 3 days post-injection, rats were culled and their brains were harvested and processed as described in Chapter II, Subsections 2.2.9.3-6. Histopathological characterisation of the generated lesion was performed by immunohistochemistry using the following primary antibodies: rabbit anti-AQP4 (1:200), rabbit anti-GFAP (1:1000), rabbit anti-C5b9 (1:500), and rabbit anti-neutrophil (1:1000). Specific binding was staining was visualised using the avidin/biotin-based peroxidase system with 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB)/H₂O₂ substrate following application of a goat anti-rabbit IgG secondary antibody (1:200). Evaluation of AQP4-IgM deposition was performed by staining brain sections directly with a biotinylated goat anti-human IgM antibody (1:200) and developing the specific signal as described above.

6.3.4 Image processing and quantification

Tissue sections were scanned with a whole image scanner connected to a brightfield microscope at 20x objective lens magnification under constant illumination. Images were imported into ImageJ, converted to 16-bit grayscale, and thresholded to isolate and quantify areas with loss of AQP4 and GFAP immunoreactivity as well as IgM and C5b9 deposition. Infiltrating neutrophils in the striatum, defined as the area inferior and medial to the external capsule, superior to the anterior commissure, and lateral to the lateral ventricle, in the injected hemisphere were also counted.

6.4 Results

6.4.1 Differences between human and rat AQP4

AQP4 is the predominant water channel protein in the mammalian brain. It is abundantly expressed on the endfoot process of astrocytes which, together with the adjacent blood vessels, make up the blood-brain and blood-cerebrospinal fluid barrier³⁰. The encoding locus of the two AQP4 isoforms, M1 and M23, has been well characterised²². The deduced amino acid sequence of human AQP4-M23 (*Homo sapiens*, UniProt ID P55087-2) is 95.68% similar to those of rat origin (*Rattus norvegicus*, UniProt ID P47863-2) with 6 of the 13 amino acid differences representing conservative substitutions (Figure 6.1). In the extracellular loops (Loops A, C, and E), two out of three substitutions involve amino acids with different physicochemical properties. Whilst information regarding the crystal structure of AQP4-M23 is not yet available, rat and human AQP4-M1 crystals have been reported to differ in their lattice contacts²¹. In lipid bilayers, human AQP4-M1 tetramers between different planes make a head-to-head contact with one another. The rat counterpart, however, contains both in-plane and between-plane contacts as the distance between tetramers in the same horizontal plane is shorter.

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6.4.2 Rat AQP4 live cell-based assay (CBA)

Representative CBA images of rat AQP4-M23 binders and non-binders are shown in Figure 6.2a. Only 9/25 (36%) human AQP4-IgMs (2 NEm, 2 MN, 2 UDNeg, 1 MDNeg, and 2 Mem) demonstrated specific binding to rat AQP4-M23. Despite their relatively high endpoint titres on human AQP4-M23 live CBA, some mAbs failed to recognise the same antigen of rat origin, for example, DR11 B4, DR26 B3, DR30 C2, DR11 D3, and DR20 D10 (Figure 6.2b). Of those that bound rat AQP4-M23, their CBA endpoint titres across both species correlated poorly with one another (Spearman $r=0.3794$, $p=0.3713$) (Figure 6.2c).

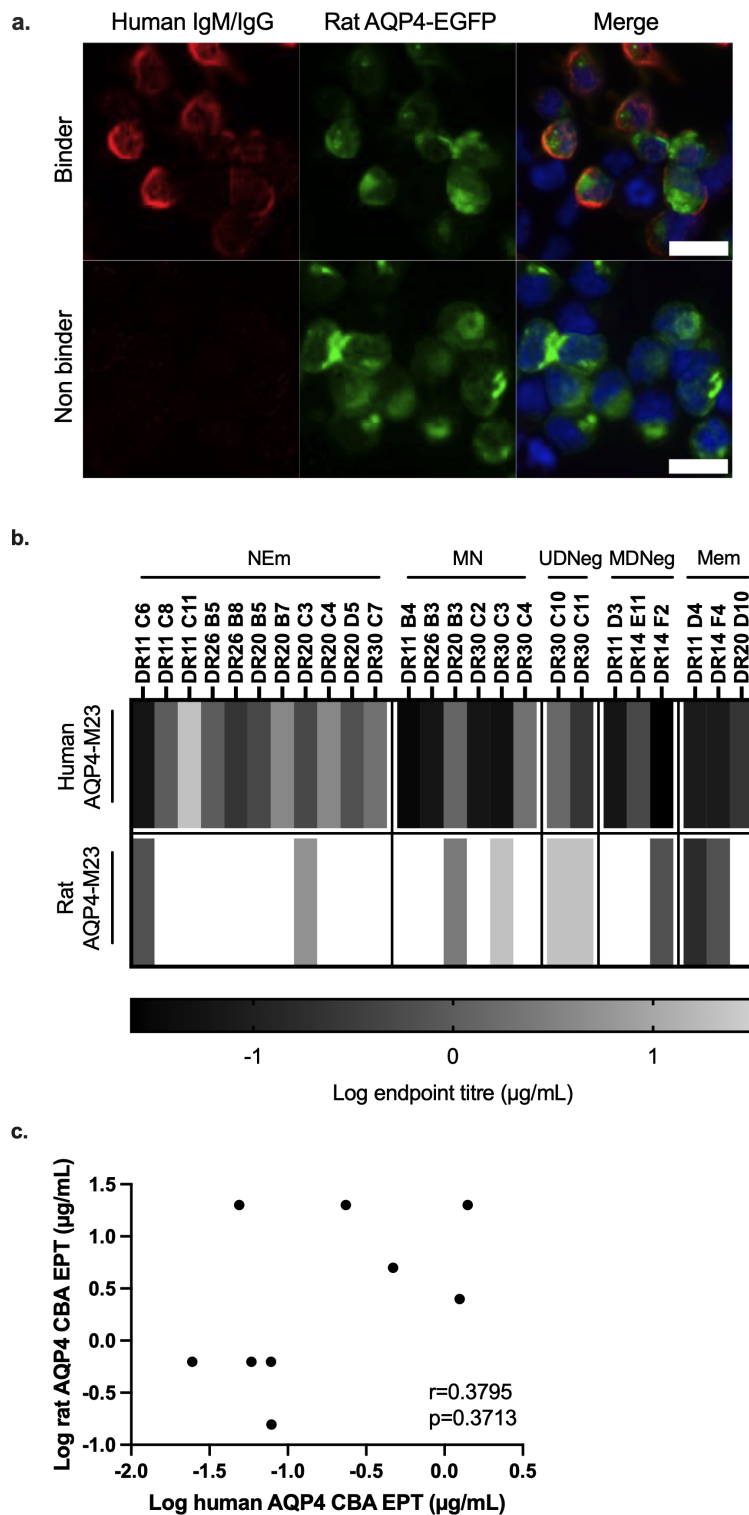


Figure 6.2. Rat AQP4 live cell-based assays (CBAs).

a. Representative CBA images of rat AQP4 binders and non-binders; **b.** A heatmap showing log endpoint titres of AQP4-IgM from different subsets on human and rat AQP4 live CBA. White colour indicates no detectable binding; **c.** Correlation of log CBA endpoint titres of human AQP4-IgM across the two species. 40x magnification, scale bar=200 μm . AQP4, Aquaporin-4; CBA, Cell-based Assay; EPT, Endpoint Titre.

Next, we examined the effect of somatic hypermutation on the ability of human AQP4-IgM to bind rat AQP4-M23 by contrasting the endpoint titres of unmutated common ancestors (UCAs) with their respective mutated parent mAbs. Only 3/6 UCAs were successfully expressed and thus, were included in the analysis. While the unmutated precursor of DR14 F4 IgM retained binding to rat AQP4-M23 at a comparable endpoint titre to its mutated parent IgM, DR11 D4 and DR14 F2 IgMs lost their antigen specificity upon reversion of somatic mutations (Figure 6.3a). An exploratory analysis to compare the differential binding of human AQP4-specific mAbs as IgM and IgG1 was also performed. Of 3 mutated mAbs which were able to recognise rat AQP4-M23 as a multimeric IgM molecule at relatively high endpoint titres, DR14 F4 and F2 mAbs did not show detectable binding to the same antigen as monomeric IgG1 antibodies (Figure 6.3b).

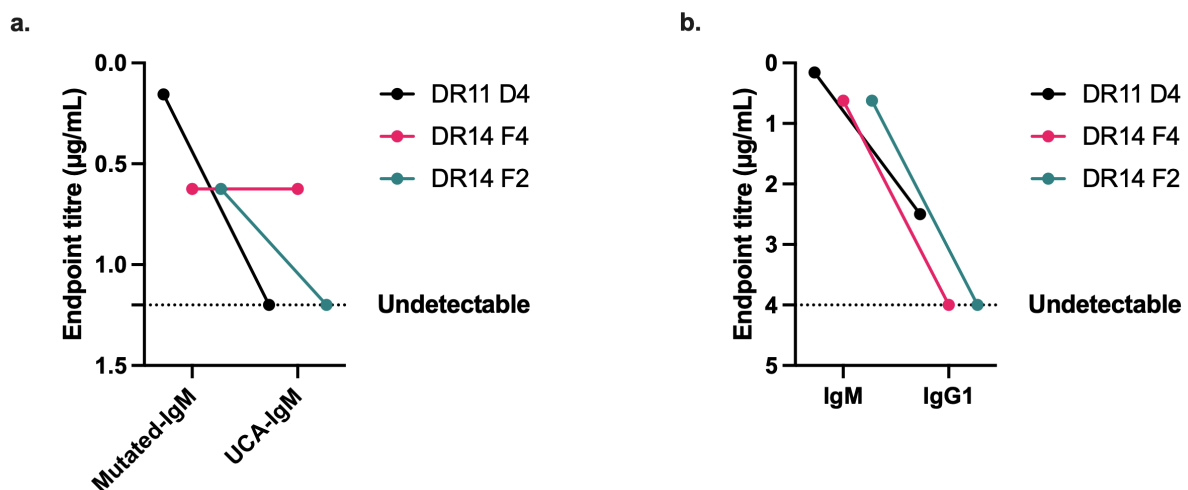


Figure 6.3. The effect of somatic hypermutation and antibody isotype on rat AQP4-M23 specificity of human mAbs.

a. CBA endpoint titres of mutated, human AQP4-IgM mAbs in comparison with their unmutated precursors; **b.** Human AQP4-specific mAbs exhibited unique rat AQP4 M23 specificity as IgM and IgG1 antibodies. UCA, Unmutated Common Ancestor.

These observations suggest that human AQP4-IgMs have different abilities to bind rat AQP4-M23 despite the very high homology between the two molecules (3/40 extracellular amino acids are different and 288/301 across the whole molecule are identical). For some mAbs, incorporation of somatic mutations confers specificity to rat AQP4-M23. Additionally,

rat AQP4-M23 binding sites may be relatively more accessible by IgM rather than IgG antibodies.

6.4.3 Rat astrocyte indirect immunofluorescence assay (IIFA)

On the astrocytic end-foot processes proximal to the perivascular membrane, AQP4 is expressed as regular arrays of intramembrane particles, termed orthogonal arrays of particles (OAPs). The size of OAPs is modified by interaction between the two AQP4 isoforms whereby M1 restrict the size of square arrays formed by M23²⁵. In the preceding experiments, we characterised the binding profiles of human AQP4-IgM on HEK293T cells exclusively expressing rat AQP4-M23. These results may not necessarily reflect their ability to recognise rat AQP4 M1-M23 heterotetramers on astrocytic membranes. Therefore, we sought to investigate their specificity to endogenous rat AQP4 by performing indirect immunofluorescence assays (IIFA) using rat primary cortical astrocyte cultures as a substrate.

Following isolation and plating of mixed cortical cell suspension, some astrocytes were seen to attach to the surface of culture flask within 24 hours (Figure 6.4a). At day-in-vitro (DIV) 3-5, the cell culture supernatant contained cell debris and dying neurons as the culture media favoured the survival of glial cells. A dense astrocyte monolayer could be seen as early as DIV 7 with some microglia and oligodendrocyte precursor cells (OPCs) sitting on top of the confluent layer. Mixed glial cultures were then separated by shaking and plated as enriched astrocyte cultures. At DIV 8, or DIV 1 post-split, astrocytes started to assume a stellate morphology. The cells were grown until they become confluent at around DIV 21. Their maturation was confirmed by immunolabelling with commercial anti-AQP4 and GFAP antibodies (Figure 6.4b).

