Patient-derived monoclonal antibodies as a precise molecular tool to understand the pathophysiology of Caspr2-antibody encephalitis

Sofija Paneva

Green Templeton College

University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2024

Посветено на баба ми Софија

И на брат ми Спасе

ACKNOWLEDGEMENTS

This thesis would have not come to fruition were it not for the contribution of some truly outstanding individuals. I will opt for a succinct ‘thank you’ here, and a lifetime of gratitude thereafter.

Prof Sarosh Irani has supervised every aspect of this project and steered the development of my research throughout. He has kindly supported my growth as a scientist and an individual, and relentlessly encouraged and pushed me towards achieving my goals. On a personal level, he has set an example of what it means to be both a remarkable clinician-scientist and a tenacious leader, and I sincerely hope to be able to channel what I have learned from him in my future life and career. Thank you Sarosh.

Prof Daniel Anthony oversaw all my *in vivo* experiments and taught me a great deal about the field of neuroimmunology, sparking what will probably be a lifelong interest in the area. His drive to continue trying even when initial experiments were not producing the desired results was often what kept me going despite failures in the lab. His research group and the regular PI And Researchers Training Initiatives were a major source of joy that I will cherish for years to come. Thanks Daniel!

Dr Mateusz Makuch’s input has been integral to the immunology work presented in this thesis, and his supervision and guidance in the lab have been second to none. Chapters 2 and 5 would have not been possible without him and his patience while discussing experimental data greatly helped in advancing my understanding of immunology. Thanks Mateusz!

Dr Adam Handel has analysed the phenotypic and genetic B-cell data presented in Chapter 5 and I am especially appreciative of his superb coding skills and readiness to discuss results and explain difficult concepts.

Dr Paddy Waters and Dr Bo Sun have provided a great deal of encouragement and insight over the years. Bo’s own PhD project laid the groundwork for my work and inspired many of my experiments, and Paddy’s questions have always managed to get me to think about the research in new and unexpected ways. Thank you both for the help and valuable advice.

The Oxford Autoimmune Neurology Group (Nuffield Department of Clinical Neurosciences) and the Experimental Neuropathology Lab (Department of Pharmacology) have been fantastic environments in which to learn and grow that have afforded me the privilege of crossing paths with people who I admire as scientists and even more so as individuals. To name but a few, Halwan, Ruby, Bryan, Paul, Sophie, and Cristine from OANG, and Karolina, Daan, Dan and Pippa from the Anthony Lab – you have been phenomenal colleagues who over the years have grown into some of the most dependable friends, inside the lab and outside of it.

I can’t go without mentioning my colleagues at the Oxford Autoimmune Neurology Diagnostic Lab, where I worked while writing my thesis in 2024. Mark, Hannah, Victor, Jake, your good spirit and energy made an otherwise challenging period that much more bearable. It has been a true pleasure to work with you.

While this is a graduate thesis, I must also acknowledge the crucial role of two mentors from my days as an undergraduate student, whose impact cannot be overstated.

Dr Melanie Ramberger was my supervisor when I first started in the lab as an FHS student in 2018. She taught me a great deal about the experimental work I would later apply in my project and kindly offered her help and guidance with my PhD application. Most importantly, she gave me the courage to apply in the first place. Thank you, Mel.

Professor Kristine Krug was a crucial influence during my studies that inspired me to pursue a career in research. Her approach to critical thinking and data interpretation have shaped me as an intellectual, and while my eventual deflection to immunology constitutes a betrayal of the highest order, it is incredible to think how much the concepts from our Neuroscience tutorials have stayed with me to this day.

The work presented here was supported by generous funding provided via the Wellcome Trust OXION Grant, and the Overseas Scholarship I received from Green Templeton College. I have especially benefited from the support of Prof Stephen Tucker and Ms Louise Samson over the years, both always just an email away.

I must not forget the contribution of the patients, without whom this work would not exist. It has been humbling to know your stories, and a privilege to play a small part in them.

On a personal note, my family and friends have been a constant pillar of love and joy. Choosing the order in which to thank them has been an ordeal on par with the writing of this thesis, and I am still unsure whether I got it right.

Prof Nensi Manuševa (University St Cyril and Methodius, Skopje) and Prof Lenče Miloševa (University Goce Delčev, Štip) have shown me incredible kindness and generosity over the years, and my life has been that much richer for knowing them. Thank you for continuously inspiring and tirelessly supporting me.

My friends from home, Živa, Oleg, Alek, Bobo, Andrej, Anastasija, Nebojša, have provided a much-needed break from the challenges of the PhD, often reminding me there is a world outside of it and being responsible for some truly amazing times while visiting Skopje. My friends from the UK, Milan, Sofia, Olivera, Melanie, Louie, Eva, Lukas, Francis, Shay, Juliet, and many others have made Oxford feel like home. I cannot thank them enough for always being able to put things into perspective, celebrating the highs and helping me restore my self-belief during the lows. Milan especially, thank you for being by my side every step of the way during my final year. The housemates at 15 Horwood Close, Ama, Tom, Dylan, Gianni, and Alex turned what would have otherwise been some very lonely nights of writing into sociable occasions accompanied by the sound of FIFA ‘15 and the occasional Harry Potter marathon.

In my culture, thanking one’s family is considered obsolete, if not slightly foolish: my grandma would always say we do not thank our parents, it’s a given they would do anything for us. So in lieu of a ‘thank you’, I chose to dedicate this thesis to my family: my brother Spase, my parents Maja and Angel, and my grandparents Sofija and Dušan. Having written over 50,000 words in this little book, I still struggle to find the right ones to give them credit for all they have done for me, but it goes without saying that all my accomplishments are theirs too.

And last but by no means least, I would like to thank myself, for sticking with it.

Statement on work done in collaboration with others

I am writing this document to clearly state which aspects of my thesis have involved work by collaborators.

In my research, I have used patient-derived monoclonal antibodies previously generated in the Oxford Autoimmune Neurology Group. I have based my own work on data generated by a PhD student in the lab previously (Dr Bo Sun). I will be using some of Dr Sun's preliminary data (with permission) in establishing the background of my thesis.

My own animal work has been guided by the work of a collaborator based at the University of Coimbra, Portugal (Dr Dominique Fernandes) and I will be using some of Dr Fernandes' data (with permission) as supporting figures in my thesis.

My immunology work I have done in collaboration with two senior Postdoctoral Scientists at the Oxford Autoimmune Neurology Group: Dr Mateusz Makuch and Dr Adam Handel. Both Dr Makuch and Dr Handel have aided with the analysis of the B-cell phenotyping and B-cell receptor sequencing data I have generated, and I will be using some of their graphs.

For characterisation of the domain specificity of B-cell receptors on my Caspr2-bait work, I have employed Caspr2 deletion constructs previously generated by a collaborator from the Oxford Neural Injury Group (Dr Teodora Trendafilova). This will be clearly stated in my thesis where data based on these constructs is presented.

My work on characterising serum samples from Caspr2 patients has involved collaboration with Dr Makuch and contributions from clinicians in the Oxford Autoimmune Neurology Group, namely Dr James Varley, Dr Christine Strippel and Dr Bryan Ceronie, who will be appropriately acknowledged in the respective chapter of my thesis.

All contributions of others and collaborations have also been stated in the Acknowledgments section at the beginning of the thesis. Wherever figures or data generated by others is used, they will be appropriately credited.

Signed:



Sofija Paneva *July 2024*
DPhil Candidate Ion Channels and Disease

Thesis Abstract

Caspr2-antibody encephalitis is a rare yet potentially treatable neurological disorder driven by autoimmunity against the neuronal cell adhesion molecule Caspr2. It holds particular importance in neuroimmunology as an exemplar of a single-antigen-driven autoimmune condition. In my doctoral thesis, I have set out to better understand both the neurological and the immunological aspect of the disorder. To achieve this, I employed patient serum samples, purified immunoglobulin G, circulating B-cells isolated from patient blood samples, and monoclonal antibodies (mAbs) cloned from these B-cells.

Patient serum samples were used to study the presence and levels of IgG1 and IgG4 subclasses of Caspr2-specific antibodies, and to correlate their concentrations to patient symptoms and disease progression. In keeping with previous findings, patient samples were found to dominantly contain Caspr2-IgG4 autoantibodies, but there was no clear association of 'subclass status' with symptom type or severity.

Using purified IgG from patients with active disease, a passive transfer animal model was developed. Stereotactic injections of the antibodies into mouse hippocampi were followed by a battery of behavioural tests seeking to examine what aspects of the human disease could be reproduced in rodents. Overall, in the rodent disease model, polyclonal IgG injections led to development of anxiety without other cognitive behavioural deficits.

To isolate the effects of Caspr2-specific reactivities, monoclonal antibodies derived from peripheral patient B-cells were used in subsequent experiments. These mAbs recapitulated some but not all aspects of the polyclonal IgG. The use of patient-derived mAbs also allowed to dissect the putative molecular mechanisms that might underlie observed behavioural deficits, and to relate these to fundamental features of the mAb, such as affinity and domain-specificity.

Finally, to investigate the immunobiological basis of Caspr2-antibody encephalitis, single B-cell cultures were used to generate a large panel of Caspr2-reactive mAbs. This work revealed both patients and healthy controls to carry Caspr2-reactive B-cells, albeit among distinct B-cell subsets. Caspr2-reactivity was limited to naïve B cells in healthy controls but observed among both naïve and memory B-cells in patients. Notably, differences in domain specificity and antibody affinity between patients and healthy controls were identified. Relating specificity to B-cell receptor sequences also helped to explore immunological features which may predispose patients, but not healthy controls, to development of disease.

My doctoral work has thus provided an in-depth and comprehensive view into the origins of Caspr2-autoantibodies and their downstream pathogenic mechanisms and has allowed for unique insights into the neuroscience and underlying immunology of this condition.

TABLE OF CONTENTS

i. Cover Page 1
ii. Acknowledgments 5
iii. Statement on work done in collaboration with others 7
iv. Thesis Abstract 8
v. Table of Contents 9
vi. Figures List 14
vii. Abbreviations 16
viii. Papers Published During DPhil 19

Chapter 1: Introduction
Preface 21
Section 1: The CNS and Immunity 22
1.1 Early Ideas on CNS Immune Privilege 22
1.2 Significance of CNS Barriers 22
1.3 The Blood Brain Barrier 23
1.4 What is CNS Immune Privilege? (And what is it not) 24
1.5 Afferent vs Efferent Immunity 25
1.6 Specialisations in Afferent Immunity: The Cellular and Soluble Route 25
1.7 Specialisations in Efferent Immunity 27
1.8 New Ideas on CNS Immune Privilege in the 21st Century 28
1.9 Dural Lymphatics and Our Understanding of CNS Immune Privilege 28
1.10 The Process of Neuroinflammation 30
1.11 Conclusion: The Exciting Field of Neuroimmunology 31
Section 2: The CNS and Autoimmunity 32
2.1 Encephalitis 32
2.2 Autoimmune Encephalitis 32
2.3 Treatment 33
2.4 Diagnostic Relevance of Autoantibodies 36
2.5 When is an Autoantibody Pathogenic? Witebsky's Criteria 37
2.6 Revisiting Witebsky's Criteria 37
2.7 Clinical associations of Caspr2-autoantibodies 38
2.8 Histopathology of Caspr2-autoantibodies 39
2.9 Are Caspr2-autoantibodies pathogenic? 39
2.10 In vitro Evidence of Caspr2-autoantibody Pathogenicity 41
2.11 Early Discoveries on Caspr2 43
2.12 Caspr2 Gene and Protein Structure 43
2.13 Caspr2 Expression and Localisation 44
2.14 Caspr2 Functions in the Nervous System 44

2.15 Conclusion: Caspr2 the Friendly Protein – should we ‘ghost’ it? 46
Section 3: Immunity 46
3.1 The Immune System 46
3.2 Early Discoveries 46
3.3 General Functions of Antibody Molecules 47
3.4 General Antibody Structure 47
3.5 The Five Classes of Immunoglobulin 48
3.6 Structure of the Immunoglobulin G (IgG) Molecule 48
3.7 IgG Subclasses 49
3.8 Monoclonal Antibodies (mAbs) 50
3.9 B-cells 50
3.10 B cell Development and Function 50
3.11 Roles of B-cells in CNS homeostasis 51
3.12 B-cells in autoimmune encephalitis 51
3.13 T-cells 52
3.14 Conclusion 52

Chapter 2: Serum studies in patients with CASPR2-autoantibodies reveal a predominance of the IgG4 subclass and potential clinical relationships
Preface 54
Introduction 55
Methods 57
Patients, Samples, and Clinical Data 57
Determination of End-Point Titre 57
CASPR2-Autoantibodies Subclass Quantification via Flow Cytometry 57
Data Analysis 58
Results 60
Breakdown of Subclass Prevalence 60
Changes in Caspr2-autoantibody Levels Over Time 60
Clinical Significance of Subclass Status 60
Subclass Status and a More Recent Classification of Caspr2-Phenotypes 64
Longitudinal Relationship Between Symptoms, Treatment, Titres, and Subclasses 64
Patients negative for both IgG1 and IgG4 Caspr2-autoantibodies 64
Discussion 66

Chapter 3: Purified IgG from sera of two patients with CASPR2-antibody encephalitis induces anxiety-related behaviours in vivo
Preface 70
Introduction 71
Methods 73

Immunoglobulin G Purification 73
Stereotactic Injections 73
Behavioural Testing 73
1. Open Field (OF) 74
2. Novel object recognition (NOR) test 74
3. Y-maze tests (YmSA and YmFA) 74
4. Light-Dark Box (LDB) 75
5. Elevated Zero Maze (EZM) 75
6. Forced Swim Test (FST) 75
Results 78
Experiment 1 78
Experiment 2 78
Experiment 3 79
Summary of Experiments 1, 2, and 3 79
Experiment 4 84
Experiment 5 84
Discussion 87

Chapter 4: *In vitro* and *in vivo* characterisation of patient-derived CASPR2 monoclonal antibodies
Preface 91
Introduction 92
Methods 93
Study approval, participants, and sample collection 93
Isolation of PBMCs 93
Cell Labelling, Fluorescence Activated Cell Sorting, and Cell Culture 93
Identification of CASPR2-reactive Immunoglobulin 94
cDNA Synthesis and Amplification of VH and VL Sequences 94
Monoclonal Antibody Production 94
1. Cloning 94
2. Expression 94
3. Purification 94
Chromogenic Endotoxin Quantification 95
Domain Specificity Mapping 95
Primary Hippocampal Neuronal Cultures 95
Immunohistochemistry 95
Field Recordings from Acute Hippocampal Slices 96
Electrophysiology on Cortical Neuronal Cultures 96
Stereotactic Injections and Behavioural Experiments 96

Results 99
Characterising patient-derived CASPR2-mAbs in vitro 99
Pilot in vivo experiment 99
Evolution of Behavioural Testing Protocols 100
Effect of CASPR2-mAbs on Memory and Anxiety Following Protocol Optimisation 100
Co-injection of mAbs E07 and E09 to Model Polyclonality 100
Injection of CASPR2 mAbs Leads to Neutrophil Recruitment in Limited Samples 104
Field Recordings from Acute Hippocampal Slices 104
Effect of CASPR2-mAbs on neuronal AMPA Receptors 104
Injection of mAb E08 Affects Anxiety-Related Behaviours 104
Comparison of mAb-injected Mice to Un-injected Naïve Controls 105
Discussion 110

Chapter 5: Studying CASPR2-reactive B-cells in patients with CASPR2-antibody encephalitis
Preface 115
Introduction 116
Methods 121
Patient Samples 121
Culturing ms40L Cells 121
Cell Labelling and Fluorescence-Activated Cell Sorting 121
Single Cell Culture and Supernatant Collection 123
Supernatant Screening 123
Cell Lysis 123
RNA Isolation 123
Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) 124
Nested PCR, Purification, and Sequencing 124
Data Analysis 125
Results 126
Screening of B-cell supernatants justifies the use of two bait constructs 126
CASPR2-reactive B-cells come from distinct subsets in patients vs. healthy controls 126
Varying domain specificities between patients and healthy controls 131
Relating B-cell phenotype of distinct domain specificities 131
Relationship between domain specificity and DRB1*11:01 status 139
UMAP Analysis allows for identification and characterisation of distinct Seurat clusters 139
Distinct BCR sequence characteristics between patients and healthy controls 145
BCR Clonal Analysis 145
Discussion 151

Chapter 6: Discussion
Preface 154
6.1 Summary of Findings 155
6.2 What have we learned? Significance for Caspr2- and CNS-(auto)immunity 157
6.3 Shortcomings of this thesis 160
6.4 Suggestions for future experiments 162

Bibliography 164

FIGURES LIST

CHAPTER 2

2.1	Summary of experiment design for Chapter 2	56
2.2	End-point titres and subclass prevalence among Caspr2-autoantibodies	59
2.3	Longitudinal levels of Caspr2-IgG, -IgG1, and -IgG4	61
2.4	Comparison of Caspr2-IgG and Caspr2-IgG4 variation over time	62
2.5	Clinical significance of CASPR2 subclass prevalence in patients	63
2.6	Clinical symptoms and immunotherapy relative to antibody levels	65

CHAPTER 3

3.1	Purified IgG Injections: Experiment 1	76
3.2	Purified IgG Injections: Experiment 2	80
3.3	Purified IgG Injections: Experiment 3	81
3.4	Purified IgG Injections: Experiments 2 and 3 Timeline	82
3.5	Purified IgG Injections: Summary of Experiments 1, 2, and 3	83
3.6	Purified IgG Injections: Experiment 4	85
3.7	Purified IgG Injections: Experiment 5	86

CHAPTER 4

4.1	Illustrated protocol for monoclonal antibody generation	92
4.2	Domain specificities of Caspr2-mAbs *in vitro*	97
4.3	Pilot *in vivo* experiment	98
4.4	Evolution of behavioural testing protocols	101
4.5	Effects of mAbs on memory and anxiety following protocol optimisation	102
4.6	Co-injection of mAbs E07 and E09	103
4.7	Neutrophil infiltration following mAb E07 injection	106
4.8	Field recordings from acute hippocampal slices following E07 injection	107
4.9	Effect of Caspr2 mAbs on neuronal AMPARs *(Dr Dominique Fernandes)*	108
4.10	Injection of mAb E08	109
4.11	Comparison of mAb-injected Mice to Un-injected Naïve Controls	110

CHAPTER 5

5.1	Illustrated protocol for Caspr2 Bait experiment	119
5.2	Relating Caspr2- and LGI1-positivity to FACS data	127
5.3	Distribution of Caspr2- and LGI1-reactive B-cells among memory and naïve B-cell compartments for healthy donors	128
5.4	Distribution of Caspr2- and LGI1-reactive B-cells among memory and naïve B-cell compartments for Caspr2 patients	129
5.5	Summary of the distribution of Caspr2-reactive B-cells for all participants	130
5.6	Domain specificity of CASPR2-positive B-cell supernatants	132

5.7	Domain specificity plotted against B-cell phenotype for Caspr2 patients	133
5.8	Domain specificity plotted against B-cell phenotype for healthy donors	134
5.9	Domain specificities superimposed upon B-cell phenotypes for patients and healthy donors segregated based on deletion construct	135
5.10	Domain specificities superimposed upon B-cell phenotypes for patients based on HLA-DRB1*11:01 haplotype	137
5.11	Seurat clusters following UMAP analysis of FACS data	140
5.12	Supernatant characteristics superimposed upon FACS Seurat Clusters	141
5.13	Further characterisation of cluster identity based on FACS parameters	142
5.14	Caspr2 bait status and BCR subclass vs. FACS Seurat cluster identity	143
5.15	Expression of IgD and CD27 in distinct clusters	144
5.16	Sample scans of DNA electrophoresis gels using nested PCR products	146
5.17	Characteristics of heavy chains and light chains across dataset	147
5.18	BCR clonotype expansion in Caspr2 patients	148
5.19	Clonal networks for Caspr2-positive and Caspr2-negative cells	149
5.20	BCR mutation rates in the IGH, IGK and IGL variable regions	150

ABBREVIATIONS

Ab – antibody

AE – autoimmune encephalitis

ALCAM – activated leukocyte cell adhesion molecule

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APC – antigen-presenting cell

AQP4 – aquaporin 4

ASC – antibody secreting cell

BAFF – B-cell activating factor

BBB – blood-brain barrier

BCG – bacillus Calmette-Guerin

BCR – B-cell receptor

BCSFB – blood-cerebrospinal fluid barrier

BEC – brain endothelial cell

BM – basement membrane

CA – Cornu Ammonis (hippocampal layer)

CAM – cell adhesion molecule

CASPR2 – contactin-associated protein-like 2

CBA – cell-based assay

CD – cluster of differentiation

CDR – complementarity-determining region

CNS – central nervous system

CP – choroid plexus

CSF – cerebrospinal fluid

DC – dendritic cell

dcLN – deep cervical lymph node

DG – dentate gyrus

DPPX – dipeptidyl-peptidase-like protein-6

DTH – delayed type hypersensitivity

EAE – experimental autoimmune encephalomyelitis

EEG – electroencephalogram

EPT – end-point titre

EZM – elevated zero maze

FACS – fluorescence-activated cell sorting

FBS – foetal bovine serum

FCS – foetal calf serum

fEPSP – field excitatory postsynaptic potentials

FPO – faecal pellet output

FST – forced swim test

GABAR – gamma-aminobutyric acid receptor

GAD – glutamic acid decarboxylase

GlyR – glycine receptor

GPCR – G protein coupled receptor

HC – healthy control

HLA – human leukocyte antigen

ICAM-1 – intercellular adhesion molecule 1 (CD54)

i.c.v. – intracerebroventricular

IgG – immunoglobulin gamma

IgLON5 – immunoglobulin-like cell adhesion molecule 5

IHC – immunohistochemistry

i.p. – intraperitoneal

ISF – interstitial fluid

IVIg – intravenous immunoglobulin

JXP – juxtaparanode

LDB – light-dark box (behavioural test)

LE – limbic encephalitis

LEC – lymphatic endothelial cell

LFA-1 – leukocyte function-associated antigen-1 ($\alpha_L\beta_2$ integrin)

LGI1 – leucine rich glioma inactivated 1

LLPC – long-lived plasma cell

LN – lymph node

LPS – lipopolysaccharide

LTP – long-term potentiation

mAb – monoclonal antibody

MACACS – membrane antigen capture activated cell sorting

MBP – major basic protein

mEPSC – miniature excitatory postsynaptic current

Mfsd2a – major facilitator super family domain containing 2a

mGluR5 – metabotropic glutamate receptor 5

MHC – major histocompatibility complex

MMP – matrix metalloproteinase

MOG – myelin oligodendrocyte glycoprotein

MoS – Morvan Syndrome

MS – multiple sclerosis

NMDAR – N-methyl-D-aspartate receptor

NMT – neuromyotonia

NOR – novel object recognition (behavioural test)

OF – open field (behavioural test)

PBMC – peripheral blood mononuclear cell

PDGFRB – platelet-derived growth factor receptor β

PLEX – plasma exchange

PMN – polymorophonuclear

PNH – peripheral nerve hyperexcitability

PNS – peripheral nervous system

PSGL-1 – P-selectin glycoprotein ligand 1

PVM – perivascular macrophage

RTX – rituximab

RT-PCR – reverse transcription polymerase chain reaction

SAS – subarachnoid space

scBCM – single-cell B-cell medium

SPR – surface plasmon resonance

TCR – T-cell receptor

TGF-β – transforming growth factor β

UMAP – Uniform Manifold Approximation and Projection

VCAM-1 – vascular cell adhesion molecule 1 (CD106)

VGAT – vesicular GABA transporter

vGLUT – vesicular glutamate transporter

VLA-4 – very late antigen-4 ($\alpha_4\beta_1$ integrin)

WT – wild type

YmFA – Y-maze forced alternation (behavioural test)

YmSA – Y-maze spontaneous alternation (behavioural test)

Papers Published During DPhil

Ramberger M, Berretta A, Tan JMM, Sun B, Michael S, Yeo T, Theorell J, Bashford-Rogers R, Paneva S, O'Dowd V, Dedi N, Topia S, Griffin R, Ramirez-Franco J, El Far O, Baulac S, Leite MI, Sen A, Jeans A, McMillan D, Marshall D, Anthony D, Lightwood D, Waters P, Irani SR. Distinctive binding properties of human monoclonal LGI1 autoantibodies determine pathogenic mechanisms. Brain. 2020 Jun 1;143(6):1731-1745. doi: 10.1093/brain/awaa104. PMID: 32437528; PMCID: PMC7296845.

Ramanathan S, Tseng M, Davies AJ, Uy CE, Paneva S, Mgbachi VC, Michael S, Varley JA, Binks S, Themistocleous AC, Fehmi J, Anziska Y, Soni A, Hofer M, Waters P, Brilot F, Dale RC, Dawes J, Rinaldi S, Bennett DL, Irani SR. Leucine-Rich Glioma-Inactivated 1 versus Contactin-Associated Protein-like 2 Antibody Neuropathic Pain: Clinical and Biological Comparisons. Ann Neurol. 2021 Oct;90(4):683-690. doi: 10.1002/ana.26189. Epub 2021 Aug 30. PMID: 34370313; PMCID: PMC8581990.

Ramirez-Franco J, Debreux K, Extremet J, Maulet Y, Belghazi M, Villard C, Sangiardi M, Youssouf F, El Far L, Lévêque C, Debarnot C, Marchot P, Paneva S, Debanne D, Russier M, Seagar M, Irani SR, El Far O. Patient-derived antibodies reveal the subcellular distribution and heterogeneous interactome of LGI1. Brain. 2022 Nov 21;145(11):3843-3858. doi: 10.1093/brain/awac218. PMID: 35727946.

Gövert F, Abrante L, Becktepe J, Balint B, Ganos C, Hofstadt-van Oy U, Krogias C, Varley J, Irani SR, Paneva S, Titulaer MJ, de Vries JM, Boon AJW, Schreurs MWJ, Joubert B, Honnorat J, Vogrig A, Ariño H, Sabater L, Dalmau J, Scotton S, Jacob S, Melzer N, Bien CG, Geis C, Lewerenz J, Prüss H, Wandinger KP, Deuschl G, Leypoldt F. Distinct movement disorders in contactin-associated-protein-like-2 antibody-associated autoimmune encephalitis. Brain. 2023 Feb 13;146(2):657-667. doi: 10.1093/brain/awac276. PMID: 35875984.