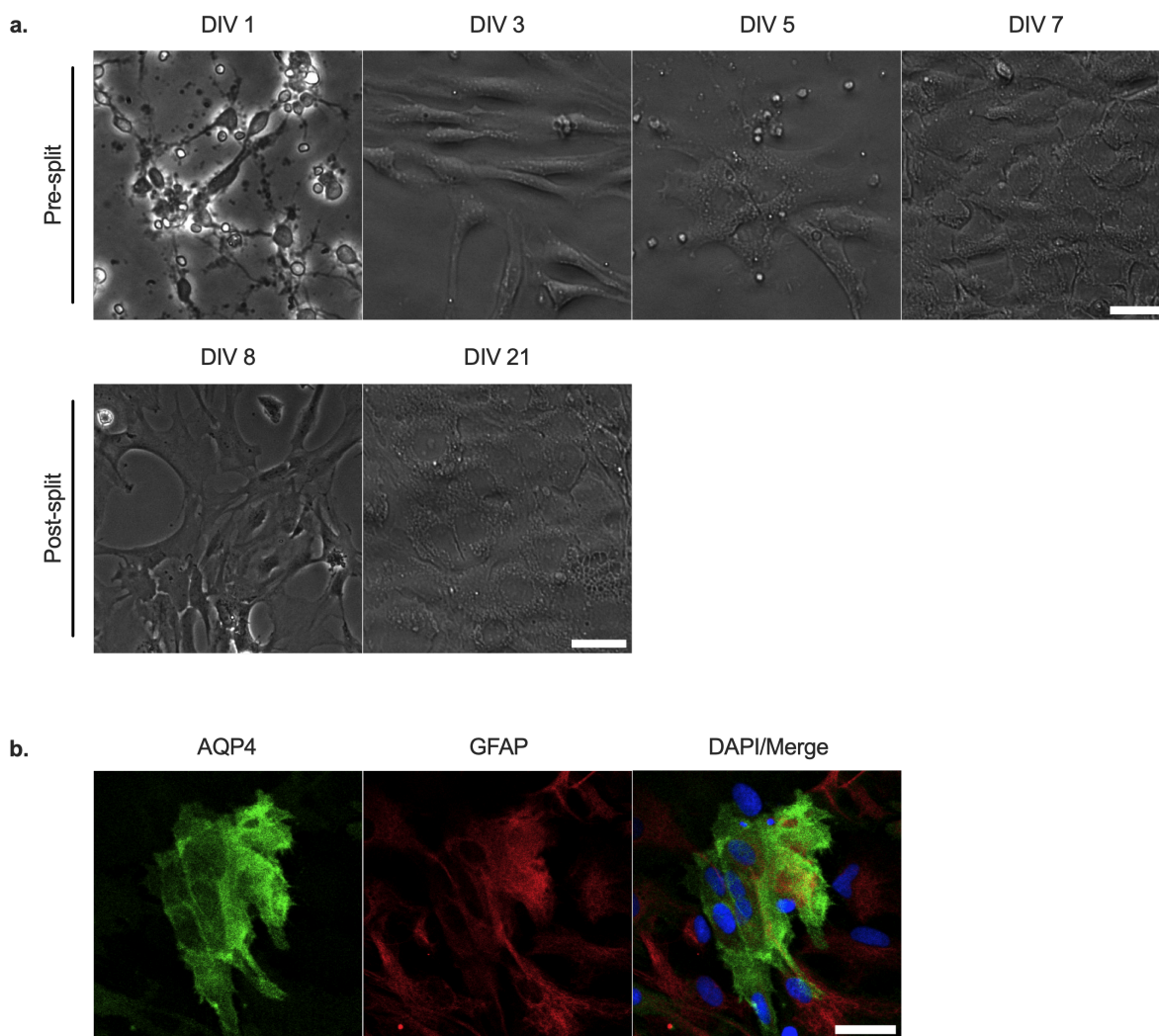


Figure 6.4. Preparation of rat primary cortical astrocyte cultures.

a. Representative brightfield microscopy images of primary astrocytes isolated from rat pup cortices over several days in vitro. DIV 1: attachment of astrocytes to the surface of culture flasks with dying neurons in the supernatant. DIV 3: formation of an early astrocyte monolayer with minimal cell debris after media change. DIV 5: globular cells which we believe to be microglia and oligodendrocyte precursor cells (OPCs) on top of the astrocyte layer⁹⁴. DIV 7: astrocyte monolayer at maximum confluence. DIV 8: plating of enriched astrocyte cultures post-separation. DIV 21: astrocyte layer shows high density 3 weeks post initial culture; **b.** Immunolabelling of mature astrocytes (DIV 21) with anti-AQP4 and anti-GFAP commercial antibodies. 40x and 100x magnification, scale bar=50 μ m. AQP4, Aquaporin-4; DAPI, 4'6-diamidino-2-phenylindole; DIV, Day In Vitro; GFAP, Glial Fibrillary Acidic Protein.

Next, we evaluated the specificity of human AQP4-IgM antibodies to endogenous AQP4 expressed on mature rat primary astrocytes. Representative IIFA images of binder and non-binder mAbs are shown in Figure 6.5a. While most of the results were consistent with the previous CBA experiments, we found some discrepancies. Only 5/25 (20%) AQP4-IgM mAbs

bound rat astrocytes, including 5/9 that also bound on rat AQP4-M23 live CBA. These comprised 1 NEm, 1 MN, 1 MDNeg, and 2 Mem IgM mAbs (Figure 6.5b). By comparison to their respective mutated parent mAbs, none of the successfully expressed UCA IgM mAbs bound rat astrocytes. Additionally, DR14 F2 mAb was shown to bind rat astrocytes as IgM and IgG1 (Figure 6.5d). One should note that it bound rat AQP4 M23-expressing HEK293T cells as IgM, but not IgG1. On the other hand, DR11 D4 mAb only bound rat astrocytes as IgM despite it demonstrated specific binding on live CBA as both antibody isotypes.

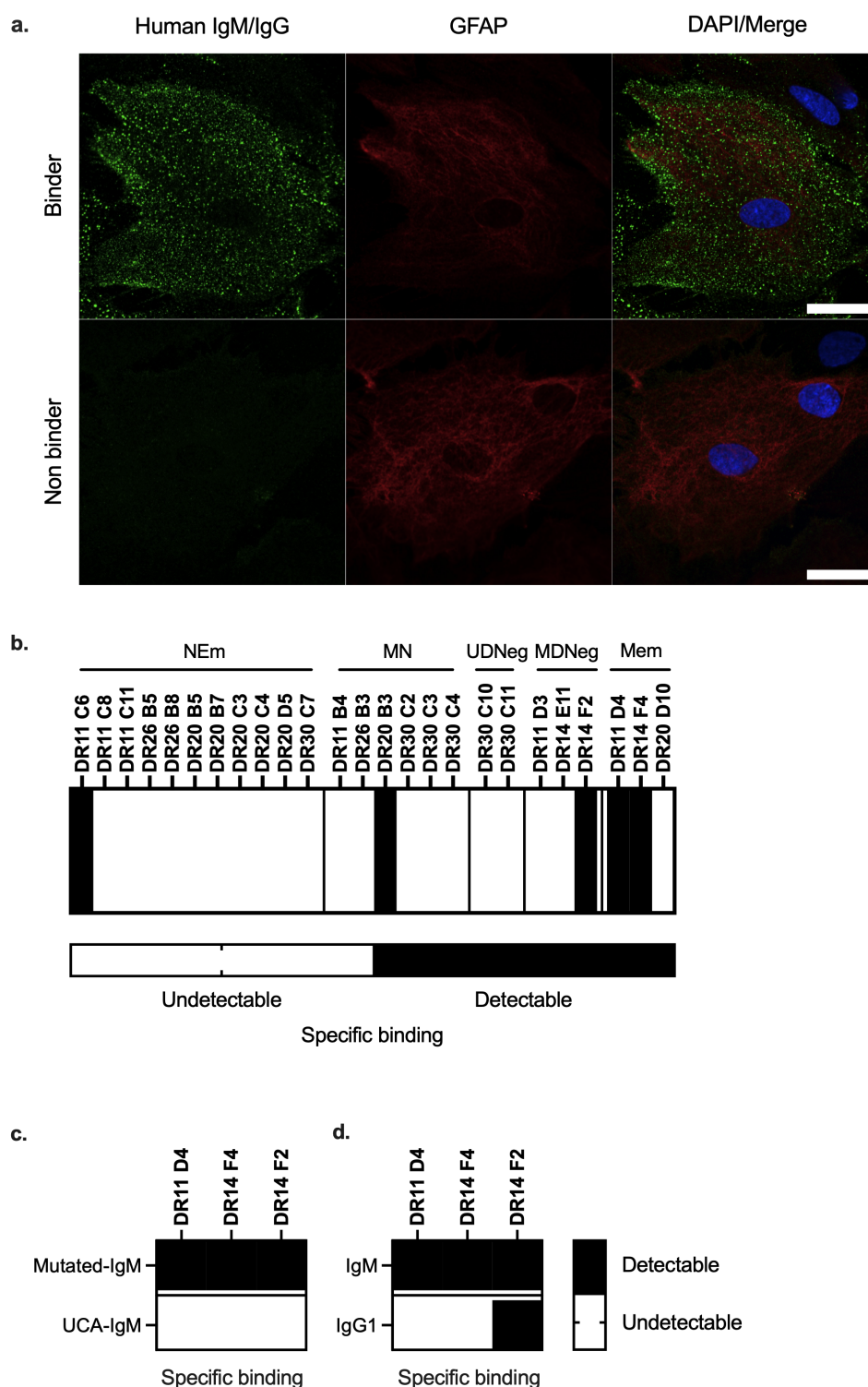


Figure 6.5. Rat astrocyte indirect immunofluorescence assays (IIFA).

a. Representative IIFA images of rat astrocyte binders and non-binders; **b.** A heatmap showing the binding patterns of different human AQP4-IgM antibodies on rat primary astrocyte cultures; **c.** Binding patterns of the mutated IgM mAbs and their respective unmutated precursors; **d.** Rat astrocyte specificity of the mutated human AQP4-specific mAbs as IgM and IgG1. 100x magnification, scale bar=20 μ m. DAPI, 4',6-diamidino-2-phenylindole; GFAP, Glial Fibrillary Acidic Protein; MDNeg, Mutated Double-Negative; Mem, Memory; MN, Mature Naïve; NEm, New Emigrant; UDNeg, Unmutated Double Negative; UCA, Unmutated Common Ancestor.

Collectively, in addition to the generally lower sensitivity of rat astrocyte IIFA compared to rat AQP4-M23 live CBA, the above observations suggest that AQP4 M23 homotetramers on transiently transfected HEK293 cells and AQP4 M1-M23 heterotetramers endogenously expressed by rat astrocytes may represent two different antigenic targets. The presence of M1 isoform may modify the AQP4 arrays to some extent that it abrogates binding of some mAbs while at the same time, makes the protein more accessible for other antibodies to bind.

6.4.4 Rat brain indirect immunofluorescence assay (IIFA)

In rodent CNS tissues, individual astrocytes exhibit highly polarised expression of AQP4 with 5 to 10-fold higher density of OAPs along endfoot processes in direct contact with capillaries than on other cell membrane domains⁹⁵. This polarisation is also observed in humans albeit to a lesser degree than rodents⁹⁵. In our *in vitro* rat astrocyte cultures, these complex cellular interactions which result in the differential distribution of AQP4 on astrocytic membranes are absent. Therefore, we sought to explore whether human AQP4-specific mAbs are able to bind AQP4 expressed on rat astrocytes in their native environment by performing IIFA using rat brain sections as a substrate.

First, we optimised the tissue processing and immunolabelling method. Non-perfused rat brains were harvested and post-fixed with 4% paraformaldehyde (PFA) at 4°C for either 1 hour (light fixation) or 24 hours (heavy fixation). Tissues were cryoprotected using 30% sucrose solution, embedded in OCT cryostat mounting medium, and sectioned along the sagittal plane at 10 µm thickness. Sections were then stained with a commercial anti-AQP4 antibody raised against a conserved intracellular epitope (amino acids 244-323) or one of the AQP4-specific mAbs which bound both human and rat AQP4 (DR14 F2 IgM). Representative IIFA images are shown in Figure 6.6. A vessel-like staining pattern corresponding to AQP4 immunoreactivity was observed in tissue sections labelled with the commercial antibody,

regardless of duration of PFA fixation. This specific pattern was also present in lightly fixed, but absent in heavily fixed sections stained with the AQP4-specific IgM mAb even with the application of heat-induced antigen retrieval process (incubation for 3 min in citrate buffer at 95°C), suggesting an irreversible loss of tissue AQP4 immunoreactivity following prolonged treatment with PFA and exposure to heat. We conclude that, in contrast to the commercial antibody, patient-derived AQP4-IgM mAbs recognise fixation- and heat-sensitive AQP4 epitopes.

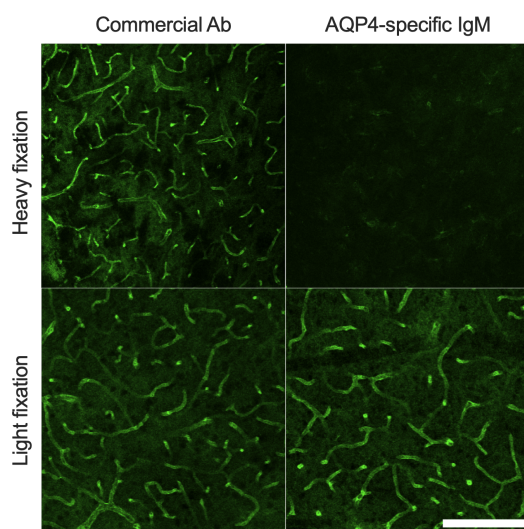


Figure 6.6. Optimisation of tissue processing and immunolabelling methods.

Rat brain sections were subjected to fixation with 4% paraformaldehyde (PFA) at 4°C for 1 hour (light fixation, bottom panels) or 24 hours (heavy fixation, top panels). For heavily fixed sections, an additional heat-induced antigen retrieval step was applied. Sections were stained with either a commercial anti-AQP4 antibody against a conserved intracellular epitope (left panels) or one of the human AQP4-specific IgM mAbs which was cross-reactive with rat AQP4 on live CBA and astrocyte IIFA (DR14 F2 IgM, right panels). While the duration of fixation did not impact the ability of the commercial antibody to recognise its tissue epitope, prolonged treatment with PFA and exposure to high temperature abrogated binding of the patient-derived AQP4-specific mAb. 40x magnification, scale bar=50 μ m. AQP4, Aquaporin-4.