Ceronie B, Strippel C, Uy C, Paneva S, Makuch M, Soleimani B, Turaga S, Binks S, Ramanathan S, Michael S, Varley J, Easton A, Themistocleous A, Dawes J, Bennett DL, Irani A, Handel AE, Irani SR. Immunotherapy-Resistant Neuropathic Pain and Fatigue Predict Quality-of-Life in Contactin-Associated Protein-Like 2 Antibody Disease. Ann Neurol. 2025 Mar;97(3):521-528. doi: 10.1002/ana.27177. Epub 2025 Jan 18. PMID: 39825737; PMCID: PMC11831874.

Sun B, Fernandes D, Kienzler AK, Paneva S, Harrison R, Ramanathan S, Harrison AL, Makuch M, Fichtner ML, Donat RF, Akdeniz D, Bayuangga H, Im MG, Williams R, Vasconcelos A, Thomsen S, Fower A, Sun R, Fox H, Mgbachi V, Davies A, Tseng M, Handel A, Kelly M, Zhao M, Bancroft J, Bashford-Rogers R, Pluvinage JV, Dandekar R, Alvarenga BD, Dustin L, Rinaldi S, Owens R, Anthony D, Bennett DL, Waters P, Davis SJ, Wilson MR, O'Connor KC, Soltys J, Carvalho AL, Irani SR. Permissive central tolerance plus defective peripheral checkpoints licence pathogenic memory B cells in CASPR2-antibody encephalitis. bioRxiv [Preprint]. 2025 Jan 14:2025.01.14.631703. doi: 10.1101/2025.01.14.631703. Update in: Sci Adv. 2025 Apr 18;11(16):eadr9986. doi: 10.1126/sciadv.adr9986. PMID: 39868113; PMCID: PMC11760777.

CHAPTER 1

INTRODUCTION

Preface

In this introduction I will aim to provide a theoretical framework for my thesis, with the different sub-sections serving as groundwork to aid in better understanding concepts covered in following chapters. As this thesis ultimately focuses on an autoimmune disease of the nervous system, I will first provide context for the research via a general overview of the field of neuroimmunology. Seminal papers that led to the brain being considered an 'immune privileged' organ, as well as more recent work that led to a re-examination of that very idea will be discussed. I will then move on to looking at the interactions between the nervous and the immune system within the context of health and disease. This will serve as a segue into the expanding clinical field of autoimmune encephalitis. The autoimmune encephalopathies, their histories, and aetiologies will be covered as examples of how disruption of otherwise finely tuned neuroimmune interactions can wreak havoc within the brain. Since a subtype of autoimmune encephalitis where the antibody target is the neuronal cell adhesion molecule Caspr2 is the focal point of the thesis, this condition will receive special attention in the introduction. I will examine the key features of the Caspr2 protein, as well as the significance of Caspr2-autoantibodies. The field is not without its controversy, for the question of whether Caspr2-autoantibodies are pathogenic at all is still not fully resolved. As this is a central theme in the thesis, I will spend some time in the introduction addressing the question of what makes an autoantibody pathogenic, my own form of re-examining and re-weighting the original Witebsky postulates delineating what constitutes autoimmunity. Finally, I will conclude the introduction with a brief look at the key immunological concepts pertaining to this thesis, focusing mostly on antibodies and B-cells, their origins, classifications, and methods of action. With this, I hope to provide the reader with both a scientific overview, clinical insights, and discussion points on the principal themes covered in my doctorate.

SECTION 1: The CNS and Immunity

1.1 Early Ideas on CNS Immune Privilege

The brain has been traditionally viewed as a sensitive organ with poor capacity for regeneration requiring special protection during infection and inflammation, and a tightly controlled environment free of toxins and pathogens to support neural function.[1] In contrast to systemic infections, wherein robust immune cell infiltration is crucial for effective pathogen clearance, potent inflammatory responses in the CNS can sometimes cause more harm than good, with excessive immune cell infiltration and targeting of cerebral vasculature leading to pathogenic brain swelling and resulting in neuronal death, thus irrevocably damaging a cell population that for the most part cannot be replaced.[2-4] With this in mind, there has been a widely-propagated notion dating back for almost a century of a special relationship between the brain and the immune system, historically classifying the brain as an 'immune-privileged' tissue.[5]

Immune privilege has been first and foremost an experimentally defined phenomenon, based on the observation that tissues that were rapidly rejected by the immune system when grafted in sites such as the skin show prolonged survival when grafted into the CNS. Traditionally, the term 'immune-privileged site' thus applies to an organ in which implanted tissue grafts are incapable of provoking an immune response.[6] The immune privileged status of the CNS was initially attributed to its supposed isolation from the immune system via several barriers, the lack of draining lymphatics, and the apparent incompetence of CNS-resident macrophages, called microglia. However, research in the last decade has rediscovered the presence of lymphatic vessels lining major venous sinuses in the dura mater, and found resident immune cells in the meninges to play a role in both innate and adaptive responses, revealing this view was completely misconstrued and the CNS is neither isolated nor passive in its interaction with the immune system.[1, 5-12] In this section, I will discuss the history of the idea of the brain as an immune-privileged site as well as how this idea came to change and evolve.

As early as the 1920s, Shirai observed that rat sarcomas would grow well when transplanted into the mouse brain parenchyma, but not when implanted subcutaneously or intramuscularly, thus implying the brain parenchyma had a higher tolerance to 'foreign' material and enjoyed special protection from the immune system.[13] A few years later, Murphy and Sturm furthered these findings by demonstrating that if spleen tissue from the recipient was co-transplanted with the foreign tumour in the brain parenchyma, this would inhibit tumour growth.[14] This suggested that the observed survival of the tumour cells in the parenchyma was likely due to a disconnection between the brain and the systemic immune response. Over later years, these preliminary observations were confirmed for tissue grafts, bacteria, viruses, and vectors, all of which evaded immune recognition when delivered to the brain parenchyma.[15-18] Notably, in the 1940s, Medawar and colleagues grafted adult rabbit skin into the rabbit brain parenchyma and saw that the rejection rate of the graft in the brain was lower than that of a similar graft in the chest.[15] This led to the idea of the brain as a tolerogenic organ unable to induce or support an immune response. Collectively, these observations gave birth to the hypothesis that antigens are sequestered within the brain, rendered invisible and inaccessible to the immune system. 'Immunological privilege' was a term coined by Billingham and Boswell in 1953 as a means of unifying these lines of thinking into a common paradigm.[19]

1.2 Significance of CNS Barriers

Around the same time as the delineation of the concept of immune privilege, the blood-brain barrier, its anatomy, and functions were being investigated in greater detail, and the two ideas gradually started to merge.[20] Anatomically, the CNS comprises several physical barriers that

help prevent contiguous spread of neurotrophic pathogens into the parenchyma, including the meninges, nasal epithelium-olfactory bulb interface, blood-cerebrospinal fluid barrier (BCSFB), and finally the blood-brain barrier.[1] Collectively, these barriers form an elegant infrastructure that impedes penetration of neurotropic organisms and tightly controls leukocyte invasion during systemic immune responses. These barriers and their roles in CNS immunity will be briefly reviewed, with special emphasis on the blood-brain barrier.

In vertebrates, the meninges is comprised of three protective layers: (1) the pia mater that intimately ensheathes the parenchyma, (2) a non-vascularised arachnoid mater, which together with the pia comprises the 'leptomeninges', and (3) a heavily vascularised dura mater.[21] The sub-arachnoid space (SAS) lies between the arachnoid and dura mater and is filled with CSF, continuously replenished via the secretory action of the choroid plexus (CP) epithelium lining each of the four ventricles.[22] Together, the SAS, CSF, and CP constitute the BCSFB, which is a specialised physical and biochemical barrier that helps maintain the CNS microenvironment by restricting entrance of toxic metabolites and facilitating removal of unwanted chemicals.[23] In the dura mater, the meninges contains lymphatic vasculature which has been shown to continuously drain CSF into the cervical lymph nodes in both rodents and humans.[24, 25] Underneath the meninges there is another barrier, the glia limitans superficialis, which is composed of surface-associated astrocytes that separate the pia mater from the brain parenchyma.[11] This kind of barrier is particularly important since the meninges is densely populated with immune cells whose access to the CNS parenchyma must be tightly regulated.[11]

1.3 The Blood Brain Barrier

The BBB establishes a separation between the blood and the CNS parenchyma, regulating CNS immune cell traffic and controlling haematogenic entry of systemic substances and other cells from the periphery.[1] Its unique anatomy is comprised of pericytes, brain endothelial cells (BEC), a basement membrane, and the glia limitans *perivascularis*. Surrounding neurons, microglia, and other immune cells also contribute to this anatomical structure, sometimes collectively called 'the neurovascular unit'.[26-28] The BECs are key components of the BBB possessing specific properties that distinguish them from endothelial cells in other organs: they have low transcytotic activity, are highly polarised, and express tight junctions that connect adjacent cells. Both tight and adherens junctions in BECs facilitate cell-to-cell crosstalk and enforce barrier functions.[26, 29-31] Furthermore, BECs express low levels of leukocyte adhesion molecules such as P-selectin under steady state conditions, limiting immune cell infiltration in the CNS, while also leaving an opportunity for these molecules to be upregulated and immune surveillance to be promoted during inflammatory states.[28, 32, 33]

In addition to the specialised capillary endothelial cells, a second key component of the BBB are the postcapillary venules which regulate entry of immune cells.[34] Perivascular macrophages (PVMs) localise to the perivascular spaces of these venules and hold immunoregulatory roles during steady state, but can be activated during inflammation or disease, thus connecting the CNS to the peripheral immune system.[35] To reach the CNS parenchyma from blood, immune cells would need to penetrate the vascular BECs, whose abluminal surface is coated by a thick basement membrane (BM) that separates BECs from pericytes and pericytes from astrocytes. This BM is formed by a fusion of the endothelial and glia limitans vascularis BM. Pericytes, which are the mural cells of blood microvessels, are embedded in the endothelial BM, whereas the glia limitans is composed of the parenchymal BM and astrocytic end-feet processes.[34, 36-39]

The association of pericytes with BECs is crucial for BBB maintenance, as it has been shown that mice deficient in a key pericyte growth factor, PDGFRB, have disruptions in tight-junction

distribution, thus leading to increased vascular permeability. Besides enhancing tight-junction formation, pericytes further limit permeability by decreasing leukocyte adhesion molecules expression via TGF-β signalling and regulating endothelial expression of Mfsd2a, which regulates BBB function by suppressing transcytosis in CNS endothelial cells.[40-44] Tight interplay between endothelial cells, astrocytes, and pericytes thus underlies the function and integrity of the healthy BBB.

Via tight junctions between brain endothelial cells, the basal lamina of these cells, and astrocyte end-processes, the BBB was envisaged to prevent immune cells patrolling the meninges from gaining access into the CNS, and to thus inhibit the efferent arm of the immune system. On the other hand, the absence of conventional lymphatic vasculature in the CNS parenchyma was thought to block the afferent arm of the immune system, as one could not imagine a way whereby CNS antigens would drain into peripheral lymphatic tissues.[5] What followed was a wrongful assumption that the immune privilege of the CNS is rigid and absolute. We know now that the brain and the immune system do in fact communicate in what constitutes a very fine-tuned delicate relationship, and immune privilege does not relate to the absolute absence of immunological components, but rather their elaborate regulation.[8, 10, 36, 45-52]

Indeed, studies have shown that circulating T-cells are able to cross the BBB in the absence of neuroinflammation, that foreign tissue grafts can in fact be rejected if transplanted into the cerebral ventricles instead of the brain parenchyma, and that CSF does drain into cervical lymph nodes, all of which have led to further re-examination of the question of how exactly immunologically privileged the brain is.[48, 49, 52-57] Whatever special immune status the brain does have, it is now clear that this is not solely facilitated by the BBB – the nature of the CNS micro-environment and specialised communication between the CNS and nearby lymphatic organs via the afferent arm of the adaptive immune system are in fact much more pertinent.

1.4 What is CNS Immune Privilege? (And what is it not)

Synthesising the ideas outlined above, a 2007 article[5] defines immune privilege as (1) relative, (2) confined to the CNS parenchyma, (3) applicable to both adaptive and innate immunity; (4) predominantly a result of specialisations in the afferent arm of adaptive immunity and (5) active as well as passive. On the other hand, immune privilege is not understood to be (1) absolute, (2) entirely explained by the presence of the BBB, (3) present in the meninges, choroid plexus, circumventricular organs, and the ventricles, (4) preserved after systemic immunization, (5) preserved at the extremes of age, (6) preserved in the inflamed CNS, (7) applicable to antibody production, and (8) entirely passive. These important caveats will be further discussed in the next few paragraphs.

Explaining the first feature of CNS immune privilege, its relativity, it is important to appreciate that all experiments performed in the 20th century compared the brain to peripheral organs and any CNS findings were therefore interpreted *relative* to the rest of the organism. We now know that immune responses in the CNS are not entirely absent, but just different to those in the periphery. For example, while injection of lipopolysaccharide in the skin elicits neutrophil and monocyte recruitment within 2 hours, in the brain such an *acute* myelomonocytic infiltration does not occur. The occurrence of any response is delayed to the third day after injection and only with 10-fold greater doses of LPS, while to approach the same density of brain-infiltrating monocytes as seen in skin, 100-fold higher doses are needed.[58]

The spatial aspect of CNS immune privilege is also important as the CNS is organised into different compartments: the parenchyma proper, the ventricles containing the choroid plexus

and cerebrospinal fluid, and the meninges (Subsection 1.2). These components show vastly different immunological responses. As early as Murphy and Sturm's experiments in the 1920s, it was shown that rejection of the foreign tumour implanted in the CNS could occur if the transplant approached the ventricles.[14] This has been demonstrated for other antigens: when injections had been made intracerebroventricularly (i.c.v.), foreign tissue grafts were rejected, Bacille Calmette-Guerin (BCG) resulted in delayed-type hypersensitivity lesions in the choroid plexus, and the influenza virus elicited humoral and cytotoxic T-cell responses.[17, 54, 59] This 'compartmentalization' of the immune privilege of the CNS was also shown for the innate immune system with i.c.v. injection of LPS into the choroid plexus inducing a myelomonocytic response identical to that in skin.[58] All these experiments taken together demonstrate that immune privilege involving both the innate and adaptive immune response is limited to the CNS parenchyma proper, implicating the remaining compartments within the CNS as potential sites for neuroimmune crosstalk.

1.5 Afferent vs Efferent Immunity

Early discussions on the immune privileged status of the CNS centred around whether this is due to special adaptations in the afferent arm of the immune response, the efferent arm, or both.[5] The afferent arm of the immune response involves antigen presentation to naïve T-cells, which in most tissues will necessitate antigen transport to draining lymph nodes. The efferent arm on the other hand involves the consequent trafficking of innate (neutrophils and macrophages) and adaptive (T- and B-) immune cells into the immuno-challenged tissue.[60, 61]

Some early experiments which addressed the afferent versus efferent question involved the intracerebral injection of BCG or adenovirus, both of which were ignored by peripheral T- and B-cells, suggesting that CNS immune privilege might be the result of afferent arm deficiency.[17, 62] A key study by Medawar done much earlier in the century which involved pre-immunizing animals with skin-to-skin grafting prior to implanting the foreign skin graft in the brain parenchyma introduced this idea by demonstrating the efferent immune arm was relatively intact.[15] In other words, 'skin homografts transplanted to the brain submit to but cannot elicit an immune response', as Medawar himself stated. An even more sophisticated set of experiments that supported this notion was conducted by Matyszak et al., who injected BCG in the brain parenchyma of rats.[16] After allowing for the mild inflammatory response to subside and the BBB to recover from the insult, the researchers challenged the animals with BCG in adjuvant subcutaneously, which resulted in a delayed-type hypersensitivity (DTH) response accompanied by demyelination and axonal damage. This further supported the hypothesis that the efferent immune system arm in the CNS was intact, and it was in fact the afferent arm that is largely responsible for the adaptive immune privilege observed in the brain, though we will see in one of the following sections that the efferent arm also confers certain specialisations.

1.6 Specialisations in Afferent Immunity: The Cellular and Soluble Route

Let's have a further look into the specialisations of the afferent arm of the immune system within the context of the CNS. As mentioned earlier, the afferent arm involves antigen presentation to naïve T-cells, leading to their priming and activation. The transport of antigen can occur via a cellular route, whereby professional antigen-presenting cells (APCs) emigrate from the immune-challenged site into draining local lymph nodes, or via a fluid route, whereby soluble antigens drain into lymph nodes via bulk flow through lymphatic vessels.[5, 60, 61]

Two extracellular fluids are associated with the CNS: cerebrospinal fluid in the ventricles and the subarachnoid space (SAS) of the meninges, and interstitial fluid (ISF) in the parenchyma. Both fluids drain to cervical and lumbar lymph nodes, albeit via different routes.[63-65]

Cerebrospinal fluid is produced by epithelial cells in the choroid plexus and flows vectorially through the ventricular system, from the lateral ventricles to the third and then fourth ventricle, finally reaching the subarachnoid space in the meninges. From the SAS, CSF drains into cervical and lumbar lymph nodes via lymphatic vessels in the nasal mucosa, the dura mater of the meninges, and lymphatics associated with the sheaths of cranial and spinal nerve roots. Both the nasal and the dural routes have been shown to allow trafficking of APCs to the draining lymph nodes. Interstitial fluid circulates within the brain parenchyma and drains into cervical lymph nodes along very narrow restricted pathways that comprise the 150 nm-thick basement membranes in the walls of cerebral capillaries, arteries, and arterioles. These intramural perivascular basement membrane pathways are too tight to permit passage of APCs, which may represent a key factor in the immune privilege of the CNS parenchyma as opposed to CSF compartments like the ventricles and SAS. For a long time, there was no evidence that immune cells can traffic from the parenchyma via these narrow intramural perivascular pathways usually 100-150nm wide, leading to the prevailing belief that unlike in other tissues, the afferent arm of the immune response in the brain parenchyma lacks a cellular pathway for antigen transport and is instead heavily reliant on the fluid route.[66-68] This was supported with several lines of evidence, discussed in the next paragraph.

The specific type of cell that usually underlies cellular transport of antigen are dendritic cells (DCs) and macrophages. Immunohistochemical studies have failed to demonstrate presence of DC cells in the uninflamed brain parenchyma or perivascular space, although they have been found in the meninges and choroid plexus.[69] Macrophages carrying myelin antigens have been described in the cervical lymph nodes of monkeys with experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS) patients, but there was no supporting evidence that these cells migrate into the lymph nodes from the CNS parenchyma.[70] In contrast to the parenchyma, immune cell trafficking within the CSF that drains via nasal and dural lymphatics has been well-characterised, and these channels are known to carry CD4+ T-cells, monocytes, and dendritic cells into cervical lymph nodes.[68, 71-76] In fact, it is precisely the presence of these APCs in the CSF and their direct drainage pathways into lymph nodes that are thought to confer CSF spaces with immunological competence and enable rapid inflammatory responses, distinct from the immunological privilege of the parenchyma.[75, 77, 78]

Compared to immune cell trafficking, soluble antigen drainage from the brain parenchyma has been well-characterised and occurs quite rapidly.[66, 67] Typically, intramural perivascular ISF-drainage pathways allow for proteins of up to 150 kDa to pass along them, but larger particles 0.2-1 mm in diameter or proteins aggregated in the shape of immune complexes usually become entrapped and can impair drainage.[65, 66, 79, 80] Thus, radiolabelled albumin, which is below this size-exclusion threshold, can be recovered from deep cervical lymph nodes once injected in deep cerebral structures like the caudate nucleus.[81] Furthermore, antigen injected within the brain parenchyma and ventricles, as demonstrated by Harling-Berg et al., drains to cervical lymph nodes where it can elicit an antibody response (in fact, far superior to that achieved via intravenous or intralymphatic administration), but failed to elicit a DTH or an effective cytotoxic T-cell response.[82] This suggests a skewing towards humoral responses following immunization in the brain. It is also possible for an antigen to elicit a humoral response without even migrating to the cervical lymph nodes: DCs present in the non-immune-privileged regions of the CNS, like the CSF and meninges, can take up antigen, reach the cervical lymph nodes, and target B-cell areas preferentially, once again indicating a skewing towards a B-cell response in the CNS.[83, 84] There is even evidence for a tolerizing effect via this route: i.c.v. infusion of major basic protein (MBP) can result in protection against MBP-induced EAE, whereas when cervical lymph node cells isolated from mice injected with

ovalbumin in the striatum are transferred intra-venously to donor mice, this conferred protection from a DTH response to the same antigen.[85, 86]

To summarise, soluble CNS antigens can drain from the SAS into regional lymph nodes with the CSF flowing through nasal or dural lymphatic vessels, and this pathway also allows for the trafficking of immune cells.[8, 10, 48, 49] ISF on the other hand drains from the CNS parenchyma directly into lymph nodes along basement membranes in the walls of capillaries and arteries, and this intramural perivascular pathway is typically too narrow to facilitate the migration of immune cells directly from the CNS parenchyma into lymph nodes, thus perhaps playing a major role in relative immune privilege of the parenchyma as compared to CSF spaces.[61, 66, 87-89] Although the two fluids drain into lymph nodes by separate pathways, there is an interconnection between the two: low-molecular-weight tracers injected into the CSF can mix in with the ISF via passage through the glia limitans on the surface of the brain. This process, termed the 'glymphatic system' is regulated via the aquaporin 4 (AQP4) water channel on astrocytic end-feet at the glia limitans. Importantly, it provides another viable avenue for clearance of solutes, although at present its contribution to the drainage of antigens from the CNS parenchyma into regional lymph nodes is unclear.[90-92]

1.7 Specialisations in Efferent Immunity

Besides the afferent arm, there are also specialisations in the efferent arm in the form of unique subsets of long-lived antigen-presenting cells that mean immune responses mounted in the CNS differ vastly from those in peripheral organs. The CNS parenchyma is rich in microglial cells derived from haematopoietic precursors in the yolk sac, while perivascular and subarachnoid spaces harbour long-lived macrophages that arise from haematopoietic precursors during embryogenesis.[93, 94] Compared to peripheral tissues, where activated dendritic cells continuously migrate to lymph nodes via lymphatic vessels, these cells rarely migrate to CNS-draining lymph nodes, as evidenced by their low turnover rates. There are also no tissue-resident T- or B-cells in the CNS parenchyma, but some adaptive immune cells can be found in the CSF of healthy individuals, although when compared to other peripheral bodily fluids like the synovial fluid, their numbers are very low and the composition of the cellular subsets is unique, with a very low fraction of innate immune cells and high fraction of effector, effector memory, and central memory T-cells.[71, 72]

Passage of B- and T-cells across the brain barriers into the CNS in the absence of neuroinflammation is rare and highly regulated, typically restricted to CD4+ and CD8+ T-cells.[38] The cells face multiple obstructions in the form of the superficial (meningeal) barriers, the blood-brain barrier, and the blood-CSF barrier in the choroid plexus. Even when these cells do reach the CNS parenchyma, they are often faced with a plethora of challenges before they can exert their effector functions. In the context of T-cells, all cells in the CNS express fas ligand (fasL), which results in the apoptosis of incoming fas-positive T-cells, irrespective of their antigen specificity.[95, 96] If they do survive, T-cells will face tight regulation by CNS-resident microglia, astrocytes, and neurons. Astrocytes secrete soluble factors that can inhibit T-cell proliferation and cytokine production or favour a regulatory T-cell phenotype. Microglia create a microenvironment rich in immunoregulatory tryptophan metabolites by upregulating indoleamine 2,3-dioxygenase and express B7-H1. The latter interacts with T-cell programmed death protein PD-1 and inhibits T-cell activation and cytokine production. Neurons secrete TGF-β and make cell-to-cell contacts with activated T-cells that can convert them into Tregs independent of antigen specificity.[97-102] Even if T-cells manage to escape all these regulations, constitutive expression of MHC molecules is minimal in the normal uninflamed CNS, thus making it very unlikely for a T-cell to survive or recognise its cognate antigen, both processes

which require MHC.[103] With all these remarkable adaptations in mind, it is truly incredible any CNS autoimmune diseases exist at all!

In contrast to T-cells, B-cells can much more easily traverse into and exert their effects in the CNS, especially within non-immune-privileged areas. It is known that B-cells in these sites can also produce antibodies, as evidenced by the presence of unique oligoclonal bands in the CSF of patients with a variety of inflammatory CNS disorders. Furthermore, germinal centre-like reactions can occur locally in the CNS: formation of lymphoid follicle-like structures has been demonstrated in the meninges of mice with relapsing EAE, and similarly ectopic B-cell follicles with germinal centres have been detected in the meninges of patients with secondary progressive MS.[104-106] Why B-cells are not regulated to the same extent as T-cells in the CNS is still not fully understood but, as we will see in one of the following sections, this has special bearings on the field of autoimmune encephalitis, where autoreactive B-cells and their antibodies are the main contributors to pathology.