Next, we examined the reactivity of AQP4-IgM mAbs using the optimised tissue-based IIFA method. Lightly-fixed rat brain sections were incubated with 20 μ g/ml of AQP4-IgM mAbs overnight at 4°C. Specific binding was visualised with a goat anti-human IgM, Alexa Fluor 488-conjugated secondary antibody (Figure 6.7a). In total, 5/25 (20%) AQP4-IgM mAbs showed detectable binding to lightly-fixed rat tissue, all of which also bound rat astrocyte

cultures in the previous experiments (Figure 6.7b). Upon reversion of somatic mutations, DR14 F4R and DR14 F2R IgM mAbs retained their specific binding to the lightly-fixed rat tissue whereas DR11 D4R IgM mAb binding was lost (Figure 6.7c). Additionally, of 3/6 mutated mAbs which bound the lightly-fixed rat tissue as IgM, all of them also exhibited reactivities as IgG1 antibodies (Figure 6.7d).

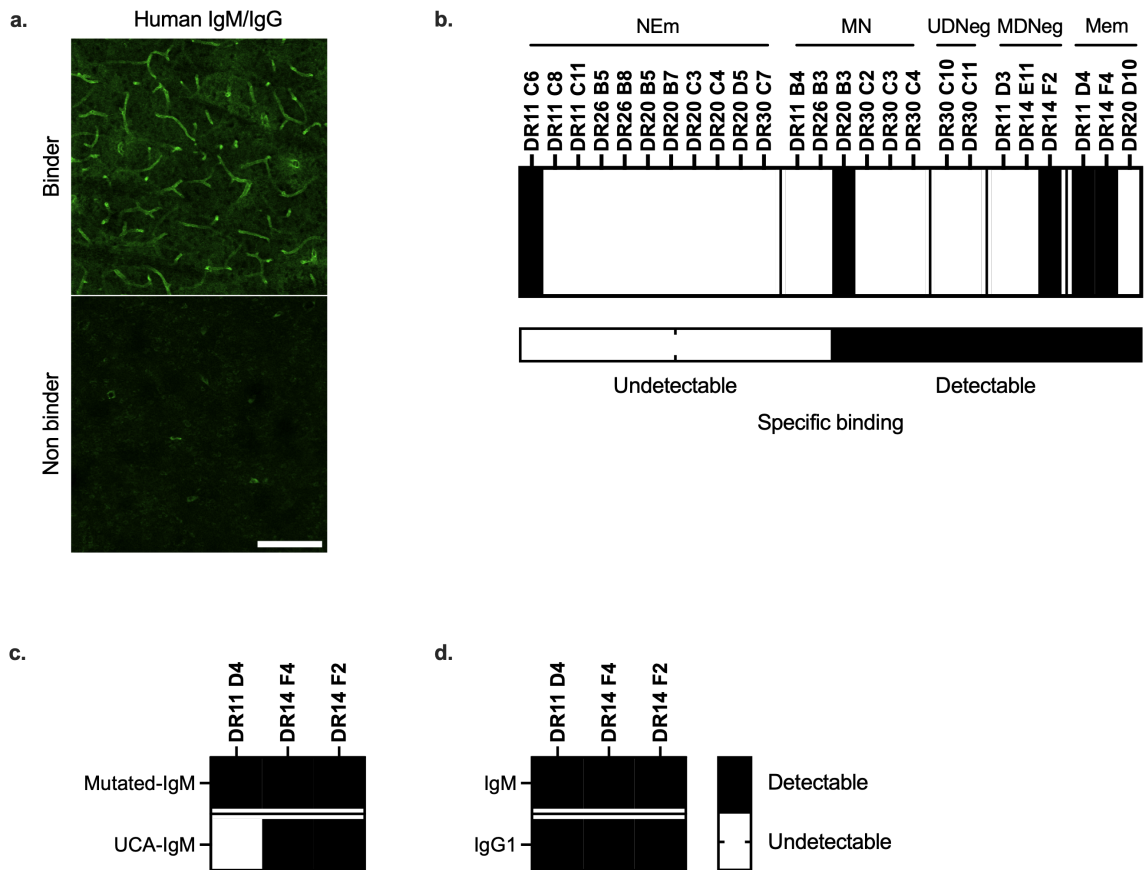


Figure 6.7. Rat brain indirect immunofluorescence assay (IIFA).
a Representative images of lightly-fixed rat tissue binders and non-binders; **b.** A heatmap showing the binding patterns of different AQP4-IgM antibodies on rat brain sections; **c.** Comparison of tissue reactivity between the mutated IgM mAbs and their respective unmutated precursors; **d.** Binding patterns of the mutated mAbs as IgM and IgG1 antibodies. 40x magnification, scale bar=50 μ m. MDNeg, Mutated Double-Negative; Mem, Memory; MN, Mature Naïve; NEm, New Emigrant; UDNeg, Unmutated Double Negative; UCA, Unmutated Common Ancestor.

6.4.5 Selection of mAbs for intracerebral injections

Up to this point, we have investigated the rat AQP4 specificity of patient-derived mAbs using 3 *in vitro* binding assay platforms, namely, rat AQP4 live CBA, rat astrocyte IIFA, and rat brain IIFA. We confirmed that not all human AQP4-specific mAbs recognise AQP4 of rat

origin. This is of particular importance as our main aim was to develop an acute rat model of NMOSD. Thus, for subsequent intracerebral co-injections of AQP4-IgM and human complement in naïve rats, 9 AQP4-IgM mAbs representing different B cell lineages were carefully chosen which include 2 NEm, 2 MN, 3 mutated, and 2 UCA mAbs. The mAb candidates and their patterns of reactivity across the 3 assays are summarised in Table 6.1. One of the UCAs, DR11 D4R, was selected as an isotype control as it did not bind any of the rat assays despite it being reactive to human AQP4. A non-AQP4 reactive mAb, DR11 E9, was also included as an additional isotype control.

mAb ID	B cell subset	Specific binding		
		Rat AQP4 live CBA	Rat astrocyte IIFA	Rat brain IIFA
DR11 C6	NEm	+	+	+
DR20 C3	NEm	+	-	-
DR20 B3	MN	+	+	+
DR30 C3	MN	+	-	-
DR14 F2	Mutated (MDNeg)	+	+	+
DR14 F4	Mutated (Mem)	+	+	+
DR11 D4	Mutated (Mem)	+	+	+
DR14 F2R	UCA	-	-	+
DR14 F4R	UCA	+	-	+
DR11 D4R	Isotype control	-	-	-
DR11 E9	Isotype control	-	-	-

Table 6.1. List of mAb candidates for animal injections.

Their binding patterns as IgM antibodies across the different in vitro rat binding assays are denoted as positive (+) or negative (-).

6.4.6 Validation of intracerebral injection protocols in rats

Building on methods previously developed in both mice and rats, we proposed a model of NMOSD using intracerebral injections of AQP4-specific antibodies supplemented with human complement in rats without pre-existing neuroinflammatory conditions. Injections of 10 µg serum NMO-IgG in naïve rats has been shown to produce robust lesions with loss of AQP4 and GFAP immunoreactivity, complement deposition as well as granulocyte infiltration⁴⁹. To validate our injection protocols, naïve rats (n=3) were injected 4 mm deep into the left striatum (1 mm anterior and 3 mm lateral to bregma) with the same amount (10

μg) of one of the mutated AQP4-IgG mAbs (DR14 F2) and 10% human complement. Rats ($n=3$) treated with 10 μg anti-hen egg lysozyme (HEL) IgG and 10% human complement were used as a control. A schematic illustration of the injection protocols is shown in Figure 6.8. A total of 2 μL were infused over 5 minutes to minimise tissue injury. Brains were harvested 3 days post-injection and serially sectioned along the sagittal plane, spanning across a length of 150 mm anterior and posterior to the site of injection. Sections were then stained with anti-AQP4 and GFAP commercial antibodies. To ensure robust and reproducible findings whilst minimising the number of animals used per experiment, sections obtained from the lesion epicentre and $\pm 100 \mu\text{m}$ away from the epicentre from each animal were considered as 3 different replicates. Thus, each treatment group was represented by 9 replicates from 3 individual animals. A human NMOSD-like lesion with a massive loss of AQP4 and GFAP immunoreactivity was observed in the AQP4-IgG, but not the control IgG group (mean area of AQP4 loss, $14.76 \times 10^4 \mu\text{m}^2$ vs $0.16 \times 10^4 \mu\text{m}^2$; mean area of GFAP loss, $14.42 \times 10^4 \mu\text{m}^2$ vs $0.18 \times 10^4 \mu\text{m}^2$, unpaired t test, $p < 0.0001$) (Figure 6.9a-d). We conclude that the dose of injected antibody and complement as well as the duration of treatment included in the injection protocols produced human NMOSD-like lesions in naïve rats.

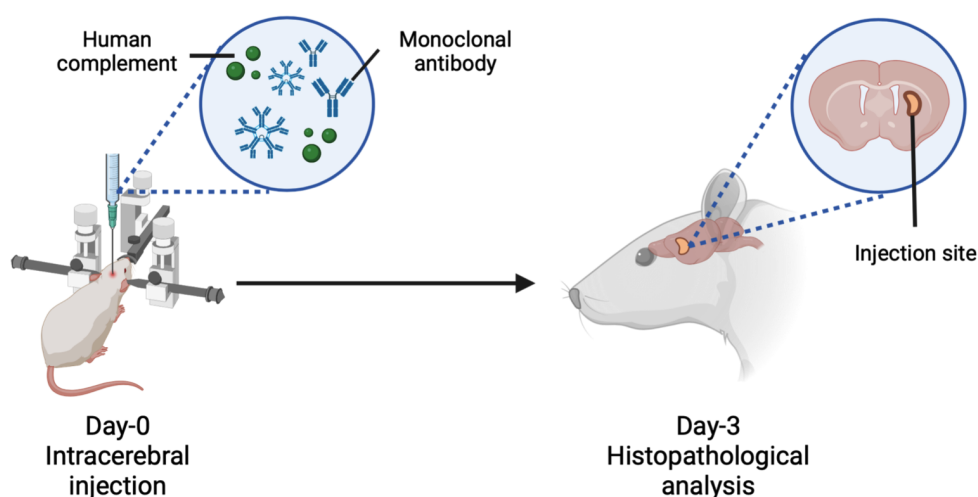


Figure 6.8. A schematic illustration of the protocols for intracerebral injection of AQP4-specific antibodies and human complement in naïve rats as an acute model of NMOSD.

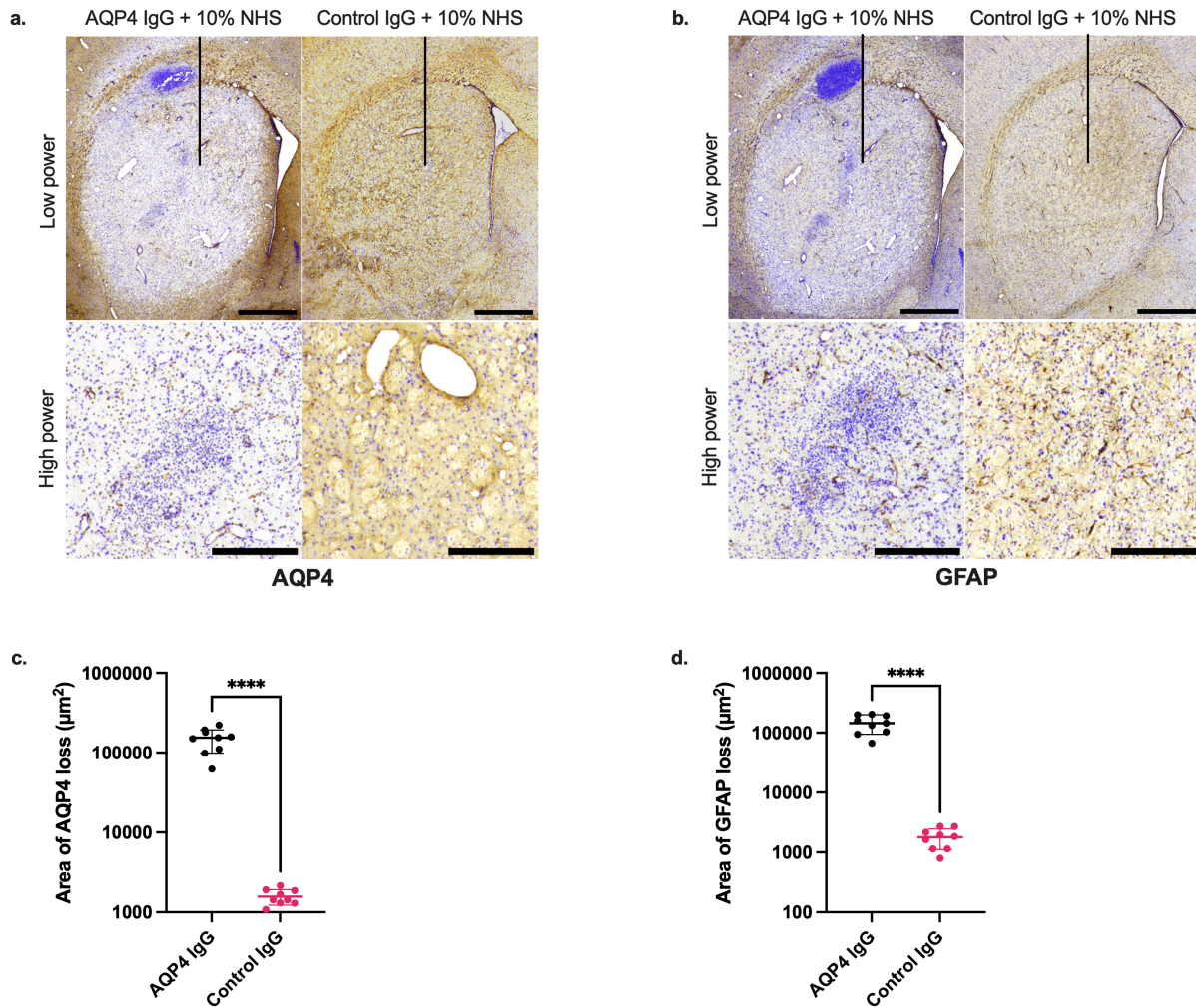


Figure 6.9. Intracerebral injection of AQP4-IgG and human complement in naïve rats produced human NMOSD-like pathology. Brains were injected with 10 μg of an AQP4-specific IgG (DR14 F2) or a HEL (control) IgG and 10% human complement in a total injection volume of 2 μL . Sections were evaluated for AQP4 (a) and GFAP (b) immunoreactivity 3 days post-injections. The needle track is shown as a vertical black line. Figures (c) and (d) summarise areas with loss of AQP4 and GFAP expression in each group, respectively. Dots represent individual replicates obtained from a total of 3 animals per group. *t*-test, mean $\pm$ SEM, $\ast=p<0.05$, $\ast\ast=p<0.005$, $\ast\ast\ast=p<0.0005$, $\ast\ast\ast\ast=p<0.0001$. 20x and 40x magnification, scale bar=200 μm . AQP4, Aquaporin-4; GFAP, Glial Fibrillary Acidic Protein; UCA, Unmutated Common Ancestor.