1.8 New Ideas on CNS Immune Privilege in the 21st Century

The CNS is still largely considered an immunologically privileged or, perhaps more accurately stated, immunologically unique site, but our understanding of the complexity of neuroimmune interactions occurring under homeostatic and pathological conditions continues to expand and thus the mechanisms of immune privilege have been further refined. In the last two decades, it has been demonstrated that (1) antigens derived from the CNS (and not just pathogens introduced into the CNS) drain into and can induce an immune response in deep cervical lymph nodes; (2) the CNS immune response can have both beneficial and detrimental effects on brain function; and (3) the CNS possesses a functional lymphatic system in the highly vascularised dura mater within the meninges that drains not just soluble but also cellular constituents from the CSF into the deep cervical lymph nodes.[36]

While CNS lymphatics were originally described as early as the 18th century (and again in the 20th), their modern age re-discovery has prompted the idea that brain ISF along with soluble neural components may drain into lymphoid tissues via subarachnoid CSF, providing a viable route for the afferent arm of the immune system in the CNS.[36, 107-109] The (re)discovery of the glymphatic system, which provides an avenue for efficient clearance of soluble compounds from the CNS parenchyma via the ISF into the CSF, has further supported this notion, thus highlighting the two fluids as important communication avenues between the CNS and the immune system.[87, 90-92, 110, 111] Substances are cleared via CSF-ISF exchange along canals made between venous walls and astrocytic end-feet, which comprise the so-called glymphatic system. This system allows the efflux of hydrophilic and lipophilic compounds, as well as proteins such as amyloid-beta (Aβ) via the water channel AQP4, suggesting an important role of this astrocytic protein in the bulk diffusion of fluid in the brain, which may be compromised with ageing.[90-92, 112-115] While the glymphatic system is known to allow for ISF and macromolecules from within the brain parenchyma to reach the CSF and eventually draining lymph nodes, it is yet unknown whether immune cells can leave the brain through the same path, although some have suggested a specific path for immune cells to leave the brain parenchyma via the rostral migratory stream.[77]

1.9 Dural Lymphatics and Our Understanding of CNS Immune Privilege

A pathway for lymphatic drainage of APCs via the CSF was recorded in rodents more than 150 years ago and in humans at the beginning of the 20th century, with the description of channels in the cribiform plate of the ethmoid bone that connect the SAS to lymphatic vessels in the nasal mucosa and ultimately drain to cervical lymph nodes.[117, 118] For over a century, this was

the only known route via which brain APCs access the peripheral immune system, but two 21st century articles have elegantly demonstrated the presence of conventional and functional lymphatic vessels located in the meningeal dura mater of the mouse, situated bilaterally along the superior sagittal sinus, and draining through the cribiform plate into the nasal mucosa.[8, 10]

Dural lymphatics were in fact probably first described by Mascagni as early as 1787, and it is worth mentioning that several past studies have also demonstrated the functionality of the dural lymphatic system.[45, 48, 49, 117] The latest works allowed for a more detailed characterisation by using a newly developed mouse meninges dissection technique and lymphatic cell reporter mice. Dural lymphatics were found to express all the hallmarks of lymphatic endothelial cells, (CD31, LYVE-1, podoplanin, VEGFR3, CCL21), to line the dural sinuses, and to exit the CNS at the base of the skull through the cribiform plate into the nasal mucosa. Importantly, T-cells, B-cells, and dendritic cells were all shown to be present in the meningeal lymphatic vessels under normal conditions, suggesting meningeal lymphatics participate in their trafficking out of the CNS and into the deep cervical lymph nodes under steady-state conditions.[10, 12]

Dural lymphatics are thought to enable fluids, macromolecules, and immune cells to drain from the CNS into deep cervical lymph nodes.[36, 75] Following their (re)discovery, several potential roles in brain immunity have been proposed, highlighting their role as a critical neuroimmune interface.[119-121] Meningeal lymphatics may be an important route for APCs as well as soluble factors from the brain to reach deep cervical lymph nodes, providing an opportunity for self-reactive T-cells in these sites to be primed against CNS antigens and initiate an autoimmune attack. Indeed, CNS-derived antigens in the CSF have been demonstrated to accumulate around the dural sinuses, where they were captured by local APCs and presented to patrolling T-cells, a process which promoted T-cell tissue resident phenotypes and effector functions in the dura mater.[12] A connection between the dural lymphatic and glymphatic systems has also been established, as transgenic mice lacking meningeal lymphatics had impaired clearance of macromolecules from the brain parenchyma into the CSF, and from the CSF into deep cervical lymph nodes.[8] These findings have generated much excitement about the possible connection between ISF clearance from the parenchyma into the CSF and later cervical lymph nodes, but the exact anatomical pathways, functionality, and significance remain to be determined.[122]

Interestingly, although perhaps in contradiction to the previous statement, the endothelial cells that line meningeal lymphatic vessels (LECs) have been proposed to play a role in maintaining CNS-reactive T-cells in meningeal spaces in an anergic state, suppressing brain-specific immune responses.[36] This idea has originated from studies in tumour-associated lymphatic vessels, where LECs express MHCI/II molecules presenting tumour-associated antigens that anergize tumour-specific CD4+ and CD8+ T-cells.[123, 124] A similar function may be performed by meningeal lymphatics and their failure to induce T-cell anergy may lead to T-cell activation in response to self-antigens and underlie the aetiology of an autoimmune attack in the brain, but experimental evidence to support this claim is still lacking. There is evidently still lots to learn about the roles of these structures in brain (auto)immunity and CNS immune surveillance, but their discovery has undoubtedly allowed us to start postulating how immune cells that drain from the brain interact with the peripheral immune system and how deep cervical lymph nodes may be involved in the tolerance to neural self-antigens. Future studies would benefit from focusing on the characteristics of the antigen presenting B-cells and dendritic cells that frequent these vessels and their potential roles in priming or preventing an immune attack on the brain. While the exact initiators of an immune attack on the brain remain to be characterised, much more is known about the resultant process of neuroinflammation, covered next.

1.10 The Process of Neuroinflammation

The movers and shapers of neuroinflammation have been extensively reviewed elsewhere,[125-129] so this section will only give a brief overview of the key players in the process. In this respect, two-photon microscopy and epifluorescence have been key developments in intravital microscopy that have allowed important translational insights into the neurovascular system of mice and rats, thus expanding our understanding of the cellular and molecular mechanisms that govern immune-cell migration across into the CNS.[129-134]

In the absence of neuroinflammation, passage of immune cells across the brain barriers is rare and typically restricted to immune cell subsets required for surveillance and detected in the CSF.[135, 136] This mostly includes CD4+ and CD8+ effector T-cells which extravasate into the parenchyma from subarachnoid venules via VLA-4/VCAM-1 mediated capture and arrest, GPCR-mediated adhesion, and LFA-1/ICAM-1 mediated diapedesis.[131, 137 71, 138] The trans-BBB migration of activated T-cells in a non-inflammatory state is, surprisingly, independent of antigen recognition on CNS endothelial cells, as demonstrated in rodent models using the framework of EAE.[130, 131, 133, 139, 140] Thus, T-cells cross the BBB in an apparently random manner irrespective of antigen specificity, MHC compatibility, cell phenotype, and TCR gene usage.[140] However, subsequent T-cell migration across the glia limitans and infiltration into the bona fide CNS parenchyma is contingent upon antigen recognition on perivascular and leptomeningeal APCs.[130, 134, 141] In the absence of CNS antigens presented by subarachnoid macrophages and dendritic cells, these T-cells are unlikely to cross the glia limitans and will probably be flushed away with the CSF.[142]

These conditions can change drastically in response to an immunogenic insult, with increased expression of adhesion molecules and proinflammatory cytokines and chemokines, combined with reduced expression of junctional molecules, all setting the stage for increased recruitment of encephalitogenic leukocytes across the BBB.[38, 63, 143] T-cells in particular may use different routes for entry into the CNS: they may cross subpial venules, subarachnoid veins, or the choroid plexus to reach the subpial space, the SAS, or the leptomeningeal spaces, respectively.[63, 144] Extravasation of T-cells across subpial venules and subarachnoid veins in the superficial brain barriers involves a multi-step cascade whereby rolling of T-cells is followed by their arrest, crawling against the direction of blood flow, and diapedesis across the vascular wall into the subpial space of the CSF-drained SAS.[38] During inflammation, T-cell rolling is facilitated by the *de novo* expression of P-selectin on leptomeningeal and parenchymal BBB endothelial cells, which interacts with PSGL-1 on T-cells, leading to a 100-fold decrease in T-cell speed.[145-147] Astrocyte-generated inflammatory chemokines are shuttled from the abluminal to the luminal side of the BBB via ACKR1, upregulated during EAE and MS.[148] GPCR-signalling leads to activation of integrins LFA-1 and VLA-4, whose interaction with endothelial ligands ICAM-1 and VCAM-1, mediates the firm arrest of T-cells on the luminal surface of inflamed BBB endothelial cells.[137, 149-151] This crucial role of the VLA-4/VCAM-1 interaction for T-cell arrest has been highlighted by the use of the humanised anti-α_4 integrin mAb natalizumab as the most effective treatment for management of relapsing remitting MS.[152]

Once arrested, T-cells polarize and begin to crawl against the direction of blood flow tethered by ICAM-1 and ICAM-2, in search of sites permissive to transcellular T-cell diapedesis, with increased levels of ICAM-1 facilitating this process.[130, 149, 153] T-cells that have successfully crossed the BBB endothelium have not yet reached the parenchyma proper but rather the CSF-drained perivascular or subarachnoid spaces.[129] To reach the parenchyma, they face yet another barrier: the glia limitans, whose biochemically distinct basement membrane constitutes of laminin α_1 and α_2 which effector T-cells do not bind.[154-156] Crossing the glia limitans requires

extravasated T-cells to be locally re-activated via recognition of their cognate antigens on perivascular APCs, a process that occurs only during neuroinflammation.[130, 141] If not, they will instead undergo apoptosis or be expelled from the CNS via CSF-drainage pathways.

If activated, T-cells will release proinflammatory cytokines that alter the characteristics and permeability of the endothelial BBB and trigger upregulation or induction of trafficking molecules, thus allowing for the recruitment of other immune cell subsets like myeloid cells across the endothelial brain barriers.[154] Local release of TNFs triggers production of matrix metalloproteinases (MMPs), specifically gelatinases MMP-9 and MMP-2 by the recruited myeloid cells, which in turn cleave β-dystroglycan on the membrane of astrocytic end-feet and lead to disintegration of the glia limitans.[157, 158] Along with the establishment of chemotactic gradients, this allows for subsequent infiltration of immune cells across the otherwise impermeable BBB into the CNS parenchyma. It is widely assumed that in the EAE model as well as in MS, clinical disease starts when immune cells cross the glia limitans.[129, 157]

Circulating B-cells also enter the CNS, either as part of normal immune surveillance or during pathologic states.[159] Under healthy steady-state conditions, B-cell numbers are very low in both the CNS parenchyma, where their density is approximately 0.1 cells/cm^2 and in the CSF, where they make up less than 1% of white blood cells.[160, 161] During CNS inflammation, these numbers can increase by at least several orders of magnitude in the parenchyma and perivascular spaces, and by severalfold in the CSF.[161, 162] In contrast to T-cells, the migration of B-cells across the BBB has been much less studied, perhaps in part due to the difficulty of isolating and maintaining B-cells in culture. A human BBB model involving human adult brain-derived endothelial cells (HBECs) has been used to study the determinants of human B-cell migration. In addition to showing that B-cells migrated across HBECs more efficiently than T-cells from the same individuals, the authors showed that blocking ICAM-1 and VLA-4, but not VCAM-1 significantly inhibited B-cell migration. This suggested that alternative ligands of $\alpha_4\beta_1$-integrin such as fibronectin or JAM-B may be distinct contributors to B-cell migration across the BBB. Blocking chemokines produced by BBB endothelial cells such as CCL2 and CXCL8, and even MMP-1, additionally reduced B-cell diapedesis.[163] Some more recent work using both mouse and human CNS barriers has highlighted the activated leukocyte cell adhesion molecule (ALCAM/CD166) as a driver of B-cell transmigration whose blockade alleviated disease severity in an animal model of B-cell dependent EAE.[164]

1.11 Conclusion: The Exciting Field of Neuroimmunology

This first section of the Introduction has outlined how the past few decades have seen a growing appreciation for the bidirectional communication that takes place between the nervous and immune systems within the context of disease and, perhaps more notably, outside of it. Not covered in this section, cells and molecules of the immune system have been shown to take part in modulating brain function via multiple signalling pathways throughout the lifespan, with cytokines and chemokines being able to affect cognitive and emotional processes, regulate neurotrophins and other molecules critical for neurodevelopment, and even influence synaptic plasticity.[165-168] Microglia have been implicated in the maintenance and regeneration of myelin, alongside their better-known roles with brain-resident macrophages during brain development and homeostasis, and the complement cascade has been unexpectedly found to guide synaptic pruning in a manner necessary for healthy brain function.[169-172] Stressors and challenges of an immune, physiological, and psychological nature will often engage cytokines and other immune signalling molecules to act as mediators of interactions with neuroendocrine, neuropeptide, and neurotransmitter systems.[173-176] Within the disease context, neuroimmune activation and neuroinflammation have both been shown to play a role in the aetiology of a

variety of neurological and psychiatric disorders such as stroke, Parkinson's and Alzheimer's disease, multiple sclerosis, chronic pain, and even major depressive and bipolar disorder.[177-184] Alongside discoveries on the role of the immune system in these established neuropsychiatric conditions, the last two decades of research have brought forth an autoimmune basis for several poorly understood neurological disorders. One such example was the establishment of an antibody-mediated autoimmune basis for some cases of encephalitis, discussed next.[185]

SECTION 2: The CNS and Autoimmunity

2.1 Encephalitis

A canonical neuroimmune condition, encephalitis is a severe inflammatory disorder of the brain parenchyma associated with neurologic dysfunction that can have many possible causes and a complex differential diagnosis.[186] The "gold standard" diagnostic test for this syndrome would, based on the definition, be a pathologic examination and testing of brain tissue, but this is rarely done premortem due to the potential morbidity of an invasive neurosurgical procedure. [187] In the absence of pathologic evidence, encephalitis has previously been defined based on clinical, laboratory, EEG, and neuroimaging criteria.[188-191] An inflammatory response in the CSF or the presence of parenchymal abnormalities on neuroimaging will often be used as surrogate markers of brain inflammation, but it is worth appreciating the added complexity of encephalitis sometimes occurring without CSF pleocytosis or neuroimaging abnormalities.[192]

In its acute form, encephalitis is a debilitating neurological disorder that develops as a rapidly progressive encephalopathy, usually in less than 6 weeks, caused by brain inflammation, with an estimated incidence of 5-10 cases per 100 000 inhabitants per year in high-income countries. [187, 193] It is important to distinguish between encephalitis and encephalopathy, two terms often used interchangeably in the literature: the latter refers to a clinical state of altered mental status manifesting as confusion, disorientation, behavioural changes, or other cognitive impairments, and this can occur with or without accompanying inflammation of the brain tissue.[187] Within the context of encephalitis, the underlying inflammation of the parenchyma that is causing the encephalopathy can be a consequence of direct infection, a post-infectious process as is the case in acute disseminated encephalomyelitis (ADEM), or a non-infectious condition, as is the case with autoimmune encephalitis.[186, 187, 191, 194, 195]

2.2 Autoimmune Encephalitis

The most frequently identified causes of encephalitis are infectious in nature, hence for a long time the diagnostic criteria and clinical guidelines for encephalitis relied on the assumption of an infectious origin.[187, 189, 191, 196] However, in the past 10 years, an increasing number of non-infectious causes of encephalitis that are autoimmune in origin have been identified, emerging as a frequent differential diagnosis when a classical infection cannot explain focal neurological symptoms.[195, 197-200] Autoimmune encephalitis normally presents with subacute onset of memory deficits and altered mental status (defined as decreased or altered levels of consciousness, lethargy, or personality change), sometimes accompanied by seizures and evidence of CNS inflammation, as well as a range of other symptoms and manifestations, depending on the subtype.[187, 201] This may include psychiatric disturbances, cerebellar or spinal cord involvement, movement disorders, autonomic dysfunction, sleep disturbances or pain.[202]

The postulated molecular mechanism is the presence of autoantibodies targeting neuroglial surface proteins, including synaptic proteins and ion channels.[203] Many autoantibody targets have been identified, including NMDAR, LGI1, CASPR2, MOG, AQP4, $GABA_BR$, $GABA_AR$,

AMPAR, DPPX, IgLON5, GAD, neurexin-3α, mGluR5, D2R and GlyR, each with their own clinical associations and biochemical intricacies.[204-219] These have been discussed extensively elsewhere.[201, 202, 220, 221] Knowledge and understanding of autoimmune encephalitis are rapidly evolving, with growth in the number of neuroglial autoantibodies discovered, identification of their target antigen and associated new syndromes, further crystallization of phenotypes, as well as expansion in the clinical spectra and diagnostic approaches. The subtype of AE that is the focus of this thesis, associated with antibodies against the neuronal cell adhesion molecule CASPR2, will be reviewed more extensively in the following subsections.

Currently, the diagnosis of possible autoimmune encephalitis relies on rapid progression of less than 3 months of short-term memory loss, altered mental status or psychiatric symptoms, fulfilment of at least one of the following four conditions: (1) new focal CNS findings, (2) seizures not explained by a previously known seizure disorder, (3) CSF pleocytosis or (4) MRI features suggestive of encephalitis, and finally, reasonable exclusion of alternative causes.[201] As can be imagined, these criteria are inherently quite vague and leave a lot at the discretion of the practicing physician. While in some cases the clinical course and MRI findings are sufficient to suggest a probable or definite AE without knowledge of antibody status, a substantial number of AE patients do not present with a well-defined syndrome and here the diagnosis will depend greatly on autoantibody test results. The significance of autoantibodies as biomarkers and/or as agents of pathogenicity will be discussed in subsection 2.4

2.3 Treatment

Acute treatment of neuroglial surface autoantibody-mediated CNS autoimmune diseases has traditionally followed a hierarchical approach of stepwise escalation of immunotherapies that tend to target the causative autoantibodies and/or B-cell biology. First-line immunotherapies include steroids, intravenous immunoglobulin, and plasma exchange. Second-line therapies include rituximab, cyclophosphamide, and bortezomib.[222, 223] Data from randomized controlled trials is generally lacking except in the case of neuromyelitis optica spectrum disorder (NMOSD), with the remaining categories of diseases in this family normally too infrequent for large-scale studies to be feasible.[224-226] While this may be true, larger cohort studies focusing on individual subtypes of AE have shown immunotherapy to be highly effective in managing symptoms: for instance, a study involving 103 patients with LGI1-antibody encephalitis has shown that while antiepileptic drugs alone stopped faciobrachial dystonic seizures (FBDS) in only 10% of patients, immunotherapy was effective in ceasing seizures in 51% of patients within 30 days of starting treatment, and in 88% within 90 days.[227] The same principle has been shown to hold true for other forms of autoimmune encephalitis.[228]

Corticosteroids are normally the first line of treatment that attenuates the immune response via genomic and nongenomic effects. They bind intracellular glucocorticoid receptors, suppressing transcription of multiple proinflammatory genes that encode cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors, and proteins.[229] These broad immunosuppressive effects lead to inhibition of B-cell proliferation, BCR-signalling, immunoglobulin gene expression, and plasma cell differentiation, but also result in depletion of T-cells, inhibition of Th1 differentiation, and dysfunction and apoptosis among innate immune cells such as macrophages and eosinophils.[230-233] Inhibition of IL-2 expression and of the transcription factor GATA3, a key driver of T-cell differentiation, could also reduce T-cell-dependent B-cell activation.[234] Corticosteroid therapeutic efficacy tends to vary across disease types, with faster effects in LGI1-antibody encephalitis than in NMDAR-antibody encephalitis or NMOSD, possibly due to higher clinical efficacy in IgG4-mediated diseases.[222, 228, 235]

Further first line therapies such as IVIg and plasma exchange (PLEX) may be considered in conjunction with corticosteroids for a more effective treatment of AE via the synergistic effects of broad immunosuppression and direct neutralization or removal of the autoantibodies from the circulation. IVIg is extracted from the collected pooled plasma of over a thousand donors and is used to provide passive immunity to immunodeficient patients.[230] High-dose IVIg can have broad and multimodal immunomodulatory effects such as autoantibody neutralization, FcγR blockade, complement, cytokine and leukocyte inhibition.[236] IVIg also accelerates IgG breakdown via competitive binding to the neonatal Fc receptor (FcRn), a homeostatic regulator of IgG catabolism.[237] Additional advantages of IVIg include the mild and transient side effects and its long half-life, with replacement therapies taking place in 3-4 week intervals.[223] PLEX on the other hand effectively removes autoantibodies from the plasma and changes lymphocyte numbers and distribution, T-cell suppression function and helper T-cell phenotype. [238] While PLEX and its more refined form 'immunoadsorption', which removes immunoglobulin from separated plasma, are not meant to affect CSF-antibodies unless the BBB is severely breached, reports have demonstrated 64% decrease in CSF-antibody titres at early follow-up.[239]

First-line immunotherapies are often insufficient in AE treatment, and observational studies have reported that second line ITs result in better functional outcomes with lower relapse rates and more manageable side-effects.[240, 241] The arsenal of later line immunotherapies available includes rituximab, cyclophosphamide, and bortezomib. Rituximab is a partially humanized monoclonal antibody directed against the glycoprotein CD20 primarily expressed on the B-cell surface. It was initially approved for the treatment of non-Hodgkin B-cell lymphomas but has since been widely used to treat various autoimmune diseases, including those affecting the CNS and PNS. It depletes both naïve and memory B-cells through antibody-mediated cellular toxicity, complement deposition and apoptosis induction.[242] Rituximab has been demonstrated to have high efficacy and safety as a second-line IT for AE, and to improve functional outcome as measured by the modified Rankin Scale (mRS) in a cohort of over 160 patients, even in cases without a proven neuronal autoantibody.[240] Substantial reduction in relapse rate has been demonstrated in patients with NMOSD, and effective depletion of short-lived plasmablasts has been reported in patients with NMDAR-antibody encephalitis.[225, 243, 244] The high effectiveness of rituximab in treating IgG4-related disease has also substantiated its use in LGI1-, Caspr2- and IgLON5-antibody encephalitis.[204, 235, 245, 246]

Despite its numerous benefits, rituximab therapy also has several shortcomings. Firstly, it does not target CD20-negative cells such as long-lived plasma cells (LLPCs), meaning that it won't affect autoantibody titres or produce severe immunosuppression.[244, 247] Furthermore, rituximab does not reset defective early B-cell tolerance checkpoints and is less efficient at depleting B-cells in secondary lymphoid organs or the CSF.[248-250] This means autoantibody-secreting cells (ASC) are spared and some tissue compartments are invulnerable to treatment, enabling newly formed autoreactive B-cells to repopulate the CNS and lead to treatment failure. NMOSD relapses have been temporally linked to the repopulation of memory B-cells, and only a small fraction of peripherally administered rituximab has been shown to reach the CNS.[225, 226, 251, 252] Finally, the B-cell depletion-repopulation cycle following long-term treatment with rituximab can result in the expansion of several enduring autoreactive B-cell clones, thus creating a clonally restricted B-cell repertoire whose persistence can drive ongoing disease.[253, 254]

Another second-line IT compared favourably to rituximab due to its low cost, and to first line ITs due to direct suppression of lymphocytic proliferation, is cyclophosphamide, an alkylating agent that crosslinks DNA via its metabolite phosphoramide mustard.[230] Cyclophosphamide prevents proliferation of activated B-cells but does not influence the non-proliferative LLPCs

which may continue secreting autoantibodies.[255, 256] Although not typically used in AE treatment due to its serious side effects, including infertility, the accumulated clinical experience of using cyclophosphamide in treating other autoimmune conditions and malignant lymphomas has justified its use in refractory cases.[223] Thus, it has comparable efficacy to rituximab in NMDAR-antibody encephalitis treatment and small studies have indicated it is effective in NMOSD.[257-259]

Despite their demonstrated efficacy as B-cell depleting agents in AE treatment, neither rituximab nor cyclophosphamide affect antibody-secreting plasma cells due to their CD20-negativity and non-proliferative nature. These populations, characterised by high rates of immunoglobulin synthesis, can be selectively targeted by bortezomib, a small-molecule proteasome inhibitor which leads to selective apoptosis of these cells and has been used in cases of severe therapy-refractory AE and highly relapsing NMOSD.[260-263]

Even with the plethora of treatments available, between 20-50% of patients have inadequate responses to second line ITs and exhibit residual impairments and persistent neurological issues.[240, 241] This not only justifies continuous research on the underlying neuroimmunology of the condition, as exemplified in this thesis, but has also prompted development of alternative approaches to treatment. Some of the options may include re-administration of first line ITs, extended use of second line ITs, or long-term maintenance with prednisolone (a corticosteroid) or steroid-sparing agents (SSAs) such as azathioprine or mycophenolate mofetil (MMF).[223] A more targeted therapy with therapeutic mAbs or direct infusion of immune mediators may also be considered, discussed next.

Tocilizumab, a monoclonal antibody that binds to both soluble and membrane-bound IL-6 receptors and blocks their interaction with inflammatory cytokine IL-6, can inhibit a variety of inflammatory cascades such as B-cell proliferation and differentiation into ASCs, development of IL-17-producing T-helper cells, and differentiation of $CD8^+$ cytotoxic T-cells.[264, 265] Its therapeutic efficacy has been demonstrated in small studies on therapy-resistant NMOSD, NMO, Caspr2-antibody encephalitis, and rituximab-refractory AE.[266-269] Direct administration of low-dose IL-2, a key regulator for the differentiation, survival, and function of regulatory T-cells (Tregs) has been proposed to selectively expand this population and promote tolerance over autoimmunity.[270, 271] However, IL-2 is a double-edged sword due to its concomitant effect activating effector and memory T-cells, hence the importance of using a low-dose to harness the lower activation threshold of Tregs compared to effector T-cells.[272, 273] This treatment avenue has been attempted in 10 patients with refractory AE, with modest but promising initial results, albeit accompanied by some serious side effects.[274]

This subsection has demonstrated the treatment options available for autoantibody-mediated neurological disorders, with first line therapies generally providing broad spectrum immune suppression and elimination of causative autoantibodies, and later line therapies targeting more specifically the immune mediators of disease, such as CD20+ B-cells, LLPCs, effector T-cells, Tregs, and the signalling cascades of IL-6 and IL-2. Despite ongoing research in the area, AE relapses have been noted even after 5-10 years due to the non-monophasic nature of the disease and relapse rates have been estimated at 10-20%, depending on antibody type.[275-278] Early aggressive immunotherapy has been shown to reduce relapse rates, but the role of maintenance therapy and its optimal duration, both key factors to attain the highest functional state possible and make sure the patient reaches complete remission, remain to be explored.[241, 278]

The rarity of AE has made conducting randomized controlled trials challenging and led to most IT agents in current use not having definite indications due to low levels of supporting evidence.[222] To make matters even more difficult, optimised regimens and dosing schedules are not yet determined, steps in acute management differ among clinicians, and there is no clear standard to rate treatment failure and estimate when to initiate a different IT agent. Finally, while studies cited in this subsection have shown that treatment response, duration, and outcome vary depending on the type of associated antibody, current AE immunotherapeutic regimens are not tailored to the individual antibody association.[223] Considering the proportion of patients with inadequate responses to first- and second line IT remains non-negligible, there is indeed considerable impetus for further research on AE immunobiology and possible biomarkers. International collaborations have proven paramount to establishing uniform treatment approaches and unifying diagnostic standards, discussed next.

2.4 Diagnostic Relevance of Autoantibodies

The initial diagnostic criteria for AE relied heavily on antibody test results and the response to immunotherapy, but more recently there has been a move away from strict and rigid adherence to these parameters.[201] The practical reasons for this have been that many institutions will not readily have access to antibody testing and obtaining test results can take up to several weeks. From a more scientific perspective, the mere absence of an autoantibody does not exclude the possibility that a neurological presentation is immune-mediated, or it may mean the antigenic target is simply unknown. On the other hand, a positive autoantibody test result also doesn't guarantee an accurate diagnosis, as one of the ongoing questions in the field is whether the antibodies are causative in nature or simply bystanders. Use of the favourable response to immunotherapy in the diagnostic criteria is also not always practical, as this is the kind of information that would only be available once treatment has already been initiated, meaning the diagnostic criterion cannot be applied at the time of symptom onset and early clinical evaluation. Furthermore, as highlighted in the previous subsection, some AE patients may not respond or have an incomplete response to immunotherapy, and response to immunotherapy alone does not indicate AE.

When relying on autoantibody test results in diagnosis, it is important to distinguish between the paraneoplastic syndromes characterised by onco-neuronal antibodies directed against linear epitopes on intracellular antigens, which show a limited response to immunotherapy, and the immunotherapy-responsive conditions typically associated with autoantibodies targeting conformational epitopes on the extracellular domain of CNS cell surface proteins. The former are thought to not be pathogenic and can be detected with ELISA, immunoblotting and rodent immunohistochemistry, while the latter are generally thought of as pathogenic and clinically significant, and are detected via techniques that harness the native conformation of the target antigen, such as cell-based assays (CBA), live rodent neurons, and immunohistochemistry of brain sections.[279, 280] Pathogenic mechanisms include recruitment of complement factors, internalisation, and direct blockade of target function.[281]

However, neuro-reactive antibodies have occasionally been detected in the absence of overt neurological symptoms, estimated to occur in 2-3% of the general population, with some even going so far as suggesting over 60% of plasma samples from living people meet the criteria for brain immuno-reactivity and declaring IgG autoantibodies to be abundant and ubiquitous in human sera depending on age, gender, and disease.[282-284] To complicate matters even further, symptoms in autoantibody-mediated neurological diseases may resolve following IT without concomitant reduction in autoantibody titres.[224-226, 285-288] Along with the non-negligible

reliance on autoantibody detection in the clinic, this inevitably leads to the question of when an antibody positivity is considered pathogenically relevant, examined next.

2.5 When is an Autoantibody Pathogenic? Witebsky's Criteria

The question of antibody pathogenicity to the self was first explored experimentally in 1957, when Ernest Witebsky and his doctoral students set out to test whether model organisms were truly unable to produce antibodies against constituents of their own body.[289] They injected experimental animals (rabbits, dogs, and guinea pigs) with thyroid extracts of their own species and found these extracts to be antigenic not only for animals of the same species, but also for the individual animal from which the thyroid extract was obtained. After demonstrating the presence of thyroid autoantibodies in the immunized animals' circulation, observing marked histopathological changes in their thyroid glands, and obtaining comparable serological and histological findings in patients with chronic thyroiditis, the authors postulated that chronic thyroiditis results from immunization against constituents of the patient's own thyroid gland.

Alongside suggesting an autoimmune aetiology for chronic thyroiditis, the authors proposed four criteria that should ideally be fulfilled to prove an autoantibody is pathogenic. Consciously modelled on the postulates of bacterial pathogenicity brought forward by Koch earlier in the decade, 'Witebsky's postulates' thus required:

(1) The direct demonstration of free, circulating autoantibodies active at body temperature or of cell-bound antibodies by indirect means
(2) The recognition of the specific antigen against which the antibody is directed
(3) The production of antibodies against the same antigen in experimental animals
(4) The appearance of pathological changes in the corresponding tissues of an actively sensitized experimental animal that are basically like those in the human disease

2.6 Revisiting Witebsky's Criteria

As initially stated in 1957, Witebsky's experimental requirements provided a scientifically rigorous framework for establishing when an antibody linked with an autoimmune disease may be considered pathogenic. Nonetheless, as the field of molecular biology developed, the original notion of autoreactivity always being pathological came to be challenged, and there was a growing understanding that self-reactive lymphocytes represent a normal population within the immune cell repertoire. In 1993, thirty-five years after their original formulation, Witebsky's postulates were updated by Noel Rose and Constantin Bona, both contributors to the original 1957 paper.[290] Considering this growing understanding of the distinction between 'physiological' and 'pathological' autoimmunity, Rose and Bona proposed that three types of evidence can be assembled to establish the autoimmune origin of a human disease:

(1) direct evidence from transfer of pathogenic antibodies or pathogenic T cells
(2) indirect evidence based on reproduction of the disease in experimental animals
(3) circumstantial evidence from clinical clues.

These 'Rose-Witebsky-Bona Postulates' move past the idea of a pathogenic autoantibody and toward the broader question of when a disease may be considered autoimmune in origin, being simultaneously open to how this definition may change with newly emerging research and permissive in terms of the type of evidence one may use to argue an autoimmune aetiology. The 'direct evidence' alludes to the so-called 'passive transfer' animal models often employed to investigate the sequelae of a pathogenic autoantibody (or pathogenic cell) in the lab. On the

other hand, the 'indirect evidence' in postulate 2 is reminiscent of the 'active immunization' animal models often employed to investigate the immunobiology of an autoimmune disease.

Rose and Bona also astutely allow for a third type of 'circumstantial evidence', outlining a few key clinical characteristics that may hint at an autoimmune aetiology to the vigilant clinician even when a laboratory-based animal model is lacking. Association with other autoimmune diseases in the same individual or family, lymphocytic infiltration in the target organ, restriction of V-gene usage, statistical association with a particular MHC haplotype, aberrant expression of MHC class II antigens on the affected organ and finally, favourably response to immunosuppression, are included as observations that may suggest an autoimmune aetiology. Their inclusion in the publication is especially prescient as laboratory evidence can take years if not decades to reliably confirm original clinical observations with experimental results, and the mere lack of a representative animal model does not necessarily exclude the possibility a disease is autoimmune. The updated postulates bear special significance for this thesis as under the original criteria, classifying Caspr2-antibody encephalitis as 'autoantibody-mediated' may be unsubstantiated. We explore this conundrum in subsequent subsections and later chapters.

2.7 Clinical associations of Caspr2-autoantibodies

Interest in the Caspr2 protein has grown exponentially after antibodies against it were described in patients with autoimmune limbic encephalitis (also known as Caspr2-antibody encephalitis), featuring characteristic limbic symptoms such as anterograde amnesia, temporal lobe seizures, and behavioural changes. Apart from patients with LE, Caspr2-autoantibodies were also found in patients with neuromyotonia, characterised by peripheral nerve hyperexcitability that manifests with cramps, stiffness, and fasciculations, and in patients with the poorly clinically delineated Morvan syndrome which combines signs of peripheral nerve hyperexcitability with CNS involvement in the form of hallucinations, insomnia, and confusion.[208, 209, 246, 291, 292]

A systematic review considering 667 patients with Caspr2-autoantibodies from 106 studies found 69% to be male, with a median age of disease onset 54 years (IQR 39-65.5). The most common reported clinical syndrome was autoimmune encephalitis, followed by limbic encephalitis, peripheral nerve hyperexcitability, Morvan syndrome, and cerebellar syndrome. Findings on MRI were reported as abnormal in over half of the cases, with the most common abnormalities being encephalitis or T2 hyperintensities in the medial temporal lobes, hippocampal atrophy, or mesial temporal sclerosis. Thymomas were also found in over 20% of patients.[293] While Caspr2-autoantibodies are considered most frequently associated with senior white male patients, more recent reports have also started examining the occurrence of Caspr2-autoimmunity in paediatric patients.[294, 295]

The clinical presentation of Caspr2-autoantibodies is thus quite complex and varied, as it can involve only the peripheral nervous system, only the central nervous system, or a combination of both, perhaps reflecting the distribution of the protein itself. It was later established that of the three Caspr2-phenotypes, Caspr2-antibody encephalitis is the most frequent and strongly correlates with the presence of Caspr2-antibodies in patient CSF.[245, 296-298] The limbic encephalitis seen in patients with CASPR2-autoantibodies can be concurrent with non-limbic features such as ataxia, other movement disorders, and neuropathic pain.[299]

It is also possible for patients with CASPR2-autoantibodies to have mono- or oligo-symptomatic presentations, for example isolated epilepsy, ataxia, or cognitive impairment.[208, 209, 246] Movement disorders are also quite prevalent (~35% of patients), and they can have versatile presentations, including ataxia, myoclonus or tremor.[300] The seizures observed are

predominantly focal (70%), with impaired awareness and limited motor components, and sometimes they can even be limited to sensory seizures. About 50% of patients will go on to develop bilateral tonic-clonic seizures later in their illness.[301]

According to two recent studies between 36-52% of patients with CASPR2-autoantibodies experience pain, which is typically neuropathic, often severe (63.6% of patients), not very responsive to immunotherapy, and sometimes even the major symptom (55.6% of patients). The pain phenotype experienced differs between patients, but the two most common types are distal-symmetric burning pain, and widespread pain with myalgia and cramps.[302, 303]

2.8 Histopathology of Caspr2-autoantibodies

Histological examination of biopsy samples can reveal a great deal about disease pathology. So far, there have been only two published reports detailing post-mortem staining of brain tissue from a patient with Caspr2 autoantibodies, perhaps due to the rarity of the disease itself and the only occasional fatalities.

The first report describes a 62-year-old male patient with short term memory loss, acoustic hallucinations, and subacute dyscognitive seizures, where resection of the right hippocampus revealed moderate parenchymal infiltration of CD8+ T-cells and mild activation of microglia. The perivascular space of blood vessels was found to contain few CD20 B-cells and CD138 plasma cells, to be expected as these cells rarely enter the parenchyma. Importantly, prominent penetration of immunoglobulin across the BBB and deposition on neuronal membranes was revealed. Where leakage of immunoglobulin across the BBB was detected, especially in the hippocampus, neurodegeneration and complement deposition could be seen. Calculation of the antibody index, i.e., the ratio between CSF and serum quotients of Caspr2 autoantibody titres and total IgG concentration, suggested the antibodies were mainly produced peripherally. Whether immunoglobulin leakage across the BBB was cause or consequence of the seizures experienced by the patient was unclear.[304]

The second study, the largest of its kind to date, used surgically resected medial temporal lobe specimens from five patients with mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) where Caspr2-autoantibodies were detected. All five samples showed significant neuronal loss and gliosis in the hippocampal region, but only two revealed abundant cellular infiltrates upon haematoxylin and eosin staining. In both patients the cell infiltrates were in the subarachnoid region, and one patient had additional cell infiltrates in the temporal cortex parenchyma. Subarachnoid infiltrates contained both polymorphonuclear and mono-nuclear cells, whereas parenchymal infiltrates contained only mononuclear cells. Histological analysis of the infiltrates revealed CD3+ T-cells, CD8+ T-cells, and CD68+ macrophages but no CD20+ or CD79a+ B-cells. When valuing this data, it is important to note that none of the patients had a history of classical autoimmune encephalitis, that the age- and sex-distribution did not reflect that normally associated with Caspr2-autoantibodies, and that the serum antibody testing was performed using commercial kits which tend to have a high false-positive rate. [305]

2.9 Are Caspr2-autoantibodies pathogenic?

With the complex clinical presentation detailed in subsection 2.7, fulfilling any of the original or revised Witebsky criteria pertaining to the replication of the disease in an experimental animal becomes elusive, especially considering the versatility and human exclusiveness of many of the symptoms experienced. While an active immunization animal model of Caspr2-autoimmunity has not been presented in the literature at the time of writing of this thesis, there

exist a handful of 'passive transfer' animal models, which have managed to replicate certain aspects of the convoluted human condition. These will be the subject of Chapter 3.

Is the limited success of the animal models to be interpreted as evidence that the autoantibodies are not pathogenic and their co-occurrence with a plethora of neurological symptoms a mere coincidence? To address this, numerous *in vitro* studies have been performed with patient Caspr2-autoantibodies throughout the years (Subsection 2.10), the outcomes of which have been slightly more reassuring. However, evidence from *in vitro* experiments is notably absent from the Rose-Witebsky-Bona criteria. Is this to say Caspr2-autoantibody pathogenicity is to be dismissed, or the postulates are yet-again due an update?

In the absence of a robust animal model, be it 'active' or 'passive', Caspr2-autoantibody-mediated diseases fall short of fulfilling two of the original Witebsky criteria and two of the revised ones. They do however enjoy some 'circumstantial evidence' from clinical clues, as required by the third revised criterion. Patients with Caspr2-autoantibodies generally respond favourably to immunosuppression, they show restriction in V-gene usage (unpublished), and there is also a known statistical association with the HLA-DRB1*11:01 MHC haplotype, all hallmarks of a disease with an autoimmune origin.[306]

It is worth appreciating however, that a more recent study has found only Caspr2-autoantibody patients with CNS involvement in the form of limbic encephalitis to bear the HLA-DRB1*11:01 haplotype more frequently than healthy controls, not those with involvement of the peripheral nervous system.[296] Combined with the observation that limbic involvement symptoms normally are the first to subside following immunotherapy, while those that affect the PNS tend to persist, this might lead us to postulate that Caspr2-antibody limbic encephalitis is the only *bona fide* autoimmune condition to be associated with these autoantibodies. But to make matters even more convoluted, it is only neuropathic pain (a PNS symptom), that has been robustly reproduced via passive transfer of patient antibodies in a model animal, the replication of CNS symptoms in other studies being far less believable.

Evidently, real-world disease scenarios are insufficiently clear-cut to elegantly fit into a neat list of criteria, sometimes even giving rise to contradictory conclusions. Caspr2-autoantibody diseases are not the only example of this, for applying the Rose-Witebsky-Bona-Criteria to the main 'culprits' in the field of autoimmune neurology leads to the disheartening conclusion that for none of them have the autoantibodies been shown to be pathogenic beyond reasonable doubt. The only notable exception is myasthenia gravis, not discussed here as it is a condition that affects the neuromuscular junction rather than the central nervous system.[307]

It does not escape my notice that perhaps the main front where most of these recently delineated conditions 'fail' is at the attempt to convincingly reproduce a multifaceted human neurological disorder in a rodent holding substantial neuroimmunological differences. Rather than going to the extremes of experimenting on primates or fully abandoning the criteria, I would argue in favour of a more 'lenient' application of the criteria to newly emerging conditions, where some arguments are given more weight than others. In our context, where laboratory evidence is lagging behind the clinical discoveries, it is perhaps reasonable to give more emphasis to the clinical clues suggesting an autoimmune aetiology, and to accept convincing evidence from *in vitro* studies on the autoantibody pathogenic mechanisms. For Caspr2-autoantibodies, the latter has allowed for some valuable insights into the potential molecular pathogenic mechanisms of the autoantibodies even in the absence of an animal model, discussed next.

2.10 In vitro Evidence of Caspr2-autoantibody Pathogenicity

Contactin-associated protein-like 2 (Caspr2) is a transmembrane protein expressed in both the CNS and PNS that belongs to the contactin-associated protein family, a distinct subgroup of the neurexin superfamily.[308, 309] Neurexins are a class of cell adhesion molecules located at the presynaptic terminal able to induce the formation of excitatory and inhibitory synapses via interactions with a diversity of neurolignin postsynaptic partners.[310-313] Caspr2-autoantibodies have been identified in individuals with a host of clinical phenotypes, but their diagnostic relevance and putative pathogenicity are a matter of ongoing debate and re-evaluation. Since the identification of the protein and even more so, since the identification of the autoantibodies, several important studies have been conducted *in vitro* as an attempt to better characterise the roles of Caspr2 in the nervous system and the way these roles are disrupted by autoantibodies.

Studies on patient serum samples have shown Caspr2-autoantibodies to be polyclonal and to target multiple epitopes distributed across the entire length of the protein, but the discoidin and laminin G1-like domains seem to be obligatory.[314] The antibodies are mainly of the IgG4 subclass, but the remaining subclasses have also been identified, with the second most common being IgG1.[245] Traditional understanding of the functional roles of these IgG subclasses in the immune system would suggest the dominant mechanisms of pathogenicity to be modulation of Caspr2 interactions by functionally monovalent bispecific F(ab)-arm exchanged IgG4, and to a lesser degree antigen internalisation and complement fixation. There has only been one report of complement factors deposition in post-mortem brain tissue [304] and no studies have been done so far to demonstrate whether patient sera can induce complement deposition and neuronal cell death *in vitro*. Serum autoantibody titres as detected by cell-based assays tend to be high, ranging from 1:200 to 1:100,000 with a median end-point titre of around 1:1000, and around half of patients also have Caspr2-autoantibodies in their CSF.[293]

Experiments have been done to see whether patient sera can induce Caspr2 internalisation, with conflicting results: earlier studies showed the autoantibodies to inhibit Caspr2 and contactin-2 interaction in solid phase binding assays, with no effect on Caspr2 surface expression in rat hippocampal neurons or Caspr2-expressing HEK293T cells following a 24-hour incubation period.[315] A second paper confirmed these findings by showing that Caspr2-autoantibodies can perturb Caspr2/TAG-1 interaction in a co-immunoprecipitation cellular model, furthermore establishing the EGF2 and L4 domains of Caspr2 as critical for this interaction via the use of deletion constructs, but did not induce Caspr2 internalisation neither in HEK cells nor in cultured primary hippocampal neurons following a 24-hour incubation.[316] However, the study did find that in Caspr2-transfected neurons, treatment with autoantibodies led to an increase in Caspr2 surface expression, suggesting the antibodies could be leading to re-distribution rather than internalisation of the target.

These findings somewhat carried forward *in vivo*, as brain histopathology on mice that had received systemic Caspr2-IgG injections combined with a temporary breach of the BBB showed only a modest and statistically insignificant increase in Caspr2 expression from controls. The same study attempted to model this phenomenon *in vitro*, showing that in both Caspr2-transfected HEK cells and in hippocampal neurons, internalisation of Caspr2-IgG could be observed, but Caspr2 expression increased only slightly.[317] In stark contrast to this, a study published in the same year found that incubating cortical neurons in culture with Caspr2-autoantibodies comprised of both the IgG1 and IgG4 subclasses affected the neuronal distribution of Caspr2 in dendrites, significantly decreasing it synaptic expression.[318] Intracellular clusters of anti-human antibodies could be seen in neurons incubated with patient sera, supporting the idea that the autoantibodies could be leading to Caspr2 internalisation. The

same study found a significant loss of surface AMPARs at the synapse in cortical neurons that had been treated with Caspr2-autoantibodies, and this was supported by *in vivo* injections in the mouse visual cortex, where the Caspr2-autoantibodies significantly decreased AMPAR-mediated currents in layer 2/3 pyramidal neurons. These last results brought forth the idea that besides blocking interaction between Caspr2 and TAG-1, autoantibodies could also be exerting their pathogenicity by impeding Caspr2 regulation over AMPAR trafficking to the synapse.

It is possible that some of the discrepancy between the earlier and later findings arose from the use of longer incubation times in the former, which might have resulted in the loss of temporal resolution, as well as from the focus on overall rather than specifically synaptic levels of Caspr2, which would have resulted in loss of spatial resolution. A more recent study using high resolution stimulated emission depletion microscopy showed that intracerebroventricular infusion of IgG from patients into mice led to hippocampal reduction of surface Caspr2 clusters and decreased Caspr2/TAG-1 colocalization. This was reinforced in cultured neurons, where Caspr2-autoantibodies from patients specifically increased intracellular levels of Caspr2 without affecting TAG-1, suggesting selective Caspr2 internalisation. In the brains of patient IgG-infused mice, levels of GluA1 and Kv1.1, two proteins regulated by Caspr2, were also decreased, lending further credibility to some of the earlier findings. Importantly, all changes were reversed following removal of the patients' IgG.[319]

Work from our own group has used anti-Caspr2 monoclonal antibodies and live cell imaging using the dye pHRodo to demonstrate in real-time that the autoantibodies can indeed internalise their target expressed on hippocampal neurons. This has been followed up with treatment of cortical neurons in culture using the mAbs to show their ability to alter both synaptic and total levels of Caspr2 and of AMPA receptors (Sun et al. 2024, *under review*). Some of these results will be presented in Chapter 4.

Other studies, rather than trying to narrow down the exact molecular perturbations, have focused more broadly on the global electrophysiological effects of Caspr2 autoantibodies and their epileptogenic potential. The only paper to look at Caspr2-autoantibodies from patient CSF samples used stereotactic injection into the dorsal hippocampi of adult male mice followed by patch-clamp and field recordings.[320] Here, Caspr2-autoantibodies induced epileptiform activity at a population level following Schaffer collateral stimulation by enhancing the probability of CA3-CA1 glutamatergic synaptic release, and lead to higher spontaneous firing in CA1 pyramidal neurons, indicating increased intrinsic excitability. The study thus supported a pro-epileptogenic modification of hippocampal neuronal activity by Caspr2-autoantibodies, revealing a potential pathogenic pathway for the seizures associated with Caspr2-antibody encephalitis.

A similar study inoculating Caspr2-autoantibodies from serum in rat hippocampi found them to increase the slope of fEPSPs at Schaffer collateral-CA1 synapses but with no effects on the presynaptic terminal paired-pulse ratio nor on long-term potentiation values.[321] This suggested Caspr2-autoantibodies could increase epileptiform activity via increase of postsynaptic potentials, but likely wouldn't affect presynaptic firing properties nor LTP at these synapses Interestingly, while the earlier study didn't measure fEPSP slope, it found a reduced paired-pulse ratio in slices from mice injected with CSF Caspr2-autoantibodies, consistent with increased probability of glutamate release from CA3 terminals. The lower severity of the second study could be connected to the use of rats rather than mice, the bigger delay between injection and recording, and the use of a sample with a lower titre from a paediatric patient

with a diagnosis of Morvan syndrome rather than definitive antibody-associated autoimmune encephalitis, as done in the earlier study.

While extensive work has been done to understand disruptions in Caspr2 function in the context of disease, these should not overshadow attempts to understand the basic features of the protein which themselves may shine new light on our understanding of Caspr2-autoantibody pathology. In the next few subsections, I will take a more detailed look at Caspr2 through the prism of structural biology, genetics, and basic neurophysiology.