6.4.7 Intracerebral injections of AQP4-IgM and human complement in naïve rats

Using the above injection protocols, we investigated the pathogenic potential of AQP4-IgM from different B cell lineages in naïve rats. Rats in the unmutated group received injections of either a new emigrant (DR11 C6) or mature naïve IgM mAb (DR20 B3) whereas those in the mutated group were injected with IgM mAbs derived from the mutated double-negative or memory B cell subset (DR14 F2, DR14 F4, or DR11 D4). The UCA group comprised rats

treated with either unmutated precursor IgM mAb DR14 F2R or DR14 F4R. All those mAbs shared the same feature whereby they bound rodent brain sections despite their varying reactivities to other rat AQP4 assay substrates. Animals injected with DR11 D4R and DR11 E9 IgM mAbs that did not react with rat AQP4-M23 on any assay were used as isotype controls. Similar to the previous approach, brain sections from 3 areas (lesion epicentre $\pm 100 \mu\text{m}$) per animal, 3 animals per mAbs were collected and compared.

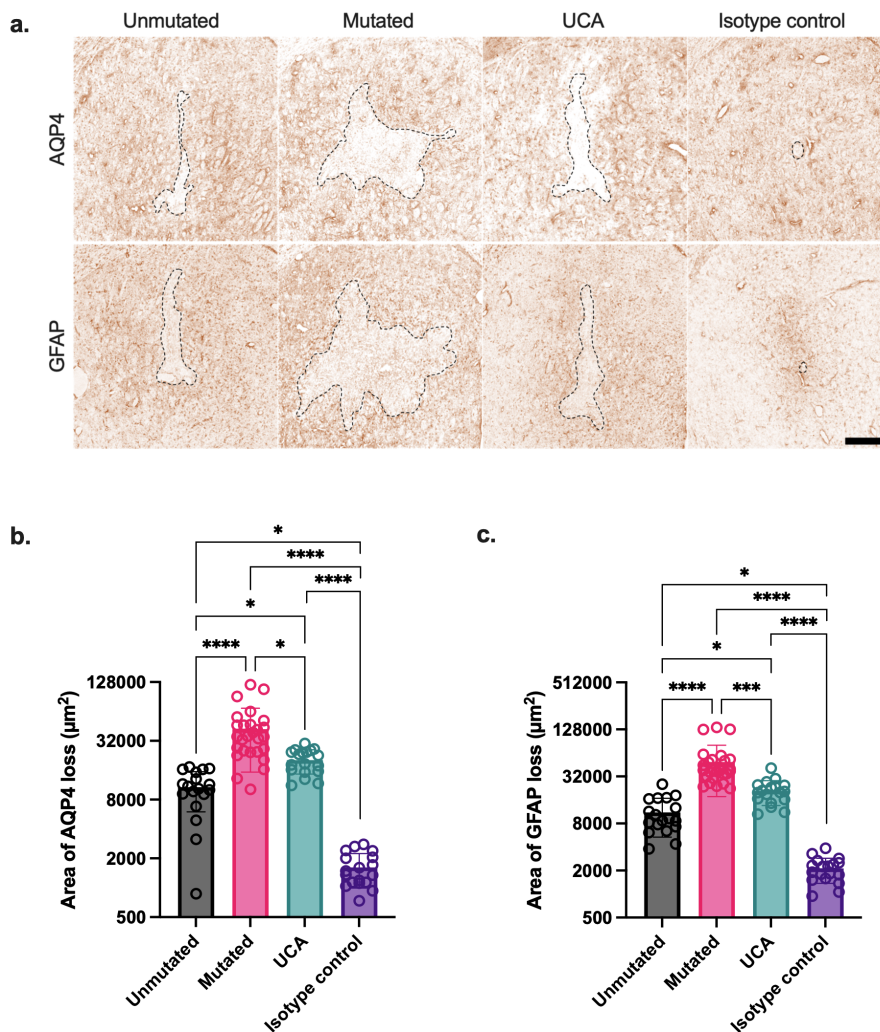


Figure 6.10. Co-injections of AQP4-IgM antibodies and human complement produced human NMOSD-like lesions in naïve rats.

Brain sections of rats injected with unmutated (NEm/MN), mutated (MDNeg/Mem), UCA, or isotype control mAbs were stained with anti-AQP4 (**a**, top panels) and anti-GFAP (**a**, bottom panels) commercial antibodies. Black dashed line demarcates lesion area. Areas of loss of AQP4 and GFAP immunoreactivity are summarised in (**b**) and (**c**). Kruskal-Wallis test, Mean $\pm$ SEM, $\ast=p<0.05$, $\ast\ast=p<0.005$, $\ast\ast\ast=p<0.0005$, $\ast\ast\ast\ast=p<0.0001$. 40x magnification, scale bar=200 μm . AQP4, Aquaporin-4; GFAP, Glial Fibrillary Acidic Protein; UCA, Unmutated Common Ancestor.

Co-injections of AQP4-IgM and human complement in naïve rats produced a well-demarcated area with loss of AQP4 and GFAP immunoreactivity around the site of injection (Figure 6.10a-c). This pathology was minimally observed in rats receiving the control IgM antibodies. The mutated mAbs induced the most severe loss in AQP4 and GFAP staining, followed by the UCA and then the unmutated mAbs, suggesting the potential contribution of AQP4-IgM in the formation of NMOSD lesions. This pathogenicity is significantly enhanced by somatic hypermutations.

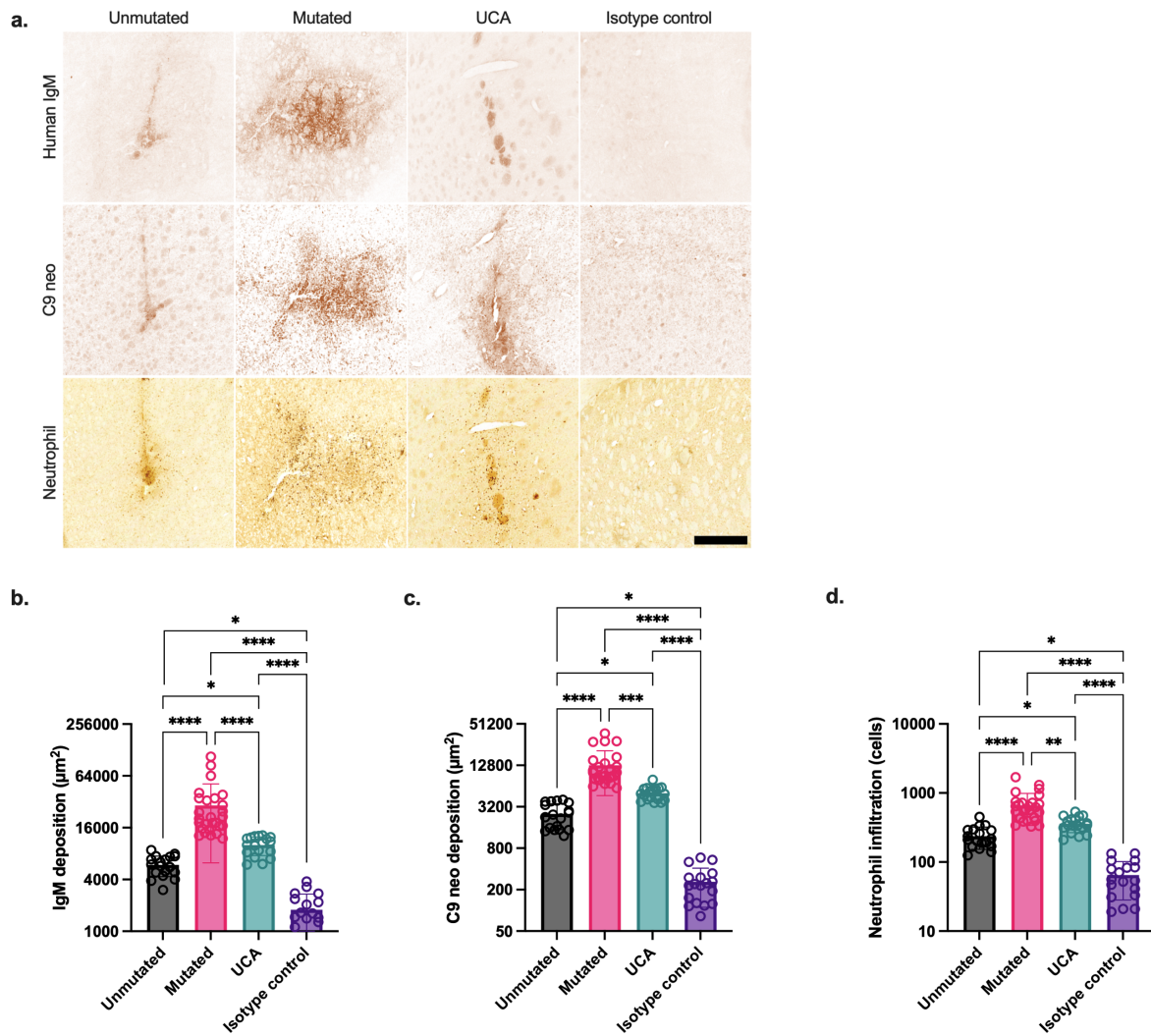


Figure 6.11. IgM and C9 neo deposition and neutrophil infiltration in following co-injections of AQP4-IgM and human complement.

Brain sections were stained with additional markers to detect human IgM, activated complement products, and neutrophils around the site of injection (**a**). Quantitative analyses of the three histopathological features are summarised in (**b**), (**c**), and (**d**). Kruskal-Wallis test, Mean $\pm$ SEM, *= $p<0.05$, **= $p<0.005$, ***= $p<0.0005$, ****= $p<0.0001$. 40x magnification, scale bar=200 μm . UCA, Unmutated Common Ancestor.

Next, brain sections were stained with additional markers to study IgM and complement product deposition as well as neutrophil infiltration. Rats injected with the mutated mAbs consistently exhibited the most pronounced histopathological findings of all treatment groups (Figure 6.11a-d). These features were slightly reduced in the UCA and the unmutated groups, and were almost absent in the control group. Additionally, the deposited IgM mostly co-localised with activated complement products, substantiating the role of the classical

complement pathway in the formation of AQP4 IgM-induced, human NMOSD-like lesions in this model.

We noticed there was a stark difference in the size of brain lesions following injections of AQP4-IgG (Figure 6.9) versus IgM (Figure 6.10). Thus, we did an additional analysis comparing areas of antibody deposition in rats receiving 10 µg of the mutated mAbs (DR14 F2, DR14 F4, DR11 D4) as IgM or IgG and 10% human complement. There was a total of 3 animals per mAb. Sections representing the lesion epicentre and ± 100 µm away from the epicentre from each animal were stained with isotype-specific secondary antibodies and analysed. At 3 days post-injections, AQP4-IgG was detected in an area of approximately 10.6×10^4 µm² (mean area from all AQP4-IgG injected rats), covering almost the entire region of striatum (Figure 6.12a, right panel). Interestingly, two distinct areas of IgM deposition were observed. There was intense IgM staining at the lesion core surrounded by a penumbra or area with less intense IgM immunoreactivity. Including the penumbra, the deposited IgM occupied an area of 2.9×10^4 µm² (mean area from all AQP4-IgM injected rats), almost one fifth the size of IgG deposition (Figure 6.12a, left panel). Larger areas of IgG than IgM deposition were also consistently shown within individual IgM-IgG mAb pairs (Two-way ANOVA, DR14 F2, $p < 0.0001$, DR14 F4, $p < 0.05$, DR11 D4, $p < 0.0001$). There were two possible explanations: a) the relatively higher molecular weight of IgM resulted in a poorer tissue penetration compared to IgG; or b) there were fewer injected IgM than IgG molecules as the injection dose was based on µg amount rather than molarity.