2.11 Early Discoveries on Caspr2

The story of Caspr2 began 25 years ago with its identification as a mammalian homolog of Drosophila Neurexin IV. Caspr2 was shown to precisely colocalise with Shaker-type delayed rectifier potassium channels Kv1.1 and Kv1.2, and their associated Kvβ2 subunit at the juxtaparanodal regions of myelinated axons. At the time, the authors suggested a role of Caspr2 in the local differentiation of the axon into distinct functional subdomains.[309]

Further work demonstrated that Caspr2 and its close relative Caspr occupy mutually excluding domains along the axon; this helped to clearly delineate the molecular composition of the nodal region in peripheral nerves, with Caspr2, Kv1.2 and 4.1B colocalising to the JXP, and neurofascin-155, Contactin, and Caspr colocalising to the paranode.[322-324] Later, it was also demonstrated that Caspr2 and Kv1 channels colocalise precisely with post-synaptic density 95 (PSD-95) at the juxtaparanode.[325] Protein 4.1B was identified as an intracellular partner of Caspr2, thought to anchor it to the actin-based cytoskeleton.[326]

A few years later, two more members of the Caspr family were identified, Caspr3 and Caspr4, and found to also have widespread expression across the CNS and PNS.[327] In the early 2000s, further insights into the potential roles of Caspr2 were gained via studies of Caspr2-null mice, which demonstrated reduced juxtaparanodal accumulation of Kv1.2 compared to wildtypes as evidenced via sciatic nerve immunofluorescence staining.[328] An important association between Caspr2 and TAG-1 (an immunoglobulin-like cell adhesion molecule) was highlighted as key for maintaining K^+ channels at the JXP, for in the absence of TAG-1, axonal Caspr2 would not accumulate at JXPs, and the normal enrichment of K^+ channels in these regions would be severely disrupted.[329]

2.12 Caspr2 Gene and Protein Structure

Caspr2 is a single-pass transmembrane protein composed of 1,332 amino acid residues, with a mass of 138 kDa.[309] It is encoded by the *CNTNAP2* gene, the largest gene in the human genome that spans 2.3 Mb of chromosomal region 7q35 and constitutes 1.5% of chromosome 7.[330] The amino acid sequence is highly conserved between species, with 98.6% homology between human and rhesus macaques and 93.8% overlap between human and mouse (UNIPROT), making animal models very useful for studying its function and disease relevance.[331] Mutations in *CNTNAP2* have been associated with a range of neurodevelopmental disorders including epilepsy, schizophrenia, autism spectrum disorder, ADHD, intellectual disability, dyslexia, and language impairment.[332] The *CNTNAP2* gene (*Cntnap2* is the mouse ortholog) is directly bound and regulated by fork-head box protein P2 (FOXP2), a transcription factor related to speech and language development.[333]

Like other classical α neurexins, Caspr2 has a short C-terminal intracellular domain, a single transmembrane helix, and a large N-terminal extracellular domain (ECD). At the C-terminus, Caspr2 contains a 4.1B-binding motif and a type II PDZ-binding motif (PDZb), which can

facilitate its interaction with cytoskeleton-associated proteins that contain a FERM or a PDZ domain respectively. Within its ECD, Caspr2 contains multiple domains not found within classical neurexins and is therefore thought to adopt a different three-dimensional structure. The extracellular portion of the protein is comprised of an N-terminal Discoidin-like domain (F58C), four Laminin G-like domains (L1-L4), two Epidermal-like growth factor domains (EGF1, EGF2) and a Fibrinogen C-terminal domain (FibC).[309] Recent papers involving electron microscopy have demonstrated a compact structure organised into three lobes that are flexibly linked by EGF1 and EGF2.[334, 335]

2.13 Caspr2 Expression and Localisation

In the adult mouse brain, Caspr2 is strongly expressed in cortex, areas related to motor activities (basal ganglia, substantia nigra, pontine nucleus, cerebellum), as well as the limbic circuitry (hippocampus, amygdala, interpeduncular nucleus, mammillary bodies). Nervous structures involved in sensory pathways like the visual, olfactory, auditory, gustatory, and somatosensory systems also express high levels of Caspr2. Specifically in the hippocampus, Caspr2 is initially detected in the dentate gyrus at embryonic day 18, where its expression gradually decreases, accompanied by concomitant increased expression in the CA1 and CA2 regions from postnatal day 7 up to adulthood.[336]

At the cellular level, Caspr2 is expressed at the soma and dendrites of pyramidal neurons in the CA3 region of the hippocampus and cortex, the distal portion of the axon initial segment (AIS), and the juxtaparanode (JXP) region of the nodes of Ranvier on myelinated axons in the CNS and PNS. In cultured hippocampal neurons, Caspr2 is preferentially associated with inhibitory synapses, with 58% of GAD65-positive inhibitory neurons expressing Caspr2 at DIV4, compared to 4% of GAD65-negative excitatory neurons. It also partially colocalised with presynaptic markers like VGLUT1 and VGAT, identifying excitatory and inhibitory synapses, respectively, and the GluA1 subunit of excitatory postsynaptic AMPARs.[309, 337-341]

2.14 Caspr2 Functions in the Nervous System

Enriched at the axon initial segment and juxtaparanodes, Caspr2 is close to the voltage-gated potassium channels (VGKCs) of the Kv1 subfamily, specifically Kv1.1 and Kv1.2. These ion channels are essential for the repolarization of the neuronal membrane following an action potential and henceforth the avoidance of repetitive firing and maintenance of the internodal resting potential. At the JXP, Caspr2 forms a cell-adhesion complex with TAG-1, a GPI-linked cell adhesion molecule closely related to contactin that is expressed by myelinating Schwann cells and oligodendrocytes. Caspr2 is thought to regulate the clustering of Kv1 channels via this interaction, as when it is knocked out in Cntnap2$^{-/-}$ mice, neither TAG-1 nor Kv1 channels are any longer enriched at the JXP.[328, 342] This relationship is bidirectional, as in TAG-1 deficient mice axonal Caspr2 did not accumulate at JXPs, and the normal enrichment of Kv1 channels in these regions was severely disrupted. In contrast to the JXPs, Caspr2 function seems to be dispensable for the clustering of Kv1 channels at the axon initial segment.[341]

It is key to appreciate that early studies focused much more on the effects of Caspr2 disruption on the molecular organisation of myelinated axons into distinct domains than the functional implications for axonal electrical activity. Thus, while the JXP complex was mislocalised in *Cntnap2*$^{-/-}$ knockout mice, both the myelination process and the action potential conduction velocity and refractory period remained unaffected, calling into question the exact significance of JXP organisation for action potential propagation.[328] A more recent study has used mutant rather than knockout Cntnap2 mice and found that profound deficiencies in Kv1 clustering at the JXP of myelinated axons preceded altered action potential repolarization, increased

postsynaptic excitatory responses, and delayed grey matter myelination in the adult mice.[343] It is possible that the global recording of axonal electrical activity via the use of acute cortical slices in the later study allowed for more subtle electrophysiological disturbances to be revealed, or that the use of mutants in lieu of complete knockouts prevented compensatory developmental mechanisms from taking shape. Collectively, these results have shaped our idea of Caspr2 as a regulator of intrinsic neuronal excitability, and perhaps even myelination, via its influence on Kv1 distribution at the neuronal membrane.

Studies on *Cntnap2*$^{-/-}$ knockouts have expanded understanding of the roles of Caspr2 beyond one as an axonal microdomain organiser, and it has also been proposed to play a role in the formation of neural networks during development. Early studies in Cntnap2-deficient mice demonstrated neuronal migration abnormalities, reduced cortical GABAergic interneurons, and abnormal network activity. At postnatal days 14 and 30, Cntnap2$^{-/-}$ knockout mice exhibit deficits in subpopulations of GABAergic interneurons in the striatum, hippocampus, and somatosensory cortex, which has led to the proposal that Caspr2 cell-autonomously regulates the electrophysiological properties of parvalbumin-positive interneurons.[344] Consistent with this idea for Caspr2 as a regulator of inhibitory function, CA1 pyramidal cells in acute hippocampal slices show altered inhibitory but not excitatory transmission.[345] It has been suggested that disruption of GABAergic signalling leads to defects in neuronal synchronization and shifts the balance between excitatory and inhibitory circuits, bringing about behavioural deficits like hyperactivity and epileptic seizures in the knockout mice.[339, 346-348]

Roles for excitatory synaptic function have also been proposed for Caspr2, with nearly half of all excitatory synapses in rat cortical neurons found to express the protein *in vitro*, and its loss found to lead to cytoplasmic accumulation of AMPA receptors.[339] More recent research has highlighted the importance of Caspr2 for AMPA receptor trafficking and AMPAR-mediated synaptic transmission. Silencing its expression led to decreased cell-surface expression of the GluA1 AMPAR-subunit *in vitro* at cortical synapses and reduced amplitude of AMPAR-mediated mEPSCs *in vivo* in the mouse visual cortex. Loss of Caspr2 also prevented cortical synaptic scaling of AMPARs *in vitro*, a form of homeostatic plasticity where the overall synaptic strength of a neuron is adjusted based on activity-dependent changes in the synaptic accumulation of AMPARs. In vivo, this manifested as hindering of visually-driven experience-dependent homeostatic plasticity, with deficits in scaling of AMPAR function in V1-layer 2/3 pyramidal neurons following visual deprivation.[318]

During development, Caspr2-loss has been found to affect neuronal migration, dendritic arborization and spine development, leading to the suggestion that Caspr2 is involved in the stabilization of newly formed dendrites.[347, 348] The underlying mechanisms via which Caspr2 may fulfil these roles in the developing brain were for a long time a mystery, especially considering its most established role was as a scaffolding protein in the peripheral nervous system via its interaction with TAG-1, and no other interaction partners had been reported specifically for the CNS. Recently, IP_3R1 was identified using a proteomics approach as a specific interaction partner for the Caspr2 ECD in the molecular layer of the developing cerebellum. This interaction was found to inhibit IP_3R1-mediated changes in cellular morphology. The first of its kind to focus exclusively on the roles of Caspr2 in the developing cerebellum, the study also found its loss in the Cntnap2$^{-/-}$ knockout mice to lead to impaired Purkinje cell dendritic development, altered intracellular signalling, and impaired motor coordination, with the interaction with IP_3R1 probably being at the heart of these changes.[349]

Caspr2-dysfunction seems to be more frequently associated with men than women, with 60.9% of patients with CNTNAP2 mutations and 89.5% of patients with Caspr2-autoantibodies being male.[331] The latter is especially surprising considering it is estimated that 80% of all individuals affected by autoimmune disorders are women, practically the inverse of the trend for Caspr2-autoimmunity.[350] Animal studies have supported the notion of a sex-dependent effect: male Caspr2-knockouts are more susceptible to dysfunction of visual cortical areas than female ones, and in the maternal-to-foetal transfer model, Caspr2-autoantibodies had an impact on male offspring only.[351, 352] A possible pharmacogenetic rationale for this trend was provided by a study in zebrafish, whereby the authors screened compounds that would reverse autism-like behaviours in a Cntnap2 knockout zebrafish model. The authors found phytoestrogen biochanin A, an agonist of oestrogen receptors to rescue zebrafish behaviour, suggesting a protective endocrine effect of oestrogen that renders females less vulnerable to Cntnap2-related pathologies. [353] Paradoxically, oestrogen is a key mediator of B-cell activation and antibody production, so this does not explain why women are less susceptible to Caspr2-AE.[354]

2.15 Conclusion: Caspr2 the Friendly Protein – should we 'ghost' it?

The second section of this introduction has covered a lot of ground, from a clinical explanation of autoimmune encephalitis to a discussion on how we assess the pathogenicity of an autoantibody found to be associated with a neurological condition, to a more detailed look on the protein Caspr2, the autoantibodies targeting it, and their clinical corollaries. It is evident that much remains to be answered in the field, but my hope is that this section has sufficiently piqued the reader's curiosity to warrant interest in the research presented in the rest of this thesis and provided a sufficiently strong theoretical foundation to assess the value thereof. In the last section of the introduction, I will present a succinct overview of the key immunological concepts relevant to this thesis.

SECTION 3: Immunity

3.1 The Immune System

To prevent the onslaught of myriad pathogens, humans have evolved general mechanisms of defence (innate immunity) and mechanisms that are specific for a given pathogen (adaptive immunity). The latter mechanism, as its name implies, can be acquired, optimized, and selectively tweaked through contact with pathogen or through vaccination. Its principal players are T-cells and antibodies. In this section I will provide a general overview of and some detail on the structure and function of antibodies, as well as the cells that produce them.[355]

3.2 Early Discoveries

As early as the eighteenth century, it was known that fluid obtained from a smallpox pustule provided immunity from acquiring the disease when injected into a recipient. Edward Jenner's work on immunizing against smallpox using fluid obtained from cowpox pustules pioneered and advanced this knowledge.[356] Even though it preceded understanding of what an antibody is, this constitutes one of the earliest ventures into experimental antibody development. Actual discovery of antibodies can be traced back to the late 1800's, when von Behring and Kitasato transferred serum from animals that had been immunized to diphtheria into animals infected with diphtheria and this provided a sort of 'passive immunity' that altered the course of disease. This work won von Behring the Nobel Prize in 1901 for 'the development of serum therapy'.[357, 358] In the early 20th century, Ehrlich pioneered work on antibody configuration predictive of our current understanding of antibody structure.[359, 360] Ehrlich also launched a theory that cells

have side chains that can bind toxins, and was the first to coin the term ‘antibody’ and describe the molecule as ‘branched’.

In 1972, Edelman and Porter won the Nobel Prize for their efforts in elucidating the chemical structure of antibodies. In the 1960s, they had performed a series of key experiments that would unravel the elegant structural organisation of the antibody.[361, 362] Earlier studies had shown that the bulk of antibody activity in serum is due to a slow electrophoretic fraction termed gamma-globulin (later renamed to immunoglobulin). The most abundant antibodies were shown to be di-valent, meaning they had two binding sites for antigen and could thus form a precipitating complex. Reduction of disulphide bonds showed that the component polypeptide chains still hang together via strong non-covalent interactions. However, once the reduced molecules were put under acidic conditions, the polypeptide chains became positively charged and the attractive forces that held them together were lost, allowing for their separation. Gel filtration revealed the existence of heavy polypeptide chains with a size of 55 kDa (for immunoglobulins G, A, and D) or 70 kDa (for immunoglobulins M and E) and light polypeptide chains with size of 24 kDa. Cleavage with proteolytic enzymes helped explain how these chains are assembled. The enzyme papain destroyed the antigen-precipitating ability of the molecule but produced two univalent Fab fragments that were still capable of binding to antigen, whereas the remaining Fc portion had no affinity for antigen. Digestion with pepsin allowed for isolation of a $F(ab')_2$ molecule which retained both binding sites and could still functionally precipitate antigen, but where the Fc portion was further degraded.

3.3 General Functions of Antibody Molecules

Antibody molecules carry out two principal functions in immune defence. First, they recognise and bind to molecular structures on the surface of foreign material that differ from molecular structures made by the cells of the host. In other words, they help distinguish ‘self’ from ‘non-self’. Antigenic determinants are usually expressed in multiple copies on the surface of foreign pathogens, like proteins or carbohydrates on a bacterial cell surface or envelope spikes on the surface of a virus. Antibody diversity is therefore essential to respond to the huge variety of molecular structures associated with highly mutable and rapidly evolving pathogens.[355]

Sometimes antibody binding on its own may be sufficient to inactivate a pathogen or render a toxin harmless: antibody binding on the surface of a virus can neutralize it and prevent entry into target cells. In many instances however a *second function* of the antibody is deployed to trigger definitive elimination of pathogen. This involves the binding of effector molecules to antibody-coated foreign material to trigger complex and versatile elimination mechanisms. These mechanisms could include: (1) activation of the complement cascade, (2) recruitment of phagocytic immune cells (neutrophils and macrophages), and (3) antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells. Usually, these powerful (and to an extent destructive) effector mechanisms are triggered only by antibody molecules being clustered together on the surface of a foreign cell and not by free unliganded antibody, which is very fortunate keeping in mind the typically high serum concentrations of antibodies.[363, 364]

3.4 General Antibody Structure

Antibodies are referred to scientifically as *immunoglobulins* due to their highly glycosylated globular nature normally consisting of around 82-96% protein and 4-18% carbohydrate.[365] When considering the functions of antibodies outlined in the previous section, it becomes apparent that the requirements imposed on an antibody molecule are in fact quite opposite. Firstly, they need to be exquisitely diverse to recognise a wide array of antigens. Secondly,

they need to have converged shared structures that allow them to recruit effector molecules. Luckily, antibody structure meets these conflicting demands.

The Y-shaped molecule has four polypeptide chains (two identical heavy and two identical light chains) arranged to span three structural units. Two of these units, the 'arms' of the Y, are identical to one another and are involved in antigen binding. They are called *fragment antigen binding (Fab)* and are encoded by sequences that vary greatly from one antibody to another and that confer on each antibody its unique binding specificity. The fact that there are two of these arms is relevant as it enhances the ability of the antibody to bind where multiple copies of antigenic determinant are present on the surface of a pathogenic molecule. The base of the Y, or third unit of the antibody, forms the *fragment crystallizable* (Fc) portion and is involved in the binding of effector molecules.[355, 366] It is important to appreciate that despite the minor alternations, all immunoglobulin molecules are constructed from one or more of these basic four-polypeptide-chain units.

3.5 The Five Classes of Immunoglobulin

There are five classes of immunoglobulin: immunoglobulin G (IgG), IgM, IgA, IgE and IgD. They all share the basic four-chain structure outlined in subsection 3.4, but differ in their heavy chains, respectively termed with Greek letters: γ, μ, α, δ, and ε. The differences are most pronounced in the Fc region, which leads to triggering of different effector functions upon binding antigen. Thus, IgM recognition of antigen can lead to activation of the complement pathway, whereas IgE recognition can lead to mast cell degranulation and anaphylaxis. The structural differences in the Fc region can also lead to differences in the polymerisation state of the monomer: IgG and IgE generally exist as monomers, IgM as a pentamer, whereas IgA will generally occur as a monomer in serum and as a dimer in seromucous secretions.[364] Of the five classes of immunoglobulin, the most prevalent one in serum is IgG, the structure of which will be further discussed in the next subsection.

3.6 Structure of the Immunoglobulin G (IgG) Molecule

The IgG molecule consists of two identical 50 kDa gamma heavy (H) chains, and two identical 25 kDa kappa (κ) or lambda (λ) light (L) chains, linked together by inter-chain disulphide bonds. In humans, κ chains are somewhat more prevalent than λ chains, whereas in mice, λ chains are rare. The heavy chain consists of an N-terminal variable domain (VH) and three constant domains (CH1, CH2, and CH3), with an additional 'hinge region' between CH1 and CH2. Similarly, the light chains consist of an N-terminal variable domain (VL) and a constant domain (CL). Thus, the molecule is organised into a total of 12 domains.[363]

The light chain (VL and CL) associates with the VH and CH1 domains of the heavy chain to form a Fab arm, the tips of which (VH and VL) are involved in antigen binding. On the other hand, the lower hinge region and CH2/CH3 domains form the Fc portion, the stem of which is involved in binding effector cells and molecules. Two heavy chain-light chain heterodimers (HL) combine into a functional antibody molecule (H_2L_2) via disulphide bonds in the hinge region and non-covalent interactions between the CH3 domains. Within a single molecule, the two heavy chains are normally identical to each other, as are the two light chains, the exception to the rule being molecules of the IgG4 subclass which in humans can exchange heavy-light pairs to produce hybrids. As the Fc part of these molecules remains the same, the net effect is 'Fab arm exchange' leading to an IgG4 antibody with two different Fab arms and thus dual specificity ('bispecific').[363, 367] The IgG subclasses are further discussed in the next subsection.

3.7 IgG Subclasses

The heavy chains of IgG can be further subdivided into groups or subclasses which vary in number depending on the species. In humans there are four subclasses named IgG1, IgG2, IgG3, and IgG4 in order of decreasing abundance. The different subclasses have highly similar primary sequences and are over 90% identical on an amino acid level, most variation being centred on the hinge region and the CH2 domain. The residues most proximal to the hinge region in the CH2 domain of the Fc part are responsible for effector functions as this part contains a binding site for C1q (part of the complement cascade) and IgG-Fc receptors (FcγR) on effector cells of the innate immune system. The interface between the CH2-CH3 domains also contains the binding site for the neonatal Fc receptor (FcRn), responsible for the prolonged half-life of IgG, placental passage, and transport to and from mucosal surfaces.[366] Next, I will briefly discuss each of the four IgG subclasses.

IgG1: Antibody responses to soluble protein antigens and membrane proteins will primarily induce IgG1 but can be accompanied by lower levels of the other subclasses, namely IgG3 and IgG4.[368] Since IgG1 is normally the most abundant IgG subclass, the lack of IgG1 that can be seen in a variety of primary and secondary antibody deficiencies can also result in lower total IgG levels, also known as hypogammaglobulinemia.[369] IgG1 deficiencies, sometimes in combination with other IgG subclass deficiencies, are associated with recurrent infections.[367]

IgG2: Immunoglobulin G-antibody responses to bacterial capsular polysaccharide antigens can be almost completely restricted to IgG2, and an IgG2 deficiency may result in the virtual absence of anti-carbohydrate antibodies.[368, 370] Increased susceptibility to certain bacterial infections is also associated with IgG2 deficiency, suggesting a role of IgG2 in the defence from these pathogens.[367, 371]

IgG3: IgG3 antibodies are particularly important for the induction of effector functions but as potent pro-inflammatory antibodies their shorter half-life could be a protective mechanism against excessive inflammatory responses.[370, 371] Viral infections will in general lead to IgG antibodies of the IgG1 and IgG3 subclasses, with IgG3 antibodies appearing first in the course of the infection, but solely IgG3-dominated responses seem to be rare.[368]

IgG4: IgG4 antibodies are often formed following repeated or long-term exposure to antigen in a non-infectious setting and may become the dominant subclass in these scenarios. Examples are long-term beekeepers and allergic individuals that have undergone immunotherapy.[372-375] In immunotherapy, relief of symptoms seems to correlate with IgG4 induction. Switching to IgG4 may be modulated by IL-10, thus linking this subclass to downregulation of immune responses and tolerance induction.[372, 376] Furthermore, high IgG4 titres can be associated with asymptomatic infections, and responses to helminth or filarial parasite infections.[377] Isolated IgG4 deficiencies are rare, and it is uncertain what the possible consequences are.

A group of disorders referred to as IgG4-related diseases (IgG4RD) are characterised by elevated serum IgG4 concentration and tissue infiltration by IgG4-positive plasma cells that can affect several organs.[378, 379] The spectrum of IgG4RD is wide and it includes patients with autoimmune pancreatitis (AIP), Mikulicz syndrome, hypophisitis, Riedel thyroiditis, interstitial pneumonitis, interstitial nephritis, lymphadenopathy, inflammatory aortic aneurysm, and inflammatory pseudomotor.[380] However, it is worth considering that 5% of the normal population also has elevated IgG4 levels, thus questioning the diagnostic significance of elevated IgG4 antibodies in these clinical scenarios.[378]

3.8 Monoclonal Antibodies (mAbs)

When attempting to generate antibodies for experimental purposes, traditional approaches have involved immunizing experimental animals with an antigen and subsequent purification of the serum to isolate the antibody fraction – a method which leads to the isolation of polyclonal antibodies.[381] However, interest in using antibodies as precise therapeutic agents led to attempts to generate homogeneous antibodies from a single plasma cell clone – called monoclonal antibodies (mAbs). This was first achieved in 1970 when Askonas reported the isolation of a single plasma cell clone and subsequent generation of a homogeneous monoclonal antibody, propagated by repeated passage of spleen cells into irradiated syngeneic mice.[382] A few years later, Kohler and Millstein published their landmark paper on the fusion of an antibody-producing plasma cell with a myeloma cell, which could be propagated indefinitely in culture and thus enable production of unlimited amounts of antibody *in vitro*.[383]

The "hybridoma" revolutionised immunology and its therapeutic implications by allowing unlimited continuous production of monospecific antibodies, earning Kohler and Millstein the Nobel Prize in 1984. Hybridomas have enabled production of engineered and fully humanised antibodies suitable for therapeutic applications in humans, culminating with the anti-CD3 mAb muromonab being the first therapeutic mAb approved for clinical use in humans in 1985.[384, 385] However, within the context of autoimmune disease, the interest has not so much been in the unlimited generation of mAbs, but rather in their sequencing and cloning. This has been harnessed to understand their underlying immunogenetic characteristics and relate these to the phenotypic features of parent B-cells. To this end, single B-cell technologies and advancement in cloning techniques have been of pivotal importance. These will be covered in more detail in Chapters 4 and 5. The last few subsections of this chapter will outline the development and key functions of B-cells, as well as their contributions to CNS physiology and pathology.

3.9 B-cells

The simplest textbook definition of B-lymphocytes is a population of cells that express clonally diverse surface immunoglobulin (Ig) receptors that recognise antigenic epitopes with high specificity. B-cells are key players in the adaptive immune response, due to their primary function as producers of antibody, however they also work as antigen-presenting cells that can shape the responses of other immune cells.[355, 363] The B-cell receptor (BCR) is a membrane-tethered antigen receptor on the surface of the B-cell that will eventually be secreted as an antibody if the B-cell differentiates into an antigen-secreting cell. There is immense diversity in the BCR repertoire within an individual and across the human species, and one of the ways that this is achieved is via quasi-random recombination of heavy-chain and light-chain gene segment during early B-cell development.[386] This diversity is a double-edged sword: on one hand, it allows B-cells to recognise a wide array of foreign antigens but, on the other, it creates an intrinsic risk of recognising self-antigens and generating autoreactive B-cells.[387, 388]

3.10 B cell Development and Function

The origin of B-cells can be traced back to the evolution of adaptive immunity in jawed vertebrates over 500 million years ago.[389] Mammalian B-cell development encompasses a complex continuum of stages beginning in primary lymphoid tissue (liver and bone marrow), followed by functional maturation in secondary lymphoid organs (lymph nodes and spleen).[387, 388] In the bone marrow, B-cells develop from haematopoietic stem cells, initially as pro-B-cells and then pre-B-cells. They undergo immunoglobulin gene rearrangement, becoming immature B-cells, and then further mature in the periphery. Mature naïve B-cells that encounter their antigen during an immune response become activated, undergo class switch recombination and somatic hypermutation, and differentiate into short-lived plasma cells (otherwise known as

plasmablasts) or germinal centre B-cells. Having migrated to germinal centres and engaged with their target antigen there, B-cells proliferate and eventually differentiate into long-lived memory B-cells and/or plasma cells.[222, 390]

3.11 Roles of B-cells in CNS homeostasis

An important question regarding the relationship between B-cells and the brain is what the roles (if any) of these cells are outside the context of disease. Mouse studies have found that B-cells are mostly abundant during development, whereupon they contribute to myelination and oligodendrogenesis, but not so much in adulthood.[391] Under normal homeostatic conditions, B-cells are rarely found in the CNS parenchyma in either mice or humans, and very small numbers have been found in the CSF, choroid plexus, or subdural meninges.[9, 46, 47, 392, 393] One of the first studies that addressed this question in the early 2000s identified very low levels of activated ($CD20^+CD23^+$) B-cells in the brain, which were observed sporadically in the parenchyma but were virtually absent from perivascular spaces.[160] Compared to their numbers in peripheral blood, B-cell numbers in the CSF are extremely low, the ratio being around 19,000 : 1 CD19+ cell in blood versus CSF.[394] However, a landmark recent study has shown that B-cells are constitutively generated in the skull bone marrow (specifically the calvarium), from where they traffic to the dura, where they were found to represent over 30% of the resident immune cells.[46] Meningeal B-cells were found to inhabit blood vessels, lymphatic vessels, or to reside in tissue. The authors postulated the calvaria-meningeal path of B-cell development to provide the CNS with a constant supply of B-cells educated by CNS antigens, that prevent the generation of immuno-globulins with high affinity for CNS epitopes, thus maintaining CNS immune privilege, but this theorised role has not yet been supported by experimental evidence.

3.12 B-cells in autoimmune encephalitis

Considering the field of CNS diseases caused by potentially pathogenic neuroglial surface autoantibodies is rapidly expanding, knowledge of the B-cell immunobiology that underlies these conditions is remarkably limited. There is an incentive to study this in more detail as despite immunotherapy, patients frequently experience relapses and residual symptoms and disability, as well as adverse effects of the immunotherapy itself, leading to high rates of long-term morbidity.

Autoreactive B-cells, thought to produce pathogenic autoantibodies, have been recurringly identified in the circulation of patients across multiple CNS autoantibody-mediated diseases and experimental modalities, alongside high serum levels of autoantigen-specific antibodies.[395-398] These cells are thought to reach the circulation by evading early B-cell tolerance checkpoints. In certain patients and certain diseases, enrichment of autoantigen-specific B-cells has also been observed in the CSF, suggesting intrathecal synthesis of the autoantibodies in the vicinity of the site of pathology.[399-401]

Considering there is robust evidence that the pathophysiology of these diseases is underpinned by autoantigen-reactive immunoglobulins, delving into the immunoglobulin genes encoded by autoantigen-specific B-cells is relevant to understand the underlying disease immunobiology. Here, most of our knowledge has emerged from the study of AQP4 autoantibodies, with a few papers focusing on NMDAR- and LGI1-autoantibodies and none on Caspr2-autoantibodies.[222] Indeed, this limitation in our understanding of the immunobiology of Caspr2 autoantibody-related diseases has been one of the main incentives behind Chapters 4 and 5. Chapter 5 in particular will also discuss the experimental approaches to studying rare autoreactive B-cells.

3.13 T-cells

T-cells are not the topic of this thesis but their integral role in the immune system and relevance for B-cell function warrant a brief overview. Along with B-cells, T-cells were characterised in the 1960s through the pioneering work of Miller, Cooper, and colleagues that defined the key roles of these two cell types in adaptive immunity, namely antibody production by the former and cell-mediated immune responses by the latter.[402-405] In contrast to B-cells, which recognise circulating antigen in its native form, T-cells recognise peptide antigens derived from proteins that were degraded intracellularly and are presented via cell surface MHC molecules, a process referred to as antigen presentation.[355]

T-cells are broadly divided into two classes based on their distinct effector mechanisms which are delineated by expression of one of two co-receptors: CD4 or CD8. CD4+ T-cells, also known as helper T-cells, detect antigen in the context of MHC class II molecules expressed on the surface of professional antigen-presenting cells. They contribute to the adaptive arm of the immune system via production of cytokines which, depending on the subtype of T-cell, can have chemotactic, proinflammatory, or immunoregulatory effects. Certain CD4+ T-cell subclasses, such as CD4+CD25+ regulatory T-cells, can also dampen the immune response following a challenge. By contrast, CD8+ T-cells detect antigen in the context of MHC class I molecules, expressed on all cells, and carry out direct cytotoxic reactions that kill infected or neoplastic cells.[363]

3.14 Conclusion

The last section of this introduction has offered a brief overview of the players that orchestrate the delicate and finely coordinated adaptive immune response. It has also given a glimpse into the risks of this response potentially going awry. The structure and function of the antibody and its parent the B-cell have been discussed alongside some key discoveries and experimental approaches in immunology. It is important to keep these in mind when reading the following chapters: while immunology has been well-established for over a century, its study within the context of neurology is relatively novel. The focus of this thesis is the interplay between the two and the exciting new insights that stem from the study of these interactions.

CHAPTER 2

SERUM STUDIES IN PATIENTS WITH CASPR2-AUTOANTIBODIES REVEAL A PREDOMINANGE OF THE IgG4 SUBCLASS AND POTENTIAL CLINICAL RELATIONSHIPS

Preface

As outlined in the second section of the introduction, systematic studies of serum antibodies in patients with Caspr2-autoimmunity are rare, most papers featuring only a handful of samples. To be able to strategically choose samples fitting for the experiments in my thesis, I therefore first set out to characterise Caspr2-Immunoglobulin G (IgG) titres and subclasses in the sera from patients in the cohort available to the Oxford Autoimmune Neurology Group (OANG) and relate these to clinical phenotypes. The study design for this chapter is summarised in Figure 2.1(A). Results from Chapter 2 helped lay the groundwork for Chapter 3, where I will purify IgG from patient serum samples and inject them into mice, Chapter 4, where monoclonal antibodies from patient B-cells will be produced and functionally characterised, and Chapter 5, where the underlying B-cell immunobiology for four patients will be investigated.

INTRODUCTION

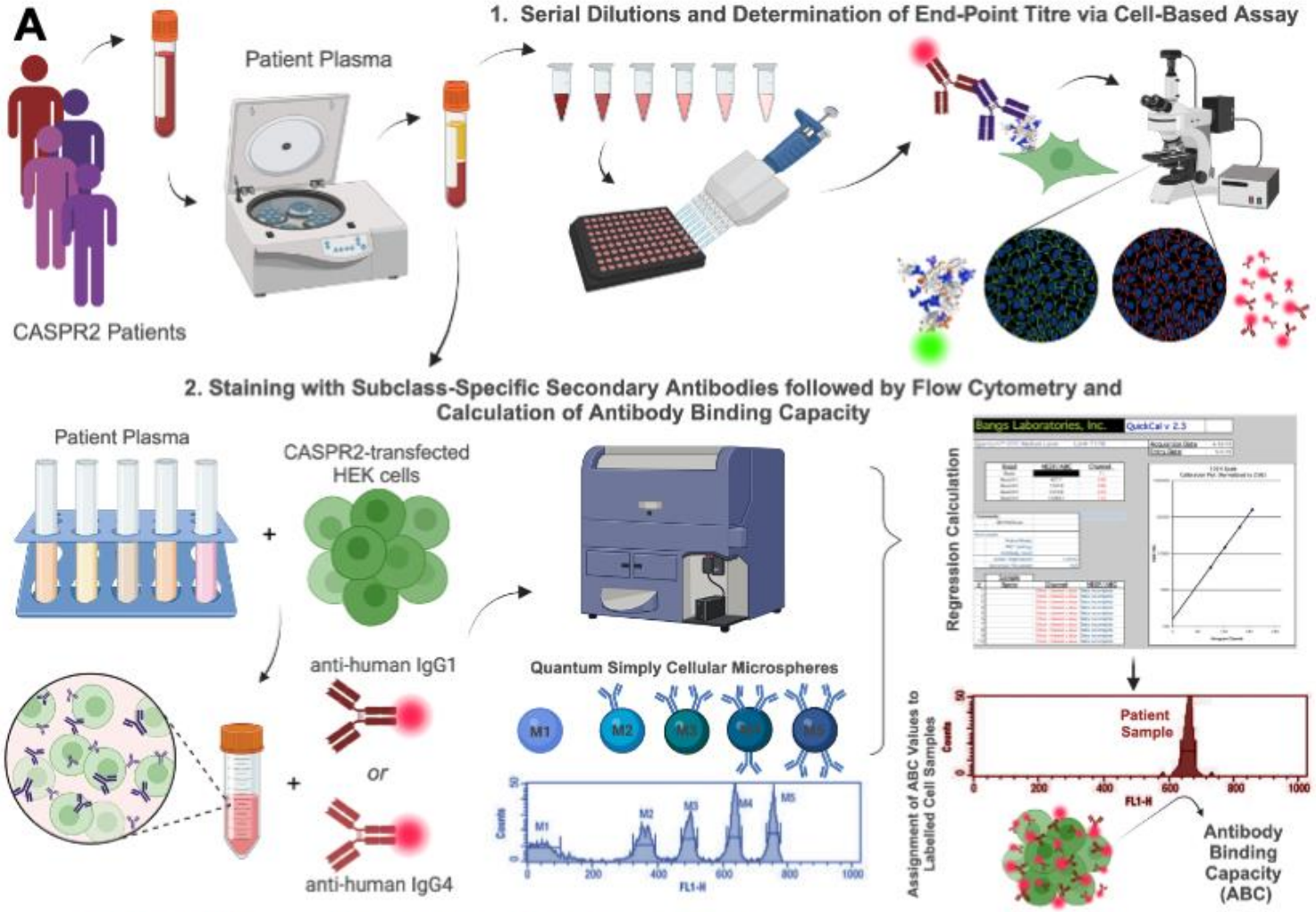
Considering individuals with Caspr2-autoantibodies are very rare (incidence estimated at 0.3 per million per year in the UK[406]), a limited number of larger cohort studies have been done to define serum autoantibody characteristics before and most knowledge is based on anecdotal evidence from diagnostic findings. Of the few studies done, only two have so far considered autoantibody subclasses and their relationship to clinical phenotypes. The first study, published in 2016, included samples from 38 patients that were confirmed to have Caspr2-autoantibodies via more than one test, including brain immunohistochemistry (IHC) and live cell-based assays (CBA)[245]. Although extensive clinical information was available on all patients, IgG subtype classification was performed for only half of the cohort (19 patients) via a CBA using subclass-specific secondary antibodies. All sera were found to be positive for CASPR2-IgG4 (100%), and 12 sera were also positive for IgG1 antibodies (63%). No notable clinical correlations with the presence of CASPR2-IgG1 antibodies were detected and the study did not feature any information on antibody titre.

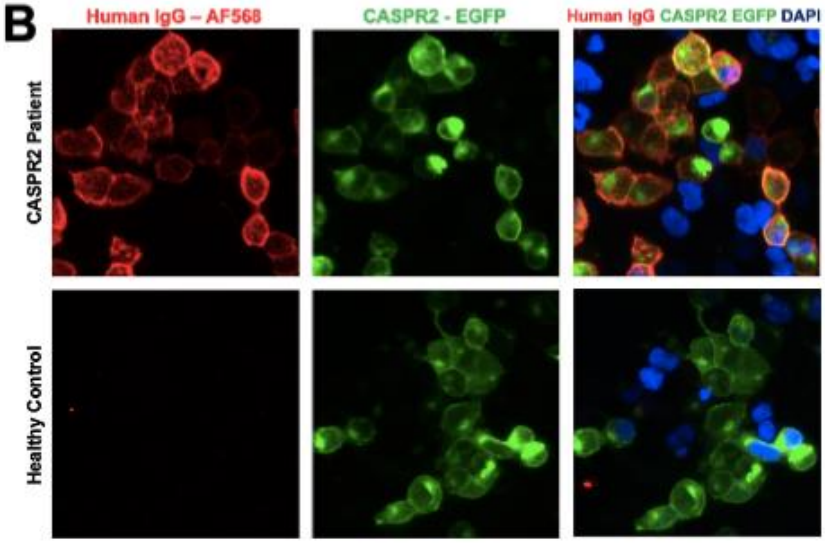
The second major study that investigated this question included a much larger cohort of 102 patients and centred on the pain phenotypes associated with Caspr2-autoantibodies. Presence of Caspr2-autoantibodies was confirmed via a live HEK293T CBA and IgG subclasses were determined for 48 of the included patients via a CBA using IgG subclass-specific secondary antibodies. Eight sera, all with low titres, were excluded as no subclass was detectable. Of the remaining 40 samples, IgG4-autoantibodies were detected in all but three sera (93%), IgG3 in 16 sera (40%), IgG2 in 26 sera (65%), and IgG1 in 15 sera (38%). Notably, IgG subclasses and titres bore no relationship to the occurrence of pain or the pain phenotype.[303]

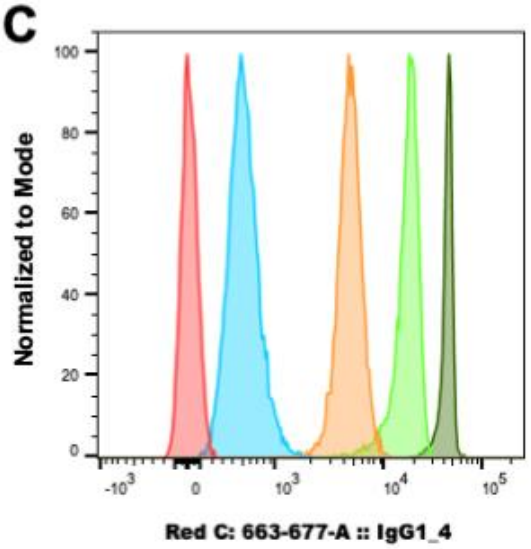
With these two previous studies in mind, the work described in this chapter aimed to use a larger cohort of patients and samples, to employ a more quantitative approach towards the Caspr2-IgG subclass classification question by utilising flow cytometry rather than a CBA with subclass-specific secondary antibodies, and to relate subclass status and levels to information on total Caspr2-IgG titres and patient symptoms over time, as permitted by the availability of longitudinal patient samples and clinical information.

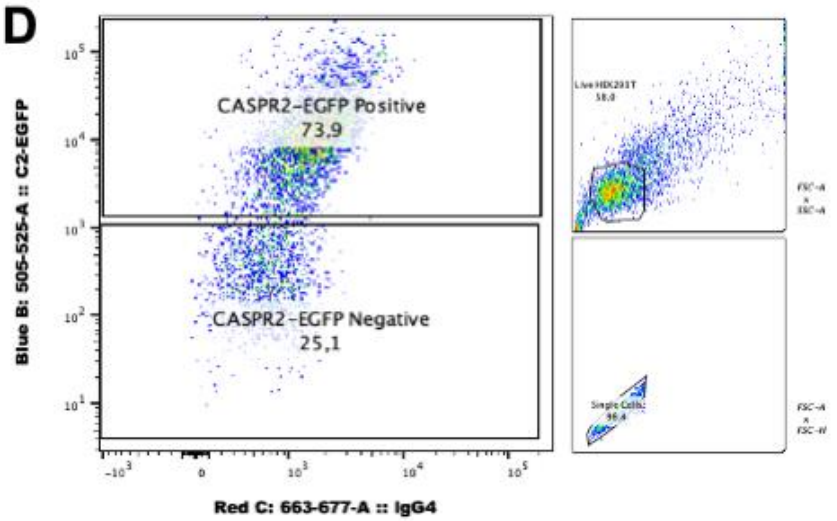
Another key piece of research from outside the Caspr2 field that guided this work came from studies on patients with neuromyelitis optica spectrum disorder (NMOSD), where shifts in the dominant AQP4-IgG subclass from AQP4-IgG1 to AQP4-IgG4, were shown to be associated with a clinical relapse.[407] However, NMOSD is considered an IgG1-dominated disease, in contrast to Caspr2-Abs-associated disease, which is IgG4-dominated. A more applicable study from the LGI1-antibody encephalitis field investigated clinical-serological relationships in 103 patients with the condition, showing that while all patients had LGI1-IgG4 antibodies (as is the case in Caspr2-AE), those with cognitive impairment had higher proportions of complement-fixing IgG1 antibodies.[227] These two previous clinical links from neighbouring fields led me to focus here on the IgG1 and IgG4 subclasses, as two opposite ends on the spectrum of IgG functionalities.[367]

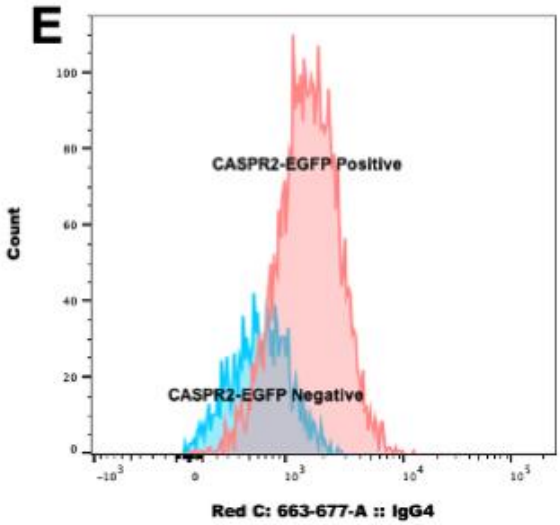
Figure 2.1 ***(next page)*** (A) Experimental design and strategy used for determining CASPR2-specific IgG subclass levels in patient sera. (B) Demonstrative images taken from a CASPR2-CBA showing staining from a strong positive patient sample compared to a negative healthy control on CASPR2-EGFP transfected HEK293T cells. (C) Fluorescence intensity of the four populations of Quantum Simply Cellular Beads (plus blank), used to design calibration curve and quantify ABC values for samples. (D) Gating strategy used for quantifying median fluorescence intensity (MFI) for CASPR2-specific subclasses. (E) Fluorescence intensity of the CASPR2-EGFP positive population and the CASPR2-EGFP negative population shown in (D). These two values were subtracted to determine the delta MFI for each sample, later converted to an ABC value.


A
1. Serial Dilutions and Determination of End-Point Titre via Cell-Based Assay
Patient Plasma
CASPR2 Patients
2. Staining with Subclass-Specific Secondary Antibodies followed by Flow Cytometry and Calculation of Antibody Binding Capacity
Patient Plasma
CASPR2-transfected HEK cells
anti-human IgG1
or
anti-human IgG4
Quantum Simply Cellular Microspheres
M1
M2
M3
M4
M5
FL1-H
Counts
Regression Calculation
Assignment of ABC Values to Labelled Cell Samples
Patient Sample
Antibody Binding Capacity (ABC)


B
Human IgG – AF568
CASPR2 - EGFP
Human IgG CASPR2 EGFP DAPI
CASPR2 Patient
Healthy Control


C
Normalized to Mode
Red C: 663-677-A :: IgG1_4


D
Blue B: 505-525-A :: C2-EGFP
CASPR2-EGFP Positive
73.9
CASPR2-EGFP Negative
25,1
Red C: 663-677-A :: IgG4


E
Count
CASPR2-EGFP Positive
CASPR2-EGFP Negative
Red C: 663-677-A :: IgG4

METHODS

Patients, Samples, and Clinical Data

Blood samples from CASPR2-autoantibody patients were collected at the John Radcliffe Hospital, Department of Neurology, after informed consent was obtained under research ethics approval committee (REC reference: 16/YH/0013, IRAS Project ID: 195152). Blood was collected in 9ml 'clot activator and serum-gel-separator' tubes and spun at 3000rpm for 10mins at room temperature as soon as possible after collection (Figure 2.1(A)). Samples were de-identified and assigned a lab number which was to be used in all future experiments in lieu of personal patient information. The serum was aliquoted and stored at -80ºC until use. Overall, a total of 88 samples from 35 different patients were included in this study. Clinical information was extracted from the Oxford Autoimmune Neurology Patient Database by two neurologists (Dr Chris Uy and Dr Bryan Ceronie) and the anonymised data regarding symptoms present and immunotherapy received at sample collection was linked to each sample. The clinical data had been collected via standardized questionnaires distributed to an international network of clinicians who had referred patients to the Oxford Autoimmune Neurology Group between 2015 and 2020.

Determination of End-Point Titre

Caspr2-autoantibodies were detected, and end-point titres of serum samples were determined using a live CBA (Figure 2.1(B)). HEK293T cells were seeded in 96-well plates at a density of 40,000 cells/well in DMEM (Dulbecco's Modified Eagle's Medium; Sigma #D6429) supplemented with 10% Foetal Bovine Serum and Antibiotic-Antimycotic (Thermofisher #15240062). The following day, 25.2µg of CASPR2-EGFP DNA was complexed with 12.6µl polyethylenimine in 420µl of non-supplemented DMEM for 10 minutes, before being added into 18mL of DMEM supplemented with FBS and AA and 150µl of this preparation being pipetted into each well of the 96-well plate. The final concentration of CASPR2-EGFP DNA was circa 250ng per well. The media was changed to fresh DMEM-FBS-AA 8 hours post-transfection to prevent cell death due to PEI toxicity and cells were left in a 37ºC 5% CO_2 incubator overnight. Approximately 24 hours post-transfection, each well was incubated with 100µl of sample diluted in DMEM buffered with 25mM of HEPES and supplemented with 1% Bovine Serum Albumin (BSA). The sample dilutions started at 1:100 and went up to 1:204,800. After a 1-hour incubation at room temperature with the samples diluted to the desired concentrations, plates were washed three times with DMEM buffered with HEPES and fixed with 10% paraformaldehyde for 10 minutes. Two more washes with DMEM-HEPES were done post-fixation. This was followed by incubation with goat anti-human IgG (H+L) AF568 secondary detection antibody (Thermofisher #A21090) diluted 1:750 (final concentration 3µg/mL) in DMEM-HEPES-BSA for 45 minutes at room temperature in the dark. Following secondary antibody incubation, plates were washed twice with DMEM-HEPES and twice with PBS, and binding was detected via visual inspection under an inverted fluorescence microscope

CASPR2-Autoantibodies Subclass Quantification via Flow Cytometry

The levels of IgG1 and IgG4 subclass antibodies in serum were quantified using a flow-cytometry antibody assay as described previously for LGI1-autoantibodies[227] (Figure 2.1(A)). Cells expressing CASPR2-EGFP were allowed to grow up to 80% confluency in 6-well plates and then re-distributed into V-bottom 96-well plates at a concentration of 50,000 cells/well in DMEM-HEPES-BSA. The V-bottom plates were spun at 1800rpm for 2mins to pellet down cells, then resuspended in diluted patient sera (1:50 – 1:800 dilutions in DMEM-HEPES-BSA to obtain accurate titrations) and left to incubate for 45mins at room temperature while shaking. Plates were then washed twice with PBS (by spinning at 1800rpm for 2mins, then resuspending

in PBS), fixed for 10mins in 4% PFA, then washed twice again in PBS. This was followed by incubation with mouse anti-human IgG1- and IgG4-subclass-specific secondary antibodies (Alexa Fluor 647-labelled, Southern Biotech), diluted 1:100 in FACS Buffer (PBS + 1% BSA) supplemented with 1% mouse serum. The secondary antibody incubation lasted for 45mins, during which samples were left shaking in the dark at room temperature. Cells were then further diluted in PBS, spun down and resuspended in FACS Buffer with DAPI added. Cells were analysed on LSR Fortessa X20 (BD Biosciences).

Data Analysis

The gating strategy using FlowJo (v10.7.2) excluded dead cells and doublets first (Figure 2.1 (D) and (E)), then gated on cells expressing CASPR2-EGFP (y-axis) against anti-human IgG binding (AF647, x-axis). To determine the relative quantities of subclass-specific CASPR2-antibodies a calibration curve was derived from Quantum Simply Cellular anti-Mouse IgG beads (Bangs Laboratories), comprised of four coated populations and one blank population of uniform microspheres that are approximately the size of human lymphocytes (7-9μm) (Figure 2.1(A)). Each of the four sets is coated with goat-anti-mouse IgG (Fc-specific) with different calibrated Antibody Binding Capacities (ABC) for mouse monoclonal antibodies (Figure 2.1(C)). When the bead populations are labelled in the same manner as the cells that are to be analysed, they provide a means for constructing a QuickCal calibration curve where ABC values are plotted against fluorescence intensity. This then allows for samples to be 'read' and for subclass-specific-IgG on HEK cells to be quantitated in ABC units. These arbitrary ABC units correspond to the "Levels determined by flow" used to express subclass-specific antibody levels throughout this chapter.

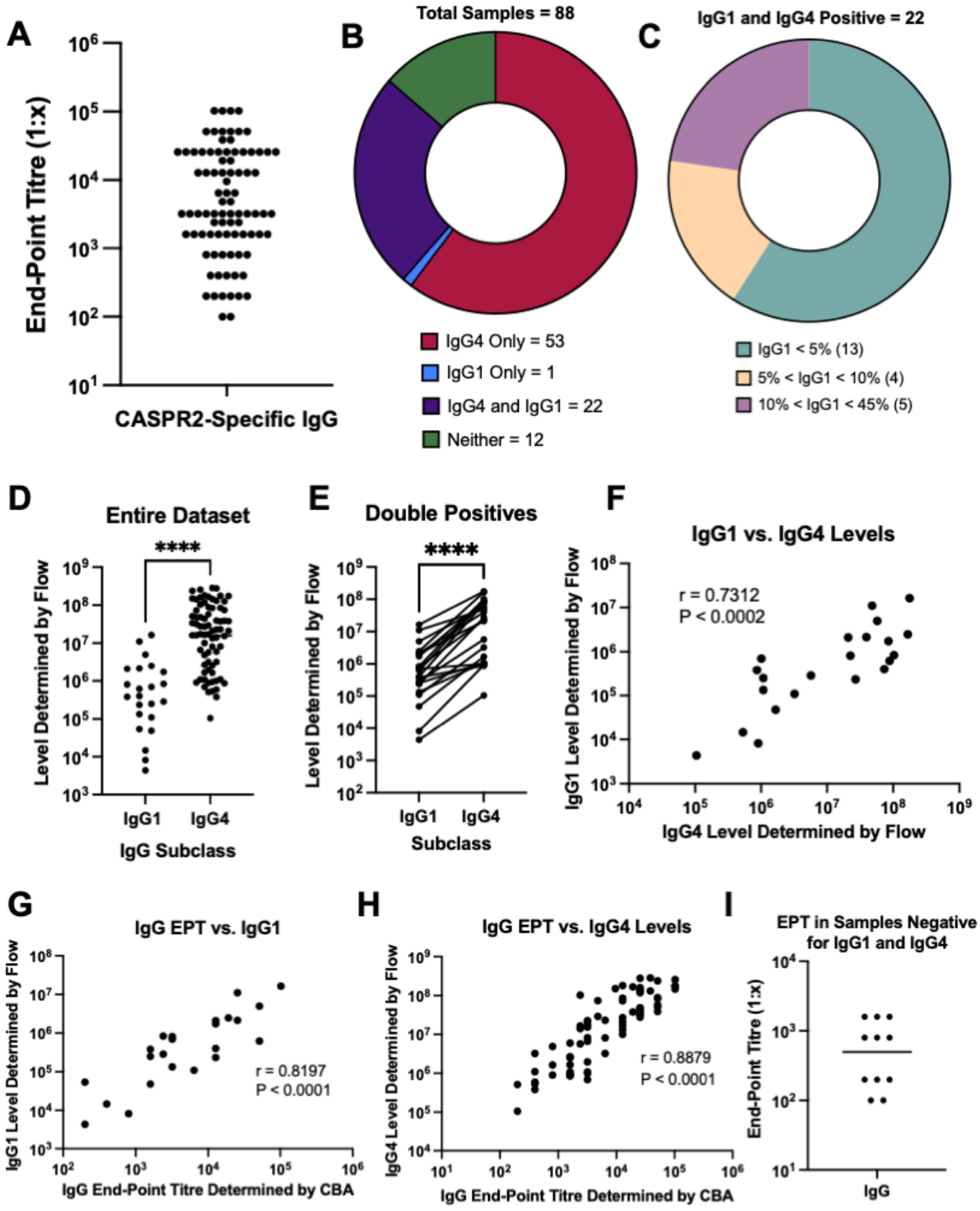


Figure 2.2 (A) Distribution of end-point titres among CASPR2 samples , as determined via serial dilutions on a CASPR2-CBA. (B) Prevalence of subclasses among patient samples, as determined by flow cytometry. (C) Percentage levels of IgG1 within samples that contained both subclasses. (D) ABC values as determined by flow cytometry demonstrate IgG4 levels across all samples were higher than IgG1 levels. (E) Within samples that contained both IgG1 and IgG4 CASPR2-specific antibodies, IgG4 levels tended to be higher than IgG1 levels by several orders of magnitude. There was a strong correlation between (F) IgG1 and IgG4 levels, (G) IgG end-point titre and IgG1 level, and (H) IgG end-point titre and IgG4 level. (Spearman's rank correlation coefficient) (I) IgG end-point titres among samples identified as having neither IgG1 nor IgG4 CASPR2-antibodies

RESULTS

Breakdown of Subclass Prevalence

A flow cytometry method was used to determine the subclass prevalence in CASPR2-auto-antibody positive sera. A total of 88 samples from across 35 patients were examined, their endpoint CASPR2-IgG titres (EPTs) ranging from 1:100 to 1:102400 (median 1:3200) as determined via live CASPR2-CBA (Figure 2.2(A)). The majority (60.2%) of samples had CASPR2-IgG4 autoantibodies only, 25% of samples contained both IgG1 and IgG4 CASPR2-autoantibodies, and only one sample had exclusively IgG1 CASPR2-autoantibodies (Figure 2.2(B)). Despite having been identified as CASPR2-positive via CBA, 12 samples contained neither IgG4 nor IgG1 antibodies, as per flow cytometry. While it is possible that these samples had presence exclusively of the IgG2 or IgG3 subclasses, this is highly unlikely[303], and a more likely explanation was that the levels of CASPR2-antibodies in these samples were below the detection threshold of our flow cytometry-based assay. This is further substantiated by these 12 samples having generally low levels of CASPR2-IgG as determined by CBA, ranging from 1:100 to 1:1600 (Figure 2.2(I)). Among samples positive for both IgG1 and IgG4, the level of CASPR2-IgG1 tended to be less than 45%, with most of these samples having less than 5% IgG1 (Figure 2.2 (C)). Across all samples, IgG4 levels tended to be higher than IgG1, and within samples that contained both subclasses, IgG4 levels were generally higher than IgG1 levels (Figure 2.2(D) and (E)). Within the 'double positive' samples, levels of IgG1 and IgG4 had a strong positive correlation (Figure 2.2(F)), while across all samples there was a strong positive correlation between CASPR2-IgG4 level and CASPR2-IgG EPT, as well as CASPR2-IgG1 level and CASPR2-IgG EPT (Figure 2.2(G) and (H)).