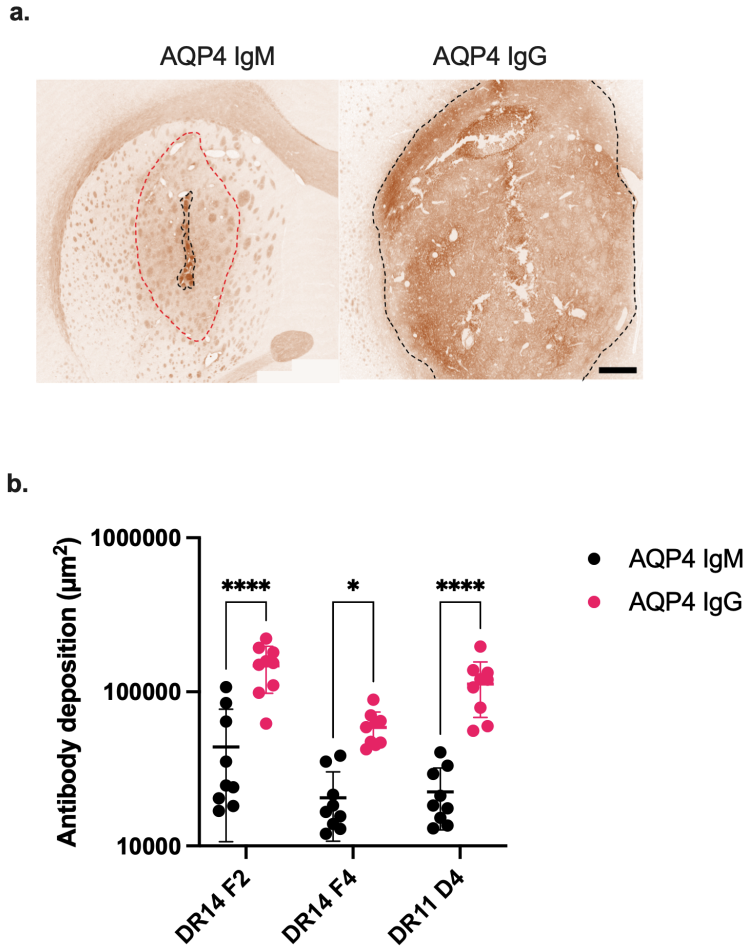


Figure 6.12. Differential tissue distribution of AQP4-IgM and IgG following intracerebral injections. Rats were injected with 3 mutated mAbs as IgM or IgG supplemented with 10% human complement. **a.** Sections were stained with isotype-specific secondary antibodies 3 days post-injections. Black dashed line demarcates an area of intense IgM or IgG staining. Red dashed line demarcates the penumbra, a wider area of less intense IgM immunoreactivity; **b.** Within individual IgM-IgG mAb pairs, larger areas of IgG deposition were consistently shown. Two-way ANOVA, Mean±SD, DR14 F2 $p < 0.0001$, DR14 F4 $p < 0.05$, D11 D4 $p < 0.0001$. 40x magnification, scale bar=200 µm.

6.4.8 Values of *in vitro* rat AQP4 binding assays in predicting *in vivo* functional activity

Human AQP4-IgM mAbs displayed unique patterns of reactivity to rat AQP4 substrates used in different binding assay platforms. We wondered if the observed *in vitro* binding profiles could predict an IgM mAb's *in vivo* functional activity. AQP4-IgM mAbs which were shown to be reactive in at least one of the assays (Table 6.1) were included in the analysis. There was a strong correlation between endpoint titres of IgM mAbs (n=8) on rat AQP4 live CBA with average areas of AQP4 loss at lesion epicentre from 3 injected animals per mAb ($r^2 = 0.7119$,

$p < 0.05$) (Figure 6.13a). The rat brain IIFA was proven valuable to predict an IgM mAb's *in vivo* pathogenic potential (binder vs non-binder, Mann-Whitney test, $p < 0.05$) (Figure 6.13c). Additionally, a trend of increasing size of areas lacking AQP4 expression was seen in animals injected with rat astrocyte-binders vs non-binders (Mann-Whitney test, $p = 0.09$) (Figure 6.13b).

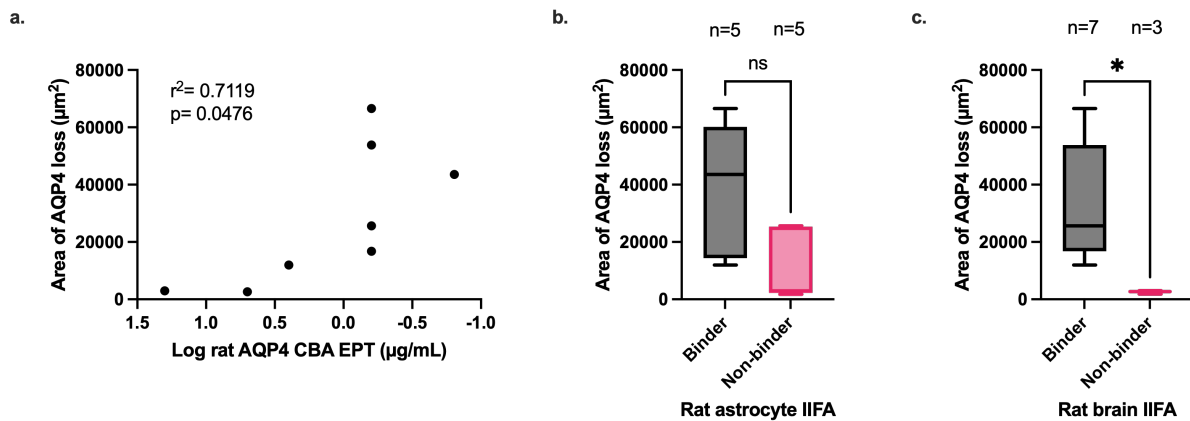


Figure 6.13. *In vitro* binding assays as a tool to predict human AQP4-IgM mAbs functional activity in an acute rat model of NMOSD.

a. Endpoint titres of mAbs ($n=8$) on rat AQP4 live-CBA correlated strongly with areas of AQP4 loss in the injected animals ($r^2=0.7119$, $p < 0.05$); **b.** There was a trend of increasing loss of AQP4 immunoreactivity in animals injected with AQP4-specific mAbs which bound rat primary astrocyte cultures (Mann-Whitney test, min to max, $p > 0.05$); **c.** Significantly more pronounced *in vivo* human NMOSD-like pathology was produced in animals injected with AQP4-IgM mAbs which were reactive to rat brain sections (Mann-Whitney test, min to max, $p < 0.05$). Numbers at the top of Figures b and c denote the total number of mAbs tested. AQP4, Aquaporin-4; CBA, Cell-based Assay; EPT, Endpoint titre; IIFA, Indirect Immunofluorescence Assay.

6.5 Summary

The use of animals in scientific research is a longstanding practice with a potential utility for advancing our understanding of human biology. In NMOSD, rodent models have allowed researchers to investigate the direct pathogenicity of IgG autoantibodies against AQP4 and the crucial role of the classical complement pathway as a disease mechanism. It supported the investigation of novel mAb therapies which disrupt antibody binding or interfere with the completion of the complement cascade, that are now approved after clinical trials. In addition to IgG and complement products, histopathological studies of human NMOSD lesions reveal significant deposits of IgM in active foci, suggesting its likely contribution to disease

pathogenesis. As a single molecule of IgM can fix complement it was thought that IgM would be highly pathogenic compared to IgG, which require multiple closely aligned Fc regions to activate complement. To address this hypothesis, we aimed to develop a robust animal model employing direct co-injections of patient-derived AQP4-IgM mAbs and human complement to naïve rat brains. The interspecies differences in AQP4 extracellular loop amino acid compositions and its surface membrane organisation prompted us to confirm the ability of human AQP4-IgM mAbs to native rat AQP4. Live CBA employing rat AQP4 M23-expressing HEK293T cells as a substrate demonstrated that not all human mAbs bound AQP4 of rat origin. IIFA using rat primary astrocytes and brain sections further discriminated mAbs which were reactive to rat AQP4 constitutively expressed *in vivo*. A few mAb candidates from different B cell lineages with rat brain section reactivity were subsequently selected for animal injections. A single intracerebral co-administration of an AQP4-IgG mAb supplemented with human complement was shown to produce human NMOSD-like lesions, validating the proposed injection protocols. By applying the same method, we demonstrated that direct co-injections of AQP4-IgM and human complement induced the formation of lesions with loss of AQP4 and GFAP immunoreactivity, complement product deposition, and neutrophil infiltration. This pathology was more pronounced in animals receiving somatically hypermutated IgM mAbs. Nevertheless, we noticed a contrast in the degree of pathological changes caused by AQP4-IgM by comparison to IgG. An exploratory analysis comparing a set of mutated mAbs as IgM and IgG showed reduced tissue penetration of the former, which may explain the slightly dampened pathological response of IgM relative to IgG. However, we could not exclude the possibility that there were fewer injected IgM than IgG molecules as the injection dose was based on µg amount rather than molarity. Lastly, we demonstrated that *in vitro* rat binding assays are a valuable set of tools to predict the functional activity of human-derived antibodies *in vivo*. In conclusion, we have developed an acute rat model of NMOSD involving direct

intracerebral co-injection of AQP4-IgM and human complement to naïve rats. Using this model, we provided evidence of the potential contribution of complement-activating, AQP4-IgM in NMOSD pathogenesis.

7 Chapter VII: Discussion

7.1 Introduction

Rational approaches to establish the autoimmune aetiology of a human disease have been drawn up since 1957 by the introduction of the Witebsky's postulates¹. The original criteria were relatively stringent as it was modelled after the Koch's postulates and thus, were revisited to accommodate the expanding concept of autoreactivity whereby some self-reactive immune cells represent a normal population of one's immune repertoire². The discovery of autoantibodies targeting exposed extracellular domains of surface-expressed neuroglial proteins over the past decades has led numerous studies to prove their direct pathogenicity and contribution to the observed, wide-ranged clinical syndromes.

Previously known as a variant of multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) has subsequently been considered as an independent entity following the discovery of autoantibodies of the IgG class against aquaporin-4 (AQP4), a water channel protein that is predominantly expressed on astrocyte end foot processes at the blood-brain barrier (BBB)^{12,13}. The acknowledged remarkable specificity of AQP4 serology status has driven revisions of the diagnostic criteria to recognise the various presentations of patients with NMOSD beyond the initially limited clinical description of the disease, for example, those presenting with extra optic-spinal CNS symptoms^{15,16,18}.

Earlier functional studies on the pathogenicity of AQP4-IgG mostly involved serum and cerebrospinal fluid (CSF) samples^{41,48,49}. The caveats of this approach are the restricted availability and the heterogenous contents of test materials, preventing detailed understanding of immunobiology which underlies the aetiology and propagation of the disease. The emerging single-cell technology involving isolation of single B cells from affected patients and generation of monoclonal antibodies (mAbs) has provided a novel and valuable tool to circumvent these limitations.

In this body of work, we adopted the single-cell approach to investigate how AQP4 specificity emerges along the B cell development and the potential contribution of AQP4-IgM to NMOSD pathogenesis. This study began with isolation and culture of single B cells from distinct B cell subsets found in the peripheral blood and thymic cell samples from four AQP4-seropositive NMOSD patients. The AQP4 specificity of the culture supernatants was evaluated to identify the earliest stage at which the specificity develops and to indirectly assess the integrity of the well-established B cell tolerance checkpoints. Additionally, the immunoglobulin gene features and the physicochemical properties of the AQP4-specific B cell receptors (BCRs) were characterised to gain information on how attempts in removing self-reactivity shape the AQP4-specific B cell repertoire in these patients. Following generation of mAbs, we characterised the evolution of binding affinity to AQP4 across B cell developmental stages. Finally, the direct pathogenicity of AQP4-IgM was examined *in vitro* and in an animal model.

7.2 Key findings

7.2.1 Defective central and partially functional B cell tolerance checkpoints in NMOSD

The numbers of clones expressing autoreactive BCRs generated by random V(D)J recombination are minimised in the early B cell repertoire through rigorous tolerance mechanisms involving clonal deletion, clonal unresponsiveness or anergy, or continued light chain gene rearrangement, also known as receptor editing^{64,84}. Studies using mAbs generated from sequential B cell subsets from healthy individuals have revealed that, in physiological circumstances, a central tolerance checkpoint in the bone marrow removes a substantial proportion of developing autoreactive early immature B cells⁶⁷. Upon egressing to the periphery, a smaller percentage of new emigrant B cells displaying reactivity to self-antigens are further eliminated by a peripheral tolerance checkpoint before entering the mature naïve compartment⁶⁷.

We identified four AQP4-seropositive NMOSD patients and two age-matched healthy controls from which peripheral blood mononuclear cell (PBMC) samples were available for this study. An additional thymic cell sample was also collected from one of the patients who was later found to carry a protein tyrosine phosphatase non-receptor type 22 (PTPN22) risk allele. PTPN22 gene polymorphisms have been associated with an increased susceptibility to developing autoimmune diseases including rheumatoid arthritis (RA), type-1 diabetes mellitus (T1D), and systemic lupus erythematosus (SLE)⁹⁶. The new emigrant and mature naïve B cell compartments of asymptomatic healthy individuals carrying one or two PTPN22 risk allele(s) of the R620W variant contained elevated frequencies of autoreactive clones compared to non-carriers, indicating a dominant effect of a single PTPN22 risk allele on altering the establishment of both central and peripheral B cell tolerance, even preceding the onset of autoimmunity⁷⁰.

Following single B cell isolation and culture of subsets downstream of each checkpoint, we reported that significantly higher frequencies of AQP4-specific new emigrant B cells were found in the PTPN22 risk allele-negative patients (0.48%) than healthy controls (0.1%). This frequency was doubled (1.15%) in the PTPN22 risk allele-positive patient. These observations suggest that there is a defective central tolerance checkpoint in NMOSD which promotes the emergence of early AQP4-specific B cells. This checkpoint defect appears to be independent to the PTPN22 gene polymorphisms and could potentially be worsened by the presence of the risk allele.