Changes in Caspr2-autoantibody Levels Over Time

Longitudinal samples were available for a select number of patients, allowing for the IgG EPT as well as IgG1 and IgG4 levels to be plotted over time (Figure 2.3). This revealed a trend whereby CASPR2-IgG4 levels, but not CASPR2-IgG1 levels seemed to follow a similar course as the CASPR2-IgG EPTs, albeit at different scales. This was especially evident for patients JR024 (Figure 2.3(A) and (B)), JR794 (Figure 2.3(E) and (F)) and to a lesser extent patient JR1284 (Figure 2.3(G) and (H). This observation prompted me to plot CASPR2-IgG EPTs alongside CASPR2-IgG4 levels for a few more patients for whom longitudinal samples were available (Figure 2.4). As well as the patients JR024, JR794, and JR1284, patients JR006 (Figure 2.4(A)) and JR041 (Figure 2.4(C)) demonstrated a similar course of change. This could suggest that changes in CASPR2-IgG4 levels, rather than IgG1, are driving fluctuations in CASPR2-IgG over time, as could be inferred based on the strong positive correlation discussed in the previous paragraph.

Clinical Significance of Subclass Status

Next, I sought to investigate whether subclass status was related to clinical presentation. I first classified all patients as having either 'only IgG4' CASPR2-autoantibodies (n=15) or having 'IgG1 Present' (n=11), meaning that at any point during their clinical course CASPR2-IgG1 autoantibodies could be detected from their samples. The latter included the one patient that only had CASPR2-IgG1 autoantibodies. Within each of the two groups I looked at the number of patients with the following symptoms: seizures, cognitive deficits, psychiatric disturbances, dysautonomia, neuromyotonia, neuropathic pain (NeP), and ataxia. These were expressed as a percentage of the total number of patients within each group. This revealed that neuromyotonia is more frequently associated with the presence of only IgG4 CASPR2-autoantibodies, whereas ataxia is more frequently associated with the presence of IgG1 CASPR2-autoantibodies. The remaining symptoms did not differentiate between subclass status groups (Figure 2.5(A)).

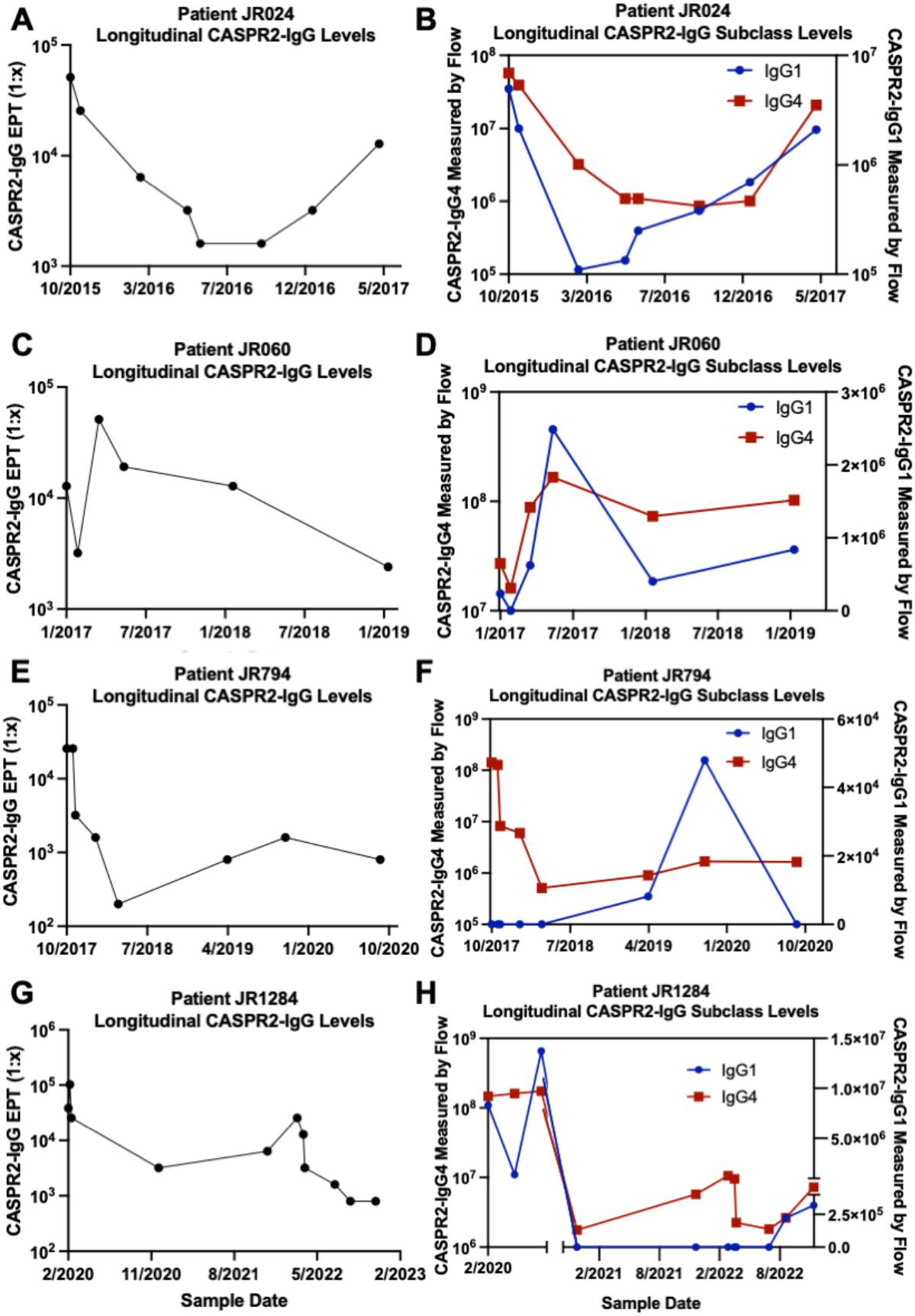


Figure 2.3 Plots showing longitudinal levels of CASPR2-IgG (left-hand graphs: A, C, E, and G), CASPR2-IgG4 (right-hand graphs: B, D, F, H; left y-axis), and CASPR2-IgG1 (right-hand graphs, right y-axis), for Patient JR024 (A, B), Patient JR060 (C, D), Patient JR794 (E, F), and Patient JR1284 (G, H).

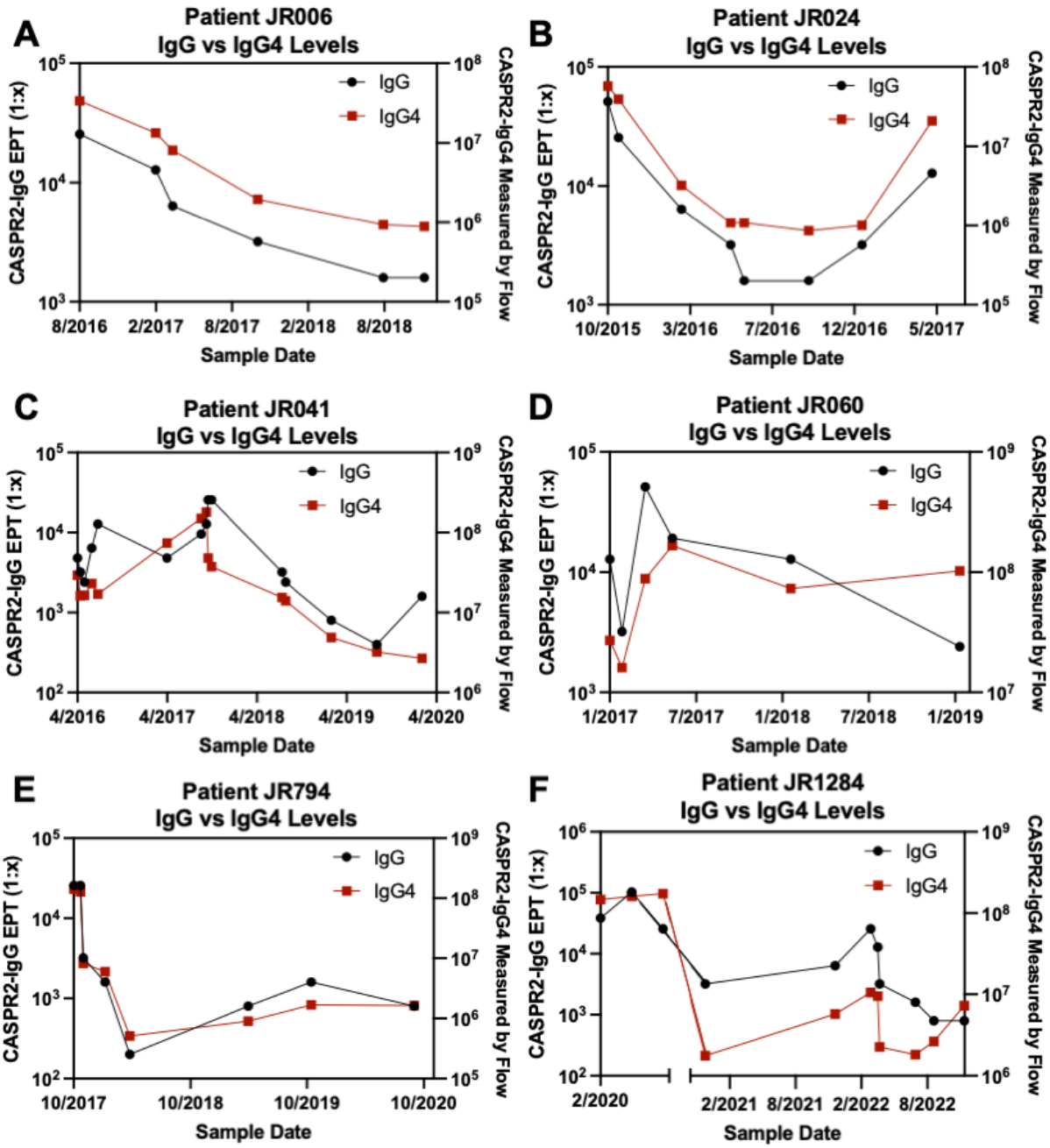


Figure 2.4 Comparison of variation of CASPR2-IgG end-point titre (left y-axis) and CASPR2-IgG4 levels (right y-axis) across time demonstrates the two tend to follow a similar course. Shown for Patient JR006 (A), JR024 (B), JR041 (C), JR060 (D), JR794 (E), and JR1284 (F)

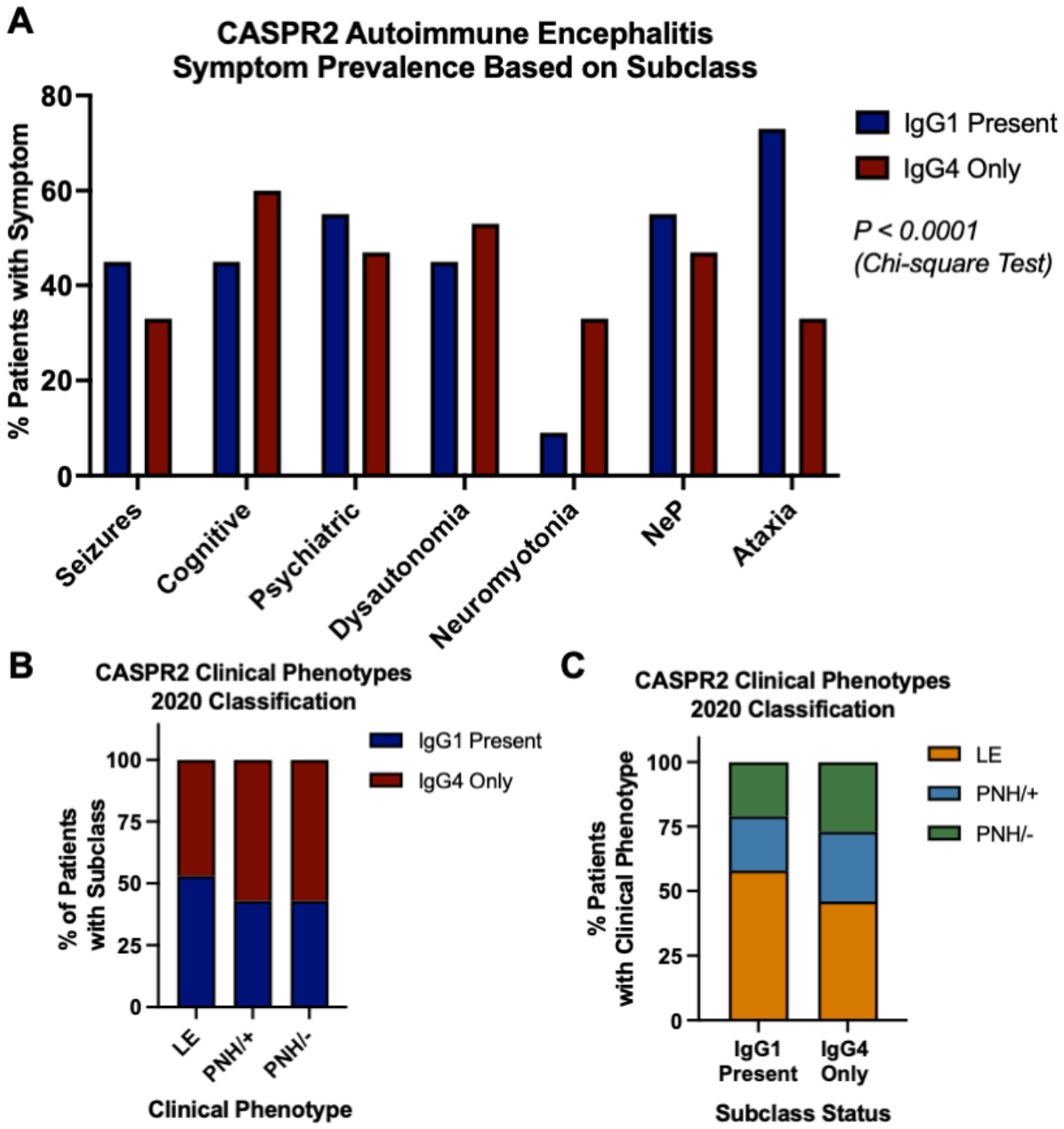


Figure 2.5 Clinical significance of CASPR2 subclass prevalence in patients (A) Patients were grouped into those who only had CASPR2-IgG4 antibodies ('IgG4 only' group, n = 15), and those who had either both IgG1 and IgG4 or IgG1 only ('IgG1 Present' group, n = 11) and the number of patients within each group that had experienced one of the key symptoms associated with CASPR2-autoantibodies (seizures, cognitive deficits, psychiatric disturbances, dysautonomia, neuromyotonia, neuropathic pain, ataxia) was expressed as a percentage. Patients were further grouped into those with prominent limbic involvement (LE), those with severe peripheral nerve hyperexcitability and extralimbic involvement resembling historical descriptions of Morvan Syndrome (PNH/+), and those with milder and usually isolated PNH (PNH/-), as per a 2020 classification of anti-CASPR2 clinical phenotypes. There was no difference in the subclass status among the LE, PNH/+ and PNH/- groups (B) nor in the clinical phenotype prevalence based on subclass status (C)

Subclass Status and a More Recent Classification of Caspr2-Phenotypes

The previous finding led me to wonder how subclass status could relate to a more recent classification of anti-CASPR2 clinical phenotypes that was introduced in 2020.[296] The new classification groups CASPR2-patients into those with primarily CNS involvement that manifests as a classical limbic encephalitis (LE group), those with combined CNS and PNS involvement that resembles the historical Morvan Syndrome definition, including peripheral nerve hyperexcitability and extra-limbic features (PNH/+), and finally those with isolated PNS involvement that manifests as less severe PNH (PNH/- group). No association was seen between subclass status and the 2020-definition of clinical phenotype (Figure 2.5(B) & (C)). This was however to be expected, as most individual symptoms mentioned in the previous paragraph bore no association with subclass status.

Longitudinal Relationship Between Symptoms, Treatment, Titres, and Subclasses

The investigation of the relationship between subclass status and clinical phenotype was extended to look at the course of symptoms, immunotherapy treatment, IgG titre and IgG4 and/or IgG1 levels within individual patients over time. A total of 8 patients for whom longitudinal data was available were included, 3 of whom had not been treated with immunotherapy (Figure 2.6). One of the first observations was that administration of immunotherapy did not necessarily reduce levels of CASPR2-IgG, -IgG1 or -IgG4, as evident for patients JR016 and JR024 (Figure 2.6(C,G)), although in some cases did this happen, as seen in patients JR041 and JR794 (Figure 2.6 (D,F)). Likewise, sometimes the antibody levels fell and certain symptoms subsided in the absence of immunotherapy, as apparent in patients JR006 and JR060 (Figure 2.6(B,H)). Looking at the relationship between autoantibody levels and symptom onset, there were no apparent trends apart from the occasional paradoxical findings. Thus, it was possible for symptoms to persist despite the levels of autoantibodies falling, as apparent for patients JR006, JR794, and JR060, but it was also possible for the symptoms to disappear despite autoantibody levels rising, as for JR061, JR024, and the initial presentation of patient JR060. Lastly, PNS symptoms (neuromyotonia, NeP, dysautonomia) were more likely than CNS symptoms to persist regardless of treatment. The exception to this trend was ataxia, which persisted in all three patients where it was present – JR002 (Figure 2.6(A)), JR794, and JR060. However, this could be by chance as two of these patients did not receive immunotherapy.

Patients negative for both IgG1 and IgG4 Caspr2-autoantibodies

Finally, I turned my attention more specifically to the patients whose samples were shown to be negative for both the IgG1 and IgG4 subclass via flow cytometry, despite positive results on the Caspr2 cell-based assay. Although the negative outcome of these samples on the flow assay was initially attributed to their relatively low Caspr2-IgG titres (Figure 2.2(I)), it was interesting to examine whether any particular features of the patient or the disease correlated with the absence of both the IgG1 and IgG4 subclasses among Caspr2-autoantibodies and perhaps pointed to the contributions of a different antibody subclass to pathology. The ages, sex, symptoms, syndrome (as per 2020 classification; Figure 2.5), and EPT ranges of these patients are summarised in Table 2.1., as well as whether or not they received immunotherapy at any point during their disease course. Some data was not available in the patient databases and this is recorded as ‘n/a’ in Table 2.1. Apart from Caspr2-IgG end-point titres being on the lower end (≤ 1600), no other clinical associations were apparent. Symptoms such as seizures, cognitive, and psychiatric disturbances were as prevalent in this patient group as they were in those identified as positive for IgG1 and/or IgG4, although interestingly, dysautonomia, ataxia, and neuropathic pain were less prevalent in this group (≤ 25%, as opposed to ≥ 40% in the positive cohort; Figure 2.5(A))

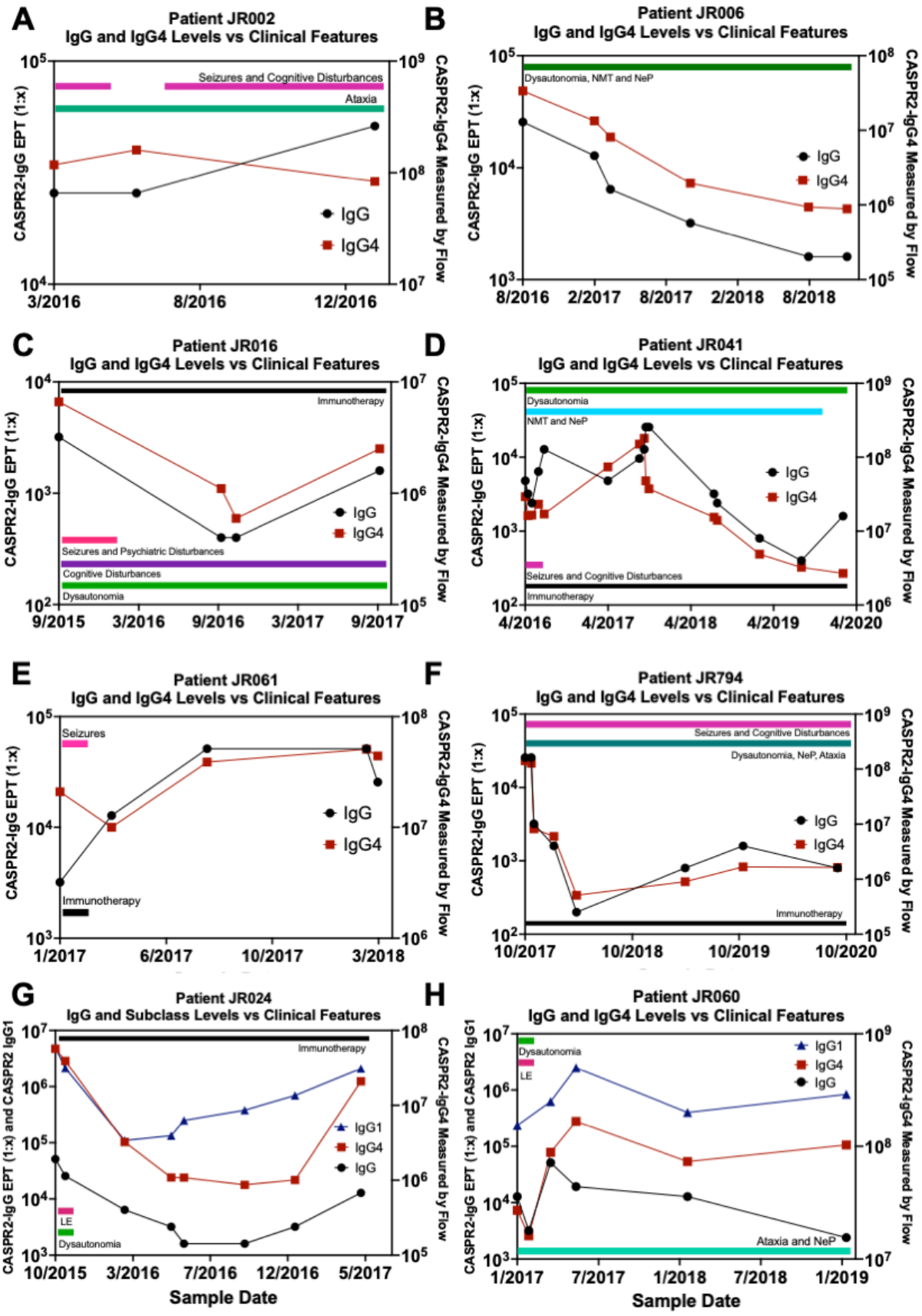


Figure 2.6 The clinical symptoms present and course of immunotherapy, where received, were plotted longitudinally in relation to CASPR2-IgG, CASPR2-IgG4 and (where present) CASPR2-IgG1 levels, for patients JR002 (A), JR006 (B), JR016 (C), JR041 (D), JR061 (E), JR794 (F), JR024 (G), and JR060 (H).

Table 2.1 Clinical data for patients negative for both IgG1and IgG4 Caspr2-Antibodies

Patient ID	Age	Sex	Symptoms	2020 Classification	EPT Range	Immunotherapy
JR030	83	M	Seizures, Cognitive and Psychiatric Disturbances	LE	1:200 - 1:1600	Yes
JR078	25	F	Cognitive and Psychiatric Disturbances	n/a	1:1600	No
JR085	68	M	Dysautonomia, Ataxia and Neuropathic Pain	n/a	1:200	Yes
JR088	37	F	Dysautonomia, Neuromyo-tonia, Neuropathic Pain	n/a	1:800	No
JR755	61	F	Seizures and Psychiatric Disturbances	n/a	1:800 - 1:1600	No
JR789	n/a	M	Seizures, Cognitive and Psychiatric Disturbances	LE	1:200	No
JR806	43	M	Seizures, Cognitive and Psychiatric Disturbances	LE	1:100	Yes
JR931	68	M	Cognitive and Psychiatric Disturbances	PNH/+	1:100	Yes

Table 2.1 Clinical data for patients whose samples were positive on a Caspr2 CBA but identified as negative for both IgG1 and IgG4 Caspr2-autoantibodies by flow cytometry (recorded as 'Neither' in Figure 2.2(B)). Clinical data was retrieved from the OANG Caspr2 samples record and the patient database (the latter done by Dr Bryan Ceronie). Fields labelled 'n/a' refer to where information was not available in the patient record. EPT range includes the end-point titre or range of end-point titres as determined using a Caspr2 CBA for Human IgG as described in Methods Section 'Determination of End-Point Titre' and shown in Figure 2.2(A). Data for one patient was excluded as it was retrospectively concluded that this was a healthy control that had been erroneously included in the Caspr2 cohort. N.B. there are eight rather than eleven patients in the table as three of the negative samples came from patient JR030 and two came from JR755. This is reflected in there being a range of EPT values given for those patients.