AQP4-specific B cells were absent in the compartments downstream of the new emigrant in healthy controls and were still detected in the NMOSD patients albeit in reducing frequencies. These collective findings demonstrate that the peripheral B cell tolerance checkpoint may be dysfunctional in these patients. Another possible scenario could be considered whereby the peripheral checkpoint may still be functional, but its counterselection

capacity is overburdened by the large number of AQP4-specific B cells in the upstream compartment, i.e., new emigrant. Nonetheless, the inefficient removal of the AQP4-specific B cells from the mature naïve repertoire in the peripheral blood may lead to an increased susceptibility to NMOSD.

7.2.2 AQP4-specific B cell repertoire in NMOSD displays unique immunoglobulin gene features

Diversification of BCRs during early B cell development in the bone marrow enables an individual to recognise a wide variety of foreign antigens with an intrinsic risk of generating non-functional and autoreactive BCRs. One of the means to achieve this is through random rearrangement of the V(D)J immunoglobulin gene segments in the heavy- and light-chain loci⁶¹. Enrichment of specific immunoglobulin heavy-chain variable (IGHV) gene segment(s) has been described in SLE (IGHV1, 3 and 4)^{97,98}, MS (IGHV1 and 4)⁹⁹, and RA (IGHV4)¹⁰⁰. Thus, we examined whether there are unique immunoglobulin gene features which may characterise the AQP4-specific B cell repertoire in NMOSD.

We found a trend towards IGHV2 being overrepresented in the AQP4-specific B cell repertoire (16.67% vs 4.15% found in the reference BCRs, Fisher's exact test $p=0.09$). In the presence of the PTPN22 risk allele(s), the predominant usage of IGHV4 gene family was almost significantly observed (46.15% vs 24.13% found in the reference BCRs, Fisher's exact test $p=0.1$). However, it should be noted that these conclusions were drawn from comparisons of IGHV gene family distributions between a limited number ($n=25$) of AQP4-specific BCR sequences in this study and 4,890 unique sequences obtained from 3 healthy volunteers as reported somewhere else⁸³. Analysis involving a larger AQP4-specific sample size and a broader range of healthy volunteers is needed to confirm these potentially interesting findings.

In contrast to the heavy chain, the light chain immunoglobulin gene repertoire is relatively constrained. This is believed to be due to the need to control self-reactivity through

receptor editing which involves repeated light chain gene rearrangements^{64,84}. Compared to the HEK-reactive and non-reactive BCRs, the AQP4-specific BCRs were predominantly formed by lambda light chains. This biased usage was more pronounced when the analysis was selectively performed in the PBMC-derived BCRs. We again observed a potential influence of the PTPN22 risk allele(s) on the light chain usage whereby kappa light chains were more frequently used regardless of AQP4-specificity.

The joining of the V and J segments during kappa light chain gene rearrangements has been demonstrated to occur in an orderly fashion with a biased utilisation of the upstream IgJK segments^{85,86}. This controlled recombination, which is governed by the proximal kappa J transcript promoter, is thought to facilitate receptor editing⁸⁶. We found that IgJK1 only contributed 20% (vs 42% in 26 human BCR sequences from a published review⁸⁵) to the total distribution of IgJK gene families with a relative expansion of the downstream gene segment usage in the AQP4-specific BCRs. Interestingly, all these IgJK1 gene-containing BCRs were exclusively derived from the PTPN22 risk allele-positive patient.

The heavy chain complementarity determining region 3 (HCDR3) is thought to be the major determinant of antibody specificity as it is formed by the joining of the distal V, all D, and proximal J immunoglobulin gene segments. Random insertions/deletions of nucleotides between the joining segments further adds sequence variations in this region. The AQP4-specific BCRs were characterised by their long HCDR3s and positively charged and hydrophobic amino acid composition. This is in line with previous findings that longer HCDR3 lengths and higher proportions of positively charged amino acids are associated with autoreactivity although the exact mechanisms remain unknown^{67,101}.

This collective evidence suggests that AQP4-specific B cells exhibit immunoglobulin gene features as a consequence of defective B cell tolerance in NMOSD. Biased usage of the IgHV2 gene family and enrichment of BCRs with certain physicochemical properties may

increase the risk of generating specificity to AQP4. Extensive receptor editing occurs as one of the mechanisms to establish self-tolerance as indicated by the predominant utilisation of lambda light chain and downstream IgJK gene segments in the AQP4-specific BCRs.

Additionally, PTPN22 risk allele(s) may operate through different mechanisms in predisposing patients with NMOSD to develop AQP4 specificity. The frequent detection of the IgHV4 gene-containing, AQP4-specific BCRs in the PTPN22 risk allele-positive patient may reflect a state of general autoreactivity as IgHV4 has been associated with many autoimmune diseases^{97–99}. Reduced receptor editing, demonstrated by the higher proportion of kappa light chain and proximal IgJK gene segment usage, may also contribute to the persistence of AQP4-specific B cells in the peripheral blood of this patient.

7.2.3 Precise development of early AQP4-specific B cells gives rise to affinity-matured AQP4-specific antibodies

We have shown that AQP4 specificities are detected as early as the new emigrant stage. This agrees with results from a prior *in vitro* study in which both the pre-germinal centre (GC) mature naïve and post-GC memory B cells were demonstrated to have the capacity to secrete AQP4-specific antibodies⁷⁸. To gain a deeper understanding on the evolution of binding affinity of the AQP4-specific BCRs, we generated mAbs from the isolated single B cells from different B cell subsets and characterise their binding kinetics.

The unmutated mAbs demonstrated AQP4 binding exclusively as IgM whereas the mutated counterparts bound the antigen as both IgM and IgG1 antibodies, suggesting the reliance of the former on the overall higher avidity of the multimeric IgM to compensate for the low affinity of the individual antigen binding-sites. Indeed, characterisation of CBA endpoint titres and binding kinetics with all mAbs tested as IgM to allow cross-subsets comparisons revealed a sequential increase in affinity in the new emigrant, mature naïve, and mutated subsets, i.e., mutated double-negative and memory.

As described above, early autoreactive B cells are removed from the peripheral blood at two distinct checkpoints on the basis of the strength of interaction of the BCRs with self-antigens⁶⁷. BCRs that recognise multivalent ligands such as those present on cell surfaces either undergo receptor editing or are eliminated^{64,102}. The induction of an anergic state has been shown to occur in B cells which BCRs bind to soluble self-antigens, for example, soluble immunoglobulin and serum lysozyme¹⁰³. Anergic B cells bear little surface IgM with normal IgD expression, but their capacity to trigger intracellular signalling is greatly diminished¹⁰³. Another potential fate is termed clonal ignorance whereby B cells with affinity for self-antigens may mature normally. They are immunologically ignorant, perhaps because their self-antigens are normally inaccessible, or present in a relatively low concentration, or not strong enough to generate an activating signal upon binding¹⁰⁴. Reflecting on these, our observations suggest that precise maturation of early AQP4-specific B cells in the settings of impaired B cell tolerance in NMOSD may give rise to high-affinity AQP4-specific antibodies.

To further confirm this hypothesis, we generated unmutated common ancestors (UCAs) by replacing the somatically mutated nucleotides of the mutated mAbs with nucleotides of their presumed corresponding germline sequences according to the IMGT database. We then tested their AQP4-specificity as multimeric IgM to replicate a natively unmutated antibody. The UCAs were shown to retain their ability to bind AQP4 albeit with reducing affinity, providing evidence that mature AQP4-specific B cells may emerge from the activation of early B cells with relatively lower initial affinity for AQP4 augmented by somatic hypermutations. Similar observations whereby UCAs bind their respective self-antigens are also obtained from studies on peptidyl arginine deiminase (PAD)-specific antibodies in RA¹⁰⁵ and broadly neutralising antibodies against influenza virus antigens¹⁰⁶. Our results, however, are discrepant with an initial study on NMOSD⁷². UCAs were reported to lose their AQP4 specificity upon reversion of somatic mutations. The expression of UCAs as IgG in that particular study may explain this

discrepancy as we have shown in one of our experiments that unmutated AQP4-specific mAbs preferentially bind as multimeric IgM than monomeric IgG antibodies.

7.2.4 AQP4-IgM antibodies display complement-dependent cytotoxicity *in vitro*

Activation of the classical complement pathway is thought to be the main molecular mechanisms by which CNS inflammation and astrocytopathy develops in NMOSD⁶⁰. Whilst the direct pathogenicity of AQP4-IgG has been extensively studied and consistently shown through numerous *in vitro*^{41,43} and rodent studies^{48,49}, the potential contribution of AQP4-IgM to disease pathogenesis remains elusive.

In this study, we reported that AQP4-IgM had the capability to induce complement deposition on the surface membrane of AQP4 M23-expressing cells *in vitro*. The half-maximal concentration (EC50) values of AQP4-IgM from different subsets were relatively diverse and did not necessarily reflect the stage of B cell development. Of all, the mutated double-negative IgM mAbs displayed the highest CDC activities whereas the memory IgM mAbs were shown to have comparable CDC activities to those from the unmutated, early mAb subsets. Our initial suspicion was that the artificial expression of the mutated memory mAbs as IgM antibodies impeded their natural CDC activity. However, upon cross-isotype comparisons of the 6 mutated mAbs (3 mutated double-negative and 3 memory mAbs), the majority of them exhibited superior complement-fixing abilities as IgM than IgG1 antibodies on a per molecule basis. This agrees with what is reported in the literature that IgM is more efficient in activating complement than IgG^{74,92,93}. It is mainly because two or more IgG molecules held within 30-40 nm to one another, as opposed to only one multimeric IgM molecule, are needed to achieve the binding energy required for C1q activation^{45,76}.

To further explore the link between B cell maturation and CDC potential, we assessed the ability of the UCAs to fix complement. Reduced CDC activities were observed in all UCA-IgMs in comparison to the corresponding mutated parental IgM mAbs. This suggests that, in

parallel with binding affinity, somatic hypermutations play a key role in enhancing CDC activity of individual AQP4-specific mAb lineages. Stabilisation of bound antibodies on the cell membrane facilitated by high affinity antibody-antigen interactions may promote efficient engagement with C1q molecules, resulting in their optimal activation.

7.2.5 Co-injections of AQP4-IgM and human complement produce human NMOSD-like lesions in naïve rats

To examine the pathogenic potential of AQP4-IgM antibodies *in vivo*, we developed an animal model employing intracerebral co-injections of AQP4-IgM mAbs and human complement in rats without pre-existing neuroinflammatory conditions. First, we evaluated the ability of human AQP4-IgM mAbs to recognise 3 different rat AQP4 substrates representing different complexities of antigen expression, namely, rat AQP4 M23-expressing HEK293T cells, rat primary astrocyte cultures, and rat brain tissues. An unexpected and interesting finding is that not all human AQP4-IgM bind rat AQP4. There are notable extracellular amino acid differences between AQP4 of human and rat origin despite the very high homology between the two proteins. Although the crystal structure of AQP4-M23 is not yet elucidated, human and rat AQP4-M1 proteins differ in their lattice contacts and inter-tetramer distances in lipid bilayers²¹. Additionally, it has been shown that the membrane distribution of AQP4 in human astrocytes is less polarised than in the rodent counterpart, being only 2-3 folds (vs 5-10 folds in rodents) enriched in the end foot processes adjacent to the capillaries than in the parenchymal membrane⁹⁵. These interspecies differences may account for the observed discrepancies of human AQP4-IgM binding to human and rat AQP4.

AQP4-IgM mAbs from across different subsets were then selected for intracerebral injections on the basis of rat brain tissue reactivity. Brain lesions with features resembling human NMOSD including loss of AQP4 and GFAP immunoreactivity, deposition of human IgM and activated complement products, and neutrophil infiltration were successfully

reproduced by co-injections of AQP4-IgM mAbs and human complement. This pathology was most pronounced in rats receiving the mutated subset, followed sequentially by the UCA and the unmutated mAb subsets. These results suggest that AQP4-IgM antibodies may contribute to the formation of NMOSD lesions through activation of the classical complement pathway. Their pathogenic potential is further improved by incorporation of somatic hypermutations.

Rats were chosen in our model due to the presence of complement inhibitor(s) in mouse serum⁵¹ and the intrinsically low mouse complement activity^{50,51} which could potentially interfere with the production of human NMOSD-like lesions. Indeed, brain lesions were only successfully generated by passive transfer of serum NMO-IgG antibodies and human complement in mice⁴⁸. The pathology was absent in mice injected with the antibodies alone. On the other hand, rat and human effectors have been reported to have comparable levels of activity⁵⁰. Administration of serum NMO-IgG antibodies without human complement was shown to induce brain lesions with human NMOSD characteristics in naïve rats⁴⁹. However, our model was focused on addressing the capacity of AQP4-IgM in activating specifically human complement *in vivo* and thus, human complement supplementation was included in our injection protocols.

7.2.6 Antibodies with HEK-reactivity may belong to a class of naturally occurring antibodies

During initial screening of single B cell culture supernatants, we found a distinct group of B cells with reactivity to HEK293T surface membrane. Despite the largely similar distributions across subsets with those of AQP4-specific, there was not any significant reduction in the frequency of HEK-reactive B cells between the new emigrant and mature naïve compartments. This suggests that HEK reactivities were not removed by the peripheral B cell tolerance checkpoint as efficiently as AQP4 specificities from the B cell repertoire in NMOSD patients.

All HEK-reactive BCRs utilised the IgHV4 gene family, specifically IgHV4-34, and their binding was only observable when they were expressed as multimeric IgM antibodies. They did not show complement-fixing ability under optimised conditions used in our assays. IgHV4-34 has been associated with a broad range of antigen reactivities including DNA, rheumatoid factors, and commensal bacteria^{107,108}. Additionally, IgM antibodies found in cold autoimmune haemolytic anaemia which target the I/i carbohydrate residues on the surface of erythrocytes are also encoded by the same exact gene segment¹⁰⁹. We hypothesised that HEK-reactive antibodies may belong to a class of naturally occurring antibodies¹¹⁰. Their functions, whether it is protective or destructive, and their antigen target(s) are yet to be elucidated.

7.2.7 Study limitations

The acknowledged limitations include the relatively low numbers of patients and healthy controls studied. Considering that patients were at varying disease states and had received some forms of immunotherapy at the time of sample collection, we managed to perform detailed characterisation of approximately of 4,000 B cells per patient and a total of 25 generated AQP4-specific mAbs. Whilst we did not anticipate the presence of a PTPN22 risk allele in one of the patients, we accounted for this potential confounding factor during data analyses. To allow cross-subsets comparisons, we adopted a study design whereby all mAbs including those of the mutated subsets expressed and tested as IgM antibodies. This artificial system may have an impact on the natural ability of some mAbs to interact with their antigens and to fix complement. Whilst we reverted identifiable mutations in the V(D)J gene segments including the CDR3 regions to generate UCAs, the mutational status of nucleotides in the non-template (N) regions was impossible to determine and thus, the reversion accuracy is unclear. Finally, our *in vivo* experiments were limited by a few select mAbs which demonstrated reactivity to rat brain tissues.

7.2.8 Future avenues

Despite our limited observations, there could be distinct mechanisms by which NMOSD develops in PTPN22 risk allele(s) carriers and non-carriers. PTPN22 gene polymorphisms have been identified as one of the strongest genetic risk factors associated with many autoimmune diseases including RA, T1D, and SLE, but not MS⁹⁶. Future studies aiming to characterise the AQP4-specific B cell repertoire from larger cohorts of patients with and without the PTPN22 risk allele(s) may provide insights on how AQP4 specificity emerges across these two different genetic backgrounds.

Models on IgG CDC revealed the links between antigens and epitopes with complement activation^{44,45}. Monoclonal antibodies recognising certain epitopes of some antigens exhibit potent CDC activity, potentially due the intrinsic properties of the target antigens (size, density, and fluidity) and the IgG orientation on surface membranes as a consequence of structural constraints imposed by epitope geometry⁴⁵. In case of AQP4, it has been shown that His151 and Leu154 epitopes facilitate non-covalent interactions between neighbouring IgG Fc domains, resulting in enhance CDC on target cells⁴⁴. As we have demonstrated that AQP4-IgM antibodies possess classical complement activity, studying their epitope specificity and developing a model of IgM CDC would provide a framework for better understanding IgM-mediated complement activation in NMOSD and other human antibody-mediated diseases with potential benefits of identifying novel therapeutic avenues.

7.2.9 Conclusions

Through application of single cell technology and characterisation of monoclonal antibodies, we have provided key insights into the B cell immunobiology underlying NMOSD. In summary, defective central and partially functional peripheral B cell tolerance checkpoints may contribute to the early emergence of AQP4-specific B cells in this disease. The associated immunoglobulin gene features may be a cause or consequence of failure in establishing B cell

tolerance. Affinity-matured and highly pathogenic AQP4-specific antibodies may originate from the precise development of early B cells with relatively lower initial affinity to AQP4. Lastly, AQP4-IgM antibodies display classical complement activity *in vitro* and *in vivo* and thus, may play a role in the formation of lesions in NMOSD.

Appendices

Appendix 1: Leader PCR-1 primers

Primer	Ig chain	Sequence (5'-3')	Tm (°C)
CHG1	IgG heavy	GTTGTCCACCTTGGTGTGCTGG	64
VHL1	IgG/M heavy	CCATGGACTGGACCTGGAG	60
VHL1 mod	IgG/M heavy	CCATGGACTGSACCTGGAG	60
VHL2	IgG/M heavy	ATGGACATACTTTGCTCCAC	54
VHL2 mod	IgG/M heavy	ATGGACATACTTTGYTCCAC	54
VHL3	IgG/M heavy	CCATGGAGTTKGGGCTGAGCTGG	66
VHL4	IgG/M heavy	ATGAAACACCTGTGGTTCTT	53
VHL4 mod	IgG/M heavy	ATGAAACAYCTGTGGTTCTT	53
VHL5	IgG/M heavy	ATGGGGTCAACCGCCATCCT	65
CHM1	IgM heavy	GAAGCCAGCACCTGTGAGG	62
CK1	IgL kappa	ACACTCTCCCCTGTTGAAGCTCTT	69
VKL1	IgL kappa	CTCAGCTCCTGGGGCTCC	64
VKL1 mod	IgL kappa	CTCAGCTCCTGGGGCTYC	63
VKL2	IgL kappa	CTGCTCAGCTCCTGGGGC	65
VKL2 mod	IgL kappa	CTGCTCAGCTCYTGGGGC	64
VKL3	IgL kappa	GGAARCCCCAGCDCAGC	60
VKL4	IgL kappa	CTCTGTTGCTCTGGATCTCTG	57
VKL5	IgL kappa	GGGGTCCCAGGTTACCTCCTC	67
CL1	IgL lambda	TGAACATTCTGTAGGGGCCAC	59
VLL1	IgL lambda	CCTCTCCTCCTCACCTCCTC	64
VLL1 mod	IgL lambda	CCTCTCCTCCTCACCCTCCTC	63
VLL2	IgL lambda	CTCCTCACTCAGGGCACAG	60
VLL2 mod	IgL lambda	CTCCTCACTCAGGRCACAG	60
VLL3	IgL lambda	ATGGCCTGGAYCCCTCTCCTSCT	65
VLL3 mod	IgL lambda	ATGGCCTGGAYCCCTCTCCTBCT	70
VLL4a	IgL lambda	CTCCTCCTCTTCCCCCTCCCCCTC	70
VLL4a mod	IgL lambda	CTCCTCCTCCACTGCACAG	60
VLL4b	IgL lambda	TGGCCTGGGTCTCCTTCTACCTAC	65
VLL569	IgL lambda	ATGGCCTGGRCTCCTCTCCTYCTC	65
VLL710	IgL lambda	ATGGCCTGGRCTCCTCTCCTYCTG	66
VLL8	IgL lambda	ATGGCCTGGATGATGCTTCTCCTC	64

Appendix 2: Circular Polymerase Cloning (CPEC) PCR-2 primers

Primer	Ig chain	Sequence (5'-3')	T _m (°C)
CPEC VH1/5/7	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCG AGGTGCAGCTGGTGCAG	75
CPEC VH2	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTACCTTGAAGGAG	72
CPEC VH3	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AGGTGCAGCTGGTGGAG	74
CPEC VH4	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTGCAGCTGCAGGAG	75
CPEC VH3-23	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AGGTGCAGCTGTTGGAG	73
CPEC VH4-34	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTGCAGCTACAGCAGTG	74
CPEC VH1- 18/69	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTTACAGCTGGTGCAG	74
CPEC VH1- 45/1-58	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGATGCAGCTGGTGCAG	73
CPEC VH1-24	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTCCAGCTGGTACAG	73
CPEC VH3- 9/30/33	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AAGTGCAGCTGGTGGAG	73
CPEC VH6-1	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTACAGCTGCAGCAG	74
CPEC VH4-39	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGCTGCAGCTGCAGGAG	75
CPEC VH3- 33/11/30	Heavy	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTCA GGTGCAGCTGGTGGAG	74
CPEC JH1/2/4/5	Heavy	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACC AG	78
CPEC JH3	Heavy	GATGGGCCCTTGGTCGACGCTGAAGAGACGGTGACC ATTG	75
CPEC JH6	Heavy	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACC GTG	79
CPEC VK1	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG ACATCCAGATGACCCAGTC	72
CPEC VK1-9/1- 13	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG ACATCCAGTTGACCCAGTCT	72
CPEC VK1D- 43/1-8	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTGTG CCATCCGGATGACCCAGTC	74
CPEC VK2	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGG ATATTGTGATGACCCAGAC	72
CPEC VK2- 28/2-30	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGG ATATTGTGATGACTCAGTC	71

CPEC VK3-11/3D-11	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAG AAATTGTGTTGACACAGTC	69
CPEC VK3-15/3D-15	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAG AAATAGTGATGACGCAGTC	70
CPEC VK3-20/3D-20	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAG AAATTGTGTTGACGCAGTCT	70
CPEC VK4-1	Kappa	CTTTTCTAGTAGCAACTGCAACCGGTGTACATTCGG ACATCGTGATGACCCAGTC	73
CPEC JK1/2/4	Kappa	ATGGTGCAGCCACCGTACGTTTGATYTCCACCTTGGT C	76
CPEC JK2	Kappa	ATGGTGCAGCCACCGTACGTTTGATCTCCAGCTTGGT C	81
CPEC JK3	Kappa	ATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGT C	72
CPEC JK5	Kappa	ATGGTGCAGCCACCGTACGTTTAATCTCCAGTCGTGT C	72
CPEC JK150/3	Kappa	ATGGTGCAGCCACCGTACGTCTGATTTCCACCTTGGT C	73
CPEC VL1	Lambda	CTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCC AGTCTGTGCTGACKCAG	71
CPEC VL2	Lambda	CTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCC AGTCTGTCCTGACCTAG	73
CPEC VL3	Lambda	CTTTTCTAGTAGCAACTGCAACCGGTTCTGTGACCTC CTATGAGCTGACWCAG	74
CPEC VL4/5	Lambda	CTTTTCTAGTAGCAACTGCAACCGTTCCTCTCTCSCA GCYTGTGCTGACTCA	77
CPEC VL6	Lambda	CTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCA ATTTTATGCTGACTCAG	73
CPEC VL7/8	Lambda	CTTTTCTAGTAGCAACTGCAACCGGTTCCAATTCYC AGRCTGTGGTGACYCAG	71
CPEC CL reverse	Lambda	GGCTTGAAGCTCCTCACTCGAGGGYGGGAACAGAGT G	73
3' CL reverse	Lambda	CACCAGTGTGGCCTTGTTGCCTTG	74
panVK forward	Kappa	ATGACCCAGWCTCCABYCWCCCTG	67
3' CK494-516 reverse	Kappa	GTGCTGTCCTTGCTGTCCTGCT	73
3' CgCH reverse tiller	Heavy	GGAAGGTGTGCACGCCGCTGGTC	78

Appendix 3: Numbers of AQP4-specific and HEK-reactive B cells

Subject	Sample type	Screening	B cell subset			
			New emigrant	Mature naïve	Double negative	Memory
			IgM	IgM	IgM	IgG
Patient 1	PBMC	Total B cells (n)	384	864	768	768
		AQP4-specific (n)	3	1	1	1
		HEK-reactive (n)	3	7	3	0
Patient 2	PBMC	Total B cells (n)	960	960	1056	960
		AQP4-specific (n)	2	0	0	3
		HEK-reactive (n)	5	5	0	0
Patient 3	PBMC	Total B cells (n)	960	960	960	960
		AQP4-specific (n)	6	2	0	0
		HEK-reactive (n)	1	0	2	0
Patient 4	PBMC	Total B cells (n)	960	960	960	864
		AQP4-specific (n)	11	1	0	0
		HEK-reactive (n)	2	2	2	0
Patient 4	Thymus	Total B cells (n)	960	960	960	960
		AQP4-specific (n)	2	4	3	1
		HEK-reactive (n)	2	10	0	1
Healthy control 1	PBMC	Total B cells (n)	960	960	960	960
		AQP4-specific (n)	2	0	0	0
		HEK-reactive (n)	2	0	0	0
Healthy control 2	PBMC	Total B cells (n)	960	960	960	960
		AQP4-specific (n)	0	0	0	0
		HEK-reactive (n)	0	0	0	0

Appendix 4: NMOSD patient-derived monoclonal antibodies

Reactivity	Patient	Subset	mAb ID	Heavy chain				Light chain		
				IgHV	IgHD	IgHJ	CDR3 (aa)	IgLJ	IgLV	CDR3 (aa)
AQP4	1	NEm	DR11 C11	IGHV1-18*04	IGHD3-10*01	IGHJ3*02	ARVPGYYGSGSYRPPPHAFDI	IGLV2-23*02	IGLJ3*02	CSYAGSSFVW
AQP4	1	NEm	DR11 C6	IGHV3-7*01	IGHD5-18*01	IGHJ4*02	ARRAVGYSYGPRGGYYFDY	IGKV3-11*01	IGKJ2*01	QQRSGT
AQP4	1	NEm	DR11 C8	IGHV4-34*01	IGHD3-3*01	IGHJ6*03	AREMAYYDFWSGYRGLGDYMDV	IGLV3-19*01	IGLJ3*02	NSRDSSGNHLGV
AQP4	3	NEm	DR26 B5	IGHV3-30*04	IGHD3-10*01	IGHJ6*02	ASPLPPPDYYGSGSYRTYYYYGMDV	IGLV3-9*01	IGLJ2*01, IGLJ3*01	QVWDSSTFVV
AQP4	3	NEm	DR26 B8	IGHV3-30-3*01	IGHD3-10*01	IGHJ6*02	ARDPRRWYTYGSGSYRYYYYGMDV	IGLV6-57*01	IGLJ3*02	QSYDSPLV
AQP4	4	NEm	DR20 B5	IGHV4-39*01	IGHD5-18*01	IGHJ6*02	ARLRRGYSPGFGLSDYYGMDV	IGKV2-28*01	IGKJ5*01	MQALQTPPA
AQP4	4	NEm	DR20 B7	IGHV3-23*01	IGHD3-10*01	IGHJ6*02	AKGYGSGSYYPYTYYYYGMDV	IGKV1-12*01	IGKJ4*01	QQANSFPLT
AQP4	4	NEm	DR20 C3	IGHV3-74*01	IGHD5-18*01	IGHJ6*02	ARDKVEYSYGYRYYYYGMDV	IGKV1-5*03	IGKJ2*01	QQYNSPYT
AQP4	4	NEm	DR20 C4	IGHV1-69*01	IGHD3-16*02	IGHJ6*02	ARARPPAYDYVWGSYRHAPYYYYGMDV	IGLV2-23*02	IGLJ1*01	CSYAGSSTFYV
AQP4	4	NEm	DR20 D5	IGHV4-39*01	IGHD5-12*01	IGHJ5*02	ARHCHSGYGLYRPPVPPNNWFD	IGLV3-1*01	IGLJ1*01	QAWDSSTGV
AQP4	4	NEm	DR30 C7	IGHV3-53*01	IGHD2-2*01	IGHJ6*02	ARDRPYRSCSSTSCYGYYYYYGMDV	IGLV2-23*02	IGLJ3*02	QQYGSSPLIT
AQP4	1	MN	DR11 B4	IGHV2-5*02	IGHD6-13*01	IGHJ4*02	AHRRIAAAGGHFDY	IGLV1-40*01	IGLJ3*02	QSYDSSLSGWV
AQP4	3	MN	DR26 B3	IGHV2-5*02	IGHD3-10*01	IGHJ4*02	AHYYYGSGSTFDY	IGLV10-54*04	IGLJ3*02	SAWDSSLSAWV
AQP4	4	MN	DR20 B3	IGHV4-4*07	IGHD3-10*01	IGHJ4*02	ARDGAATYYYGSGSYLRFDY	IGLV1-47*01	IGLJ3*02	AAWDDSLSGWV
AQP4	4	MN	DR30 C2	IGHV5-51*01	IGHD5-24*01	IGHJ4*02	ARHLGRDGYNRSPRHLDY	IGLV3-1*01	IGLJ1*01	QAWDSSTYV
AQP4	4	MN	DR30 C3	IGHV4-59*01	IGHD6-13*01	IGHJ6*02	ARDIRGAAAGFYYYYYGMDV	IGLV1-40*01	IGLJ1*01	QSYDSSLSGYV
AQP4	4	MN	DR30 C4	IGHV4-34*01	IGHD5-12*01	IGHJ6*02	ARVVPRQWLRFNQVYYYGMDV	IGKV1-5*03	IGKJ1*01	QQYNSYSRT
AQP4	4	UDNeg	DR30 C10	IGHV3-23*01	IGHD5-18*01	IGHJ5*02	AKDFPRLYSYGRLNWFD	IGKV3-11*01	IGKJ1*01	QQRSNWPPTWT
AQP4	4	UDNeg	DR30 C11	IGHV3-73*02	IGHD6-13*01	IGHJ4*02	TRHGYSSSWDDGGRTTPEPL	IGKV4-1*01	IGKJ4*01	QQYYSTPLT

AQP4	1	MDNeg	DR11 D3	IGHV5- 51*01	IGHD5- 18*01	IGHJ4*02	ARRAFTYGGHHFDS	IGLV2- 14*03	IGLJ2*01	SSFPSSNTVL
AQP4	2	MDNeg	DR14 E11	IGHV3- 23*04	IGHD3- 10*01	IGHJ6*02	AKDFLSYYGSGNYPPLGIDV	IGLV6- 57*03	IGLJ3*02	QSYGSSNWW
AQP4	2	MDNeg	DR14 F2	IGHV4- 39*01	IGHD3- 9*01	IGHJ4*02	ARRHDYDLSTGRRYYFDY	IGKV1- 39*01	IGKJ3*01	HQTYNIPGT
AQP4	1	Mem	DR11 D4	IGHV3- 48*03	IGHD3- 9*01	IGHJ6*03	ARDPLNMLTGYHRSPYYKSYMDV	IGKV3- 20*01	IGKJ2*01	QVYGASPGT
AQP4	2	Mem	DR14 F4	IGHV3- 7*01	IGHD3- 10*01	IGHJ6*01	ARIRDQDLYGTGKSNNYSSGLDV	IGLV1- 47*01	IGLJ2*01	AAWDNSLSRLL
AQP4	4	Mem	DR20 D10	IGHV4- 59*11	IGHD3- 22*01	IGHJ4*02	ARVGFVGRYYYYDSSGYFYFAY	IGLV3- 1*01	IGLJ1*01	QAWDSDTFYV
HEK	1	NE	DR11 C3	IGHV4- 34*01	IGHD5- 18*01	IGHJ4*02	ARGGRGYSYGYGFDY	IGKV1- 39*01	IGKJ3*01	QSYSTPFT
HEK	1	NE	DR11 C5	IGHV4- 34*01	IGHD1- 26*01	IGHJ4*02	ARVPSGSYRRYFDY	IGKV1- 39*01	IGKJ2*01	QSYSTPYT
HEK	1	NE	DR11 C9	IGHV4- 34*01	IGHD3- 22*01	IGHJ4*02	ARGRGYYYDSSGYCYFDY	IGLV2- 11*01	IGLJ2*01	CSYAGSYTVV
HEK	3	NE	DR26 B4	IGHV4- 34*01	IGHD2- 15*01	IGHJ4*02	ARVVPRQWLRFNQVYYYGMDV	IGLV6- 57*04	IGLJ2*01	QSYDSSIVV
HEK	4	NE	DR20 C5	IGHV4- 34*01	IGHD6- 19*01	IGHJ6*02	ARGRRSSGWYYYGMDV	IGLV1- 44*01	IGLJ3*02	AAWDDSLNGPHWV
HEK	4	NE	DR20 C8	IGHV4- 34*01	IGHD5- 24*01	IGHJ4*02	ARGPKMATIKGLDY	IGKV4- 1*01	IGKJ1*01	QYYSTPRT
HEK	4	NE	DR20 C9	IGHV4- 34*01	IGHD2- 15*01	IGHJ4*02	ARGSRDYCSGGSCYPLHARTYFDY	IGKV1- 39*01	IGKJ5*01	QSYSTPPIT
HEK	1	MN	DR11 B2	IGHV4- 34*01	IGHD5- 18*01	IGHJ4*02	ASTAPRGRYSYGKRDFDY	IGLV2- 18*02	IGLJ2*01, IGLJ3*01	SSYTSSSTVV
HEK	1	MN	DR11 B3	IGHV4- 34*01	IGHD1- 1*01	IGHJ3*02	ARGRHGRLRGAFDI	IGKV1- 39*01	IGKJ1*01	QSYSTPWT
HEK	1	MN	DR11 B6	IGHV4- 34*01	IGHD1- 14*01	IGHJ5*02	ARRPGGRWFDP	IGKV4- 1*01	IGKJ1*01	QYYSTPWT
HEK	1	MN	DR11 B7	IGHV4- 34*01	IGHD5- 18*01	IGHJ4*02	ARGLRGYSYGPRPFDY	IGKV3- 20*01	IGKJ1*01	QYGSSPVA
HEK	1	MN	DR11 B11	IGHV4- 34*01	IGHD5- 18*01	IGHJ6*02	AGGSYGFPYYYYYGMMDV	IGKV1- 17*01	IGKJ1*01	LQHNSYPRT
HEK	2	MN	DR14 E4	IGHV4- 34*01	IGHD3- 10*01	IGHJ4*02	ARDRKLYGPYYYGSGSYGGGWYFDY	IGLV1- 44*01	IGLJ2*0	AAWDDSLNGRV
HEK	2	MN	DR14 E5	IGHV4- 34*01	IGHD3- 10*01	IGHJ4*02	ASLRVKYYYGSGRVPADY	IGLV2- 14*03	IGLJ1*01	SSYTSSSPYV
HEK	4	MN	DR20 B2	IGHV4- 34*01	IGHD6- 13*01	IGHJ6*02	ARGVQQRVYYYYYYYGMMDV	IGKV3- 20*01	IGKJ1*01	QQSRT

HEK	4	MN	DR20 B4	IGHV4- 34*01	IGHD3- 9*01	IGHJ3*02	ARWSRRLRYFDWSPSHAFDI	IGLV2- 14*01	IGLJ1*01	SSYTSSSYV
HEK	4	UDNeg	DR20 D7	IGHV4- 34*01	IGHD6- 19*01	IGHJ6*02	ARISSGWHYYYYYGMVDV	IGKV1- 9*01	IGKJ4*01	QQLNSYPLT
HEK	4	UDNeg	DR20 D8	IGHV4- 34*01	IGHD4- 17*01	IGHJ4*02	ARGRLYGDYFPY	IGKV1- 8*01	IGKJ4*01	QQYYSYPLT
Non reactive	1	NEm	DR11 F5	IGHV4- 59*01	IGHD3- 3*01	IGHJ6*03	ARGGDFWSGYSQHYYYMDV	IGLV2- 8*01	IGLJ2*01, IGLJ3*01	SSYAGSNNLV
Non reactive	1	NEm	DR11 F6	IGHV3- 23*04	IGHD1- 26*01	IGHJ6*03	AKGSGSYYYYYYMDV	IGKV1- 12*01	IGKJ4*01	QQANSFPPT
Non reactive	1	NEm	DR11 F7	IGHV3- 30-3*01	IGHD2- 15*01	IGHJ4*02	VSGGSLIDY	IGKV3- 15*01	IGKJ1*01	QQYNNWLTWT
Non reactive	1	NEm	DR11 C4	IGHV3- 11*06	IGHD1- 26*01	IGHJ2*01	ASRESGSYDWYFDL	IGLV1- 40*01	IGLJ1*01	QSYDSSLGWW
Non reactive	1	NEm	DR11 C7	IGHV4- 39*01	IGHD5- 18*01	IGHJ6*03	ASEERIQLHDYYYYYMDV	IGKV3- 11*01	IGKJ4*01	QQRSNWPLT
Non reactive	1	NEm	DR11 C10	IGHV1- 3*01	IGHD6- 19*01	IGHJ6*02	ASSGGSSGWYHDYYGMDV	IGLV3- 1*01	IGLJ2*01, IGLJ3*01	QAWDSSTALVV
Non reactive	1	NEm	DR11 D2	IGHV3- 23*04	IGHD1- 1*01	IGHJ4*02	ASATVLTWNPGAY	IGKV2- 30*01	IGKJ1*01	MQGTHWPPA
Non reactive	4	NEm	DR20 B8	IGHV3- 48*02	IGHD3- 16*02	IGHJ4*02	ARRYDYVWGSYRYTGHHYFDY	IGKV3- 15*01	IGKJ3*01	QQYNNWPLT
Non reactive	1	MN	DR11 E2	IGHV3- 30*18	IGHD3- 3*01	IGHJ6*02	AKDRLRFLMDEYGMVDV	IGKV1- 33*01	IGKJ4*01	QQYDNLPLT
Non reactive	1	MN	DR11 E3	IGHV1- 46*01	IGHD2- 15*01	IGHJ6*02	ARDSVVAATGGVYGMVDV	IGKV1- 9*01	IGKJ2*01	QQLNSYPRT
Non reactive	1	MN	DR11 E4	IGHV3- 7*01	IGHD3- 10*01	IGHJ6*02	AREMVRGVIVYYYYGMDV	IGKV1- 12*01	IGKJ4*01	QQANSFPPT
Non reactive	1	MN	DR11 E5	IGHV3- 15*01	IGHD3- 3*01	IGHJ3*02	TTGNLYDFWSGYHDAFDI	IGLV2- 14*01	IGLJ2*01	SSYTSSSTHV
Non reactive	1	MN	DR11 E7	IGHV4- 34*01	IGHD6- 13*01	IGHJ4*02	ARRAAAYTRAVPLDY	IGKV3- 20*01	IGKJ4*01	QQYGSSPPLT
Non reactive	1	MN	DR11 E9	IGHV1- 3*01	IGHD3- 22*01	IGHJ3*02	ARNKPPRYYYDSSAYGAFDI	IGLV3- 21*03	IGLJ1*01	QVWDSSSDHLYV
Non reactive	1	MN	DR11 E10	IGHV1- 18*04	IGHD3- 22*01	IGHJ4*02	ARSAMIETSTAYFDY	IGKV1- 12*01	IGKJ4*01	QQANSFPPT
Non reactive	1	MN	DR11 E11	IGHV4- 34*01	IGHD2- 15*01	IGHJ3*02	ARGGRQDAFDI	IGKV1- 5*04	IGKJ4*01	QQYNSYSRT
Non reactive	1	MN	DR11 F2	IGHV1- 8*01	IGHD2- 8*01	IGHJ3*02	ARPGVPPQYDAFDI	IGLV3- 1*01	IGLJ2*01	QAWDSSTVV
Non reactive	2	MN	DR14 B2	IGHV3- 30*18	IGHD1- 26*01	IGHJ4*02	AKDAPSGDYFDY	IGLV1- 47*01	IGLJ3*02	AAWDDSLGWW

Non reactive	2	MN	DR14 B3	IGHV3- 30*02	IGHD3- 22*01	IGHJ4*02	AKDYDSSGYPGYFDY	IGKV3- 15*01	IGKJ2*01	QQYNNWPPYT
Non reactive	2	MDNeg	DR14 B10	IGHV4- 59*08	IGHD3- 10*01	IGHJ6*02	ARTLLWFGDSEVYGMDV	IGKV1- 39*01	IGKJ3*01	QQSYITGFT
Non reactive	2	MDNeg	DR14 C2	IGHV1- 2*06	IGHD4- 17*01	IGHJ4*02	CARGVAVKTVTKFDS	IGKV1- 6*01	IGKJ1*01	LQDYNYPWT
Non reactive	2	Mem	DR14 C4	IGHV1- 2*06	IGHD2- 8*01	IGHJ4*02	ARGVFDH	IGKV2- 30*01	IGKJ5*01	MQGAHWQGPIT

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