DISCUSSION

In this chapter I have characterised the subclasses of CASPR2-autoantibodies in patient sera, their levels and their relationships to CASPR2-IgG titre and clinical phenotype. At the time of writing of this thesis, there are only two published studies that have examined this, both characterising CASPR2-autoimmunity as IgG4-dominated and neither having identified a relationship between subclass status and clinical characteristics[245, 303]. The results reported in this chapter largely support this claim, with some caveats. Indeed, all samples apart from one contained CASPR2-IgG4 antibodies and most samples (53/88) only had the IgG4 subclass. Furthermore, in the 23 samples where IgG1 was present, this was usually at very low level, with most of these samples having less than 5% IgG1. Considering that the total IgG in human serum is composed of roughly 60% IgG1 and 4% IgG4,[367] the fact that these relative abundancies are practically reversed for CASPR2-specific IgG is especially astonishing and reflects the idea that IgG4 autoantibodies are the primary driver of CASPR2-related disease.

Notably, 12 samples that were identified as CASPR2-autoantibody positive via CBA were shown to have neither subclass on the flow cytometry assay. This could be explained due to their low IgG titres not being detectable via flow cytometry, suggesting that CBA-methods based on visual inspection might tend to have higher sensitivity than flow cytometry assays, or indeed that they are more prone to 'false positives'. Alternatively, it is possible that pathology in these patients is mediated by the other IgG subclasses. To further inquire into the latter possibility, I tried to see whether there are any features of the disease or patient that relate specifically to the negative flow cytometry assay outcomes. One possible guess was that the negative patients has received a longer course of treatment and were in fact in remission, but

this was not reflected in the patient data as 4/8 patients had not received any treatment. The sex prevalence and age-range of the patients reflected what is normally seen in Caspr2 cohorts, but certain symptoms (dysautonomia, neuropathic pain, and ataxia) were less prevalent than they were in the positive cohort. Unfortunately, information on the 2020 syndrome classification was not available for 4/8 patients, making it very difficult to draw further conclusions about an association between symptoms/syndrome and IgG1/IgG4 negativity.

The only two other studies that have investigated the subclass prevalence of CASPR2-autoantibodies both relied on a Cell-based assay method, although surprisingly in one of them a fifth of patients (8/40, all low titre) had no subclass detectable, whereas the other study determined subclass status in only half of the patients in the study, albeit it is unclear whether this was due to the remaining samples having no subclass detectable or simply the study design. In this sense, our method outperforms both previous published ones and it would be interesting to see whether our 12 samples where no subclass had detectable via flow could have their subclass status determined via CBA. In terms of subclass prevalence however, both studies reported higher incidence of CASPR2-IgG1 compared to our findings (63% in one study, and 37% in the other, compared to 26% in our study). This could be put down to the fact that our study included multiple samples per patient taken at different points in time where available, and it just so happened that several patients where multiple samples were available had either only CASPR2-IgG4 or mostly CASPR2-IgG4. By contrast, the other two published studies typically included one sample per patient. Indeed, if we look at patients rather than samples in our study as was done for the results in Figure 2.5(A), we find there are roughly as many patients with IgG1 present as those with only IgG4 autoantibodies, in fact putting out CASPR2-IgG1 patient prevalence at 42%, resembling what has been previously published.

In contrast to the previous studies which merely looked at the occurrence of each subclass, or used a semi-quantitative CBA-scoring approach, our study was the first to express subclass levels in terms arbitrary ABC units via the use of Quantum Simply Cellular beads system. This allowed us to draw parallels in terms of subclass levels, revealing a positive correlation between CASPR2-IgG1 levels and CASPR2-IgG4 levels, as well as between each and the CASPR2-IgG end-point titre. While it is perhaps unsurprising that higher overall levels of CASPR2-specific immunoglobulin are a positive predictor of higher subclass-specific CASPR2-autoantibody levels, this finding is especially interesting considering the two sets of results came from two different detection modalities: cell-based assay serial dilution and flow cytometry, further confirming the validity of both methods. Furthermore, the availability of quantitative data on subclass levels allowed for plotting this information longitudinally and revealing a similar trend in CASPR2-IgG and CASPR2-IgG4 levels, as well as allowing to explore the relationship between IgG titre, subclass levels, symptom onset, and treatment. Thus far, no CASPR2-autoantibody study has looked at this type of information either on a cohort level nor at the more fine-grained level of individual patients.

It is worth acknowledging our experiments didn't consider CASPR2-specific autoantibodies of the IgG2 and IgG3 subclass. We chose to focus on IgG1 and IgG4 due to previous reports of the clinical significance of these two subclasses.[227, 407] However, unlike NMOSD, CASPR2-associated syndromes generally don't follow a relapsing-remitting course, henceforth the AQP4-IgG subclass findings may not be very applicable. By contrast, the LGI1 study might be more applicable as LGI1- and CASPR2-autoantibodies related syndromes are relatively congruent from the medical perspective, were once considered part of the same disease aetiology,[208, 292] and even today are often lumped together in clinical reviews and discussions.[302, 406, 408-410] The only study so far that has looked at CASPR2-autoantibodies of

the IgG2 and IgG3 subclass found their presence to be approximately as frequent as that of IgG1, and furthermore found no association between the presence of these subclasses and the clinical presentation.[303] The same study also found no association between the autoantibody titres and IgG4 or IgG1 subclasses and pain in CASPR2-positive patients. In this sense, our finding that some but not all symptoms bore association with the presence or absence of CASPR2-autoantibodies of the IgG1 subclass is a new contribution to the field. Our work confirms what has been previously known about the persistence of neuropathic pain in CASPR2-patients despite treatment,[302] but is the first to note a similar trend for ataxia, whose association with CASPR2-autoantibody titre and subclass has not been investigated in detail in previous clinical reports.[411, 412]

The findings reported in Chapter 2 substantiate choices made in experiment design in the following thesis chapters. Firstly, in Chapter 3, sera from patients JR002 and JR1284 are chosen for isolation of IgG that is then injected into mice. As shown here, JR002 is an CASPR2-IgG4-only patient, whereas JR1284 has both subclasses among CASPR2-IgG. It will be interesting to examine whether the two different types of sera will produce different behavioural deficits. Then, in Chapter 3, monoclonal CASPR2-specific antibodies are cloned from patients JR002 and JR060. These mAbs are cloned both on an IgG1 and IgG4 backbone and injected into mice as both modalities, which furthers the exploration begun in Chapter 2 on the significance of antibody subclass, alongside specificity, for pathogenicity. Finally, considering this chapter showed no clear relationship between levels of CASPR2-IgG, immunotherapy, and symptom onset or resolution, the final data chapter will turn towards the autoantibody-producing B-cells. This study will include two of the patients examined more closely in this chapter, namely patients JR794 and JR1284, as well as two more patients included in the subclass study, to look at the unique features which distinguish autoreactive B-cells in these patients from those in healthy controls.

CHAPTER 3

PURIFIED IgG FROM SERA OF TWO PATIENTS WITH CASPR2-ANTIBODY ENCEPHALITIS INDUCES ANXIETY-RELATED BEHAVIOURS *IN VIVO*

Preface

Caspr2-autoantibodies were identified nearly 15 years ago, and the clinical conditions they are now associated with (neuromyotonia, Morvan syndrome, and limbic encephalitis) have been characterised for much longer within the context of voltage-gated potassium channel (VGKC) antibodies, now no longer considered diagnostically relevant.[208, 209, 291] With this in mind, it is surprising how little work has been done in terms of understanding the pathogenic effects of Caspr2-autoantibodies in the living organism. In the thesis introduction, I summarised how this has led to the question of whether Caspr2-autoantibodies are even pathogenic at all and gave an in-depth account of most *in vitro* studies performed to date. The notable rarity of successful animal studies has made it harder for Caspr2-autoantibodies to meet the Rose-Witebsky-Bona criteria for pathogenicity. In this chapter, I will attempt to address this by systematically investigating the behavioural consequences of injecting Caspr2-autoantibodies from patient sera into the adult mouse brain. Before diving into the outcomes of my own experiments, I will provide an in-depth summary of the *in vivo* studies available in the literature and analyse their successes and limitations.

At the time of writing of this thesis, there are only three published studies that have attempted *in vivo* injection of Caspr2-autoantibodies from patients, with varying degrees of success.[317, 319, 413] While one might argue this attests to their lack of pathogenic/diagnostic relevance, I have postulated that it was the delivery methods used, especially when studying effects of Caspr2-autoantibodies on the central nervous system, that stood in the way of successfully replicating patient clinical phenotypes. In the work presented here, I will opt for delivery of a small volume of highly concentrated immunoglobulin G directly into the mouse hippocampus, examining its effects on memory and anxiety.

The study that has been most successful in producing a robust behavioural deficit focused on the peripheral nervous system and used a delivery method compatible with the phenotype being developed.[413] Mice were administered intraperitoneal injections of purified IgG from patient plasma or from a healthy control. One of the patients had Morvan syndrome with the typical features of neuromyotonia, dysautonomia, pain, and severe insomnia, while the other presented with cerebellar ataxia and neuropathic pain; both had very high end-point titres of IgG in their sera, as detected via live CBA. Mice were dosed with either 6mg or 10mg of IgG per day for either 14 or 22 days. Throughout the treatment regime, withdrawal threshold and latency were tested for using von Frey fibres to examine mechanical sensitivity. Mice treated with CASPR2-IgG developed a significant delayed-onset mechanical hypersensitivity when compared to control IgG-treated mice, with the effect being observed after 11 days of IgG injections and becoming most prominent after 14 days. No differences between groups were seen in the open field test in terms of locomotor activity and rearing behaviour. The mechanism via which CASPR2-autoantibodies elicit pain was shown to be enhancement of excitability in dorsal root ganglion (DRG) neurons through regulation of Kv1 channel expression at the soma membrane. Importantly, these results were confirmed in *Cntnap2*$^{-/-}$ mice where full-length CASPR2 had been genetically ablated.

While being the most extensive and clinically relevant study on the in vivo effects of Caspr2-autoantibodies, this paper is simultaneously the one least applicable to the work in this thesis, as it focuses on the effects of Caspr2-Abs on the PNS, and furthermore shows no cognitive behavioural deficits. The first study that did focus on the CNS effects of Caspr2-IgG relied on an intraperitoneal route combined with LPS administration to temporarily breach the blood-brain barrier and permit CNS access to the IgG injected systemically.[317] Mice received 1-2ml of 20mg/ml IgG per day for 8 days, accompanied by behavioural tests for memory, anxiety, locomotion, coordination, and social interactions from Day 5 (two days after BBB breach) until Day 11 of the experiment. Despite the large amounts of IgG injected, the invasive breach of the BBB, and the extensive testing, the behavioural deficits were quite mild, casting serious doubt on whether CASPR2-Abs could at all be pathogenic in the CNS. Mice given CASPR2-IgG displayed slightly decreased spontaneous alternation on the Y-maze test and longer latency to first interaction on the reciprocal social interaction test, associated with more freezing behaviour, and reduced non-social activities like rearing and grooming compared to controls. Neuropathology showed that CASPR2-IgG injected mice showed signs of glial activation, increased CASPR2 expression, and a modest loss of Purkinje cells.

With the previous publication giving little hope that CASPR2 CNS pathology can be modelled in a mouse, a more recent paper provided slightly more convincing evidence of CASPR2-IgG pathogenicity.[319] Here, an intracerebroventricular (i.c.v.) injection was used, whereby bilateral catheters were inserted in the lateral ventricles and connected to subcutaneous osmotic pumps

filled with pooled IgG from six patients. The IgG was administered over the course of 11 days, at unspecified concentrations and volumes. Mice were tested on the novel object recognition test and demonstrated a decreased novel object location (NOL) index compared to controls on day 18, subsequently reverting to normal performance by day 25. No differences were seen in anxiety-related behaviour, motor and cerebellar functions, anhedonia, and pain sensitivity compared to controls. The spatial memory deficit was accompanied by hippocampal reduction in surface CASPR2 clusters, decreased CASPR2/TAG1 colocalization, and reduced surface levels of Kv1.1 and GluA1, two proteins considered to be CASPR2-regulated. Despite very strong histological evidence of pathogenicity, it is important to note that the novel object test performance was beneath standards even among controls, with NOL indices of 0.2 versus the usually accepted 0.5 value.[414, 415] Furthermore, the novel object test is intended to test for visual recognition and there have been certain criticisms of it being used as a spatial memory test, as done in this study. In turn, there are multiple other tests designed to assess hippocampal-based spatial memory like the Morris water maze, Barnes maze, T-maze or Y-maze tests that would have been more appropriate.[416]

Although slightly different in terms of aims and methods used from the previous three, another study that warrants mentioning in this section attempted an *in utero* CASPR2-IgG exposure method. Pregnant dams received intraperitoneal injections of 20mg daily of either CASPR2-IgG from two patients or IgG from healthy controls. This was done from day E12.5 to E18.5, and the long-term effects of maternal-to-foetal antibody transfer were studied.[417] CASPR2-IgG, but not control IgG, was shown to reach the foetal brain parenchyma. Although reaching developmental milestones similar to their control counterparts, as adults the neonates exposed to CASPR2-IgG *in utero* showed marked social interaction deficits, abnormal distribution of glutamatergic neurons in somatosensory cortex, increased microglial activation, and decreased glutamatergic synapses in the prefrontal and somatosensory cortices. CASPR2-IgG was thus shown to profoundly affect CNS development, although the behavioural deficits were once again restricted to social interactions.

Taken together, the studies discussed support an effect for CASPR2-autoantibodies on pain sensitivity, social behaviour, and spatial memory. Across all studies, high levels of IgG needed to be administered over a prolonged course for any effect to be observed, perhaps reminiscent of the high titres of CASPR2-antibodies seen in patients.[418] Yet even when administration was successful, the effect was often relatively mild. The studies looking at CASPR2-IgG effects in the PNS and *in utero*, while experimentally very sound, are tangential to the topic of this thesis. By comparison, the two studies that directly address the effects of CASPR2-Abs on the CNS rely on loading the CNS with supraphysiological levels of CASPR2-Abs to ultimately achieve an effect that is either mild or reversible. Furthermore, the LPS-mediated permeabilization of the BBB used in one of the studies and the pooling of multiple (already) polyclonal patient samples in the other are both significant departures from how the disease occurs naturally. Notably, there have been no reports of BBB breach in patients with the condition.

In my work, I have opted for an approach whereby immunoglobulin was delivered to the CNS directly via a stereotactically-mediated intrahippocampal injection, as done previously for LGI1 autoantibodies.[396] A very small volume of IgG close to physiological IgG concentrations in mouse serum was used.[419, 420] Behavioural testing was performed very close to injection time using a comprehensive array of tests for anxiety-related and depressive-like behaviours, spatial- and recognition-based memory, and locomotion. Two samples from patients discussed in the previous chapter were used. One sample, from patient JR002, was collected while the patient was still untreated, while the other, from JR1284, was collected once immunotherapy

had been long underway. Both samples had very high titres of CASPR-IgG: 1:25600 for JR002 and 1:102400 for JR1284, and both patients were clinically classified as having limbic encephalitis. Patient JR002 had only IgG4-CASPR2 antibodies, whereas patient JR1284 had both the IgG1 and IgG4 subclass. Equipped with these well-characterised samples, I designed a series of simple experiments to examine the behavioural effects of CASPR2-IgG in vivo, applying what was learned in each experiment onto the next one.

METHODS

Immunoglobulin G Purification

Immunoglobulin G was purified from patient sera using Protein G Sepharose™ Beads 4 Fast Flow as per the manufacturer's instructions. Beads were washed 3 times in PBS by spinning them at 12,000g for 20s. Each serum was incubated with an equal volume of beads overnight at 4°C while spinning on a tube rotator. The next day beads were transferred to spin columns provided by the manufacturer and centrifuged at 12,000g for 20s to separate unbound serum in the supernatant, then washed 3 times with 200µl PBS. Bead-bound IgG was eluted using 50µl of 0.1M glycine-HCl, pH 2.7 into 5µl of 1M Tris-HCl, pH 8.4, to immediately neutralise the glycine-HCl. The elution step was repeated 5 times to maximise yield, after which spin-concentration and buffer exchange were performed using Amicon® Ultra-0.5ml Centrifugal Devices with a 3kDa cut-off, leaving the serum-purified IgG at a final concentration of 5mg/ml in DPBS. Maximum care was taken to avoid any contamination.

Stereotactic Injections

All animal work for this study was performed under project license P996B4A4E, personal license I11739608. Adult female C57BL/6J mice (Charles River), 5 weeks of age, were housed in cages of five in a room maintained at a controlled temperature (21°C) and humidity (5-10%) with illumination in 12h cycles; food and water were available *ad libitum*. All injections were performed during the light phase, and animals were habituated to the experimental room for one hour prior to beginning injections and 15 minutes prior to behavioural testing. Procedures were conducted in compliance with the Animal Scientific Procedures Act (ASPA) 1986, revisited 2012, and the European Directive 63/2010 on the protection of animals, as well as in accordance with the Institutional Animal Care and Use Committee (University of Oxford). For the surgery, mice were anaesthetised with isoflurane and placed in a stereotactic apparatus. A mid-sagittal incision was made to expose the cranium and two burr holes were drilled over the hippocampi to the following coordinates from bregma: anteroposterior -1.5 mm; lateral ±1.8 mm. A glass microcapillary containing the solution to be injected (purified IgG, concentration 5 mg/ml in DPBS) was lowered 1.5 mm ventral to bregma and, depending on the experiment, either 0.5µl or 1µl of solution was injected per hemisphere over the course of 5 minutes. The incision was then cleaned and closed with resolvable sutures. Mice were monitored post-surgery and re-sutured, where necessary. They were allowed to recover a minimum of 3 hours post-surgery prior to behavioural testing. Where two rounds of injections were performed, the second injection was performed 24 hours after the first, using the exact same procedure. Mice were regularly handled prior to commencing behavioural testing.

Behavioural Testing

All behavioural tests were performed at the Department of Pharmacology Animal Facility in accordance with institutional guidelines. Animals were handled regularly prior to testing, and tests were designed to minimise animal discomfort. No more than three tests were performed per day, and animals were left to rest for at least 15mins between tests. Wherever possible,

behavioural results were analysed by two independent observers and the average was reported. Where this was not possible, results were analysed by a single blinded observer or using ANY-Maze software. The protocols for individual behavioural tests are summarised below.

1. *Open Field (OF)*

A white wooden box with 4 separate fields, each with dimensions 50cm x 50cm x 50cm was used. A 25 x 25cm square outline was made with red tape in each cube to mark the inner zone. Mice were placed in the box facing one of the four walls and allowed to explore for 5mins, after which the mouse was removed, the faecal pellets were counted, and the box was cleaned with water and 70% ethanol before the next mouse was put in. A mouse was considered to be in the inner zone when 90% of mouse body area was in the red square. Time spent in the inner zone and rearing count were measured manually, while mobility parameters (distance travelled, average speed, and time immobile) were measured using the ANY-Maze software.

2. *Novel object recognition (NOR) test*

Mice were allowed to explore the open field box for 5mins preceding testing (Habituation). In the training phase, mice explored two identical objects for 15mins (Familiarization). Then, in the test phase, performed 5mins after Familiarization, one of the objects from the training phase was replaced with a novel object and the mouse was left to explore for 5mins. Assignment of objects as novel or familiar, and the placement of the novel object was randomized, and objects were cleaned thoroughly with water and 70% ethanol between trials. A mouse was considered to explore an object when its snout was directed towards the object within a 2cm distance. Discrimination ratio (DR) was calculated with the following formula:

$$DR = \frac{T_{NEW} - T_{OLD}}{T_{NEW} + T_{OLD}}$$

Where "T" stands for time spent exploring an object. Mice that did not interact with the objects for at least 20s during the testing period were excluded from the analysis.

3. *Y-maze tests (YmSA and YmFA)*

A standard wooden Y-maze apparatus with three arms, each arm 30cm in length was used. In the spontaneous alternation test, mice were placed in one arm facing the centre of the maze and allowed to freely explore the maze for 8mins. A spontaneous alternation was defined as a mouse entering a different arm of the maze in each of 3 consecutive arm entries. The percentage spontaneous alternation (SA%) was then calculated using the following formula:

$$Spontaneous\ alternation\ \% = \frac{\#\ spontaneous\ alternations}{total\ number\ of\ arm\ entires - 2} \times 100$$

The forced alternation (FA) test consisted of two sessions: in the training session (5mins) one of the arms of the maze was blocked and the mouse was allowed to explore the remaining 2 arms. After a 1min break the test session was performed, where the mouse could freely explore all three arms for 5mins. The blocked arm was randomized, and arms were marked with black-and-white signpost patterns to provide spatial cues. Novel arm preference was calculated for either the first minute or the first two minutes of testing as shown:

$$\frac{time\ spent\ in\ novel\ arm}{total\ time\ spent\ in\ all\ three\ arms} \times 100$$

4. *Light-Dark Box (LDB)*

A wooden box with dimensions 60cm x 15cm x 20cm, where one-third of the box was covered from light (dark section) and two-thirds were exposed to light was used. The light section had one side covered with plexiglass, allowing to observe mouse behaviour. Mice were first placed in the dark section and allowed to habituate to the environment for 10s, their entry into the light section prevented via a closing of the gate between the two sections. A bright light was placed at the far end of the light section, to further deter the mouse from light zone exploration. Once the gate was opened and the mouse could freely move between sections, testing was performed for 5mins where the time it took for the mouse to first enter the light section, time spent in the light section, and exploratory behaviours in the light section were manually quantified.

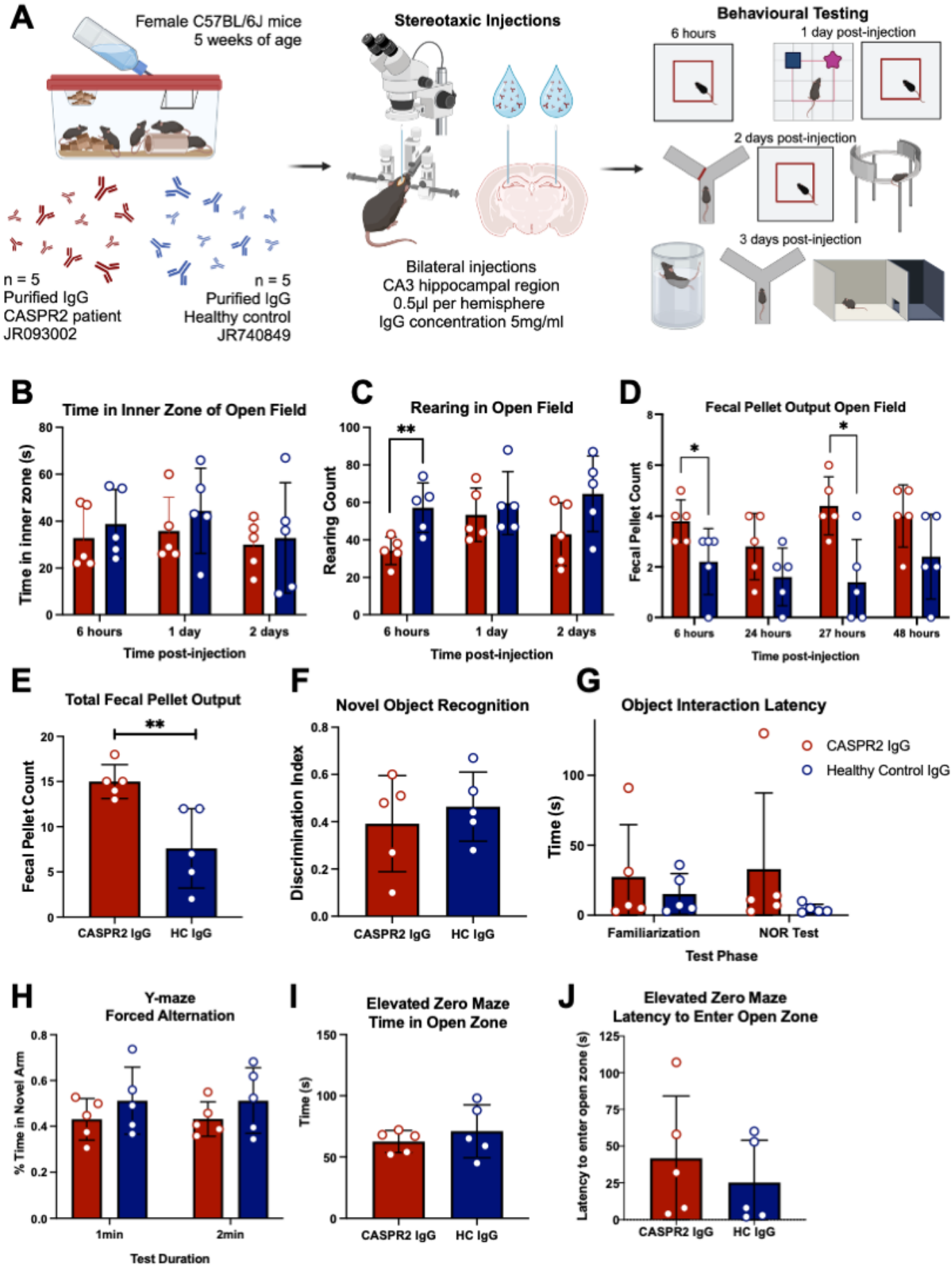
5. *Elevated Zero Maze (EZM)*

A circular plastic maze (diameter 50cm) standing on 50cm-tall support columns was used, with half of the maze flanked by 10cm-tall dark borders (closed area) and the other half completely exposed (open area). Mice were placed facing inwards in one of the two closed zones and left to explore the maze for 5mins. The time it took for a mouse to first enter the open zone (latency) and total time spent in the open zone were recorded manually.

6. *Forced Swim Test (FST)*

A 60cm-tall cylinder, 40cm in diameter, made from plexiglass was used. Prior to testing, two-thirds of the cylinder were filled with water at 37°C (to match the mouse body temperature) and mice were carefully placed and left in the water for 5mins, while monitored by two vigilant observers. The behaviour of the mice was recorded from the side of the cylinder. Time spent immobile (mouse showing no activity for at least 2s) and time taken to first immobility episode were determined manually.

Purified IgG Injections Experiment 1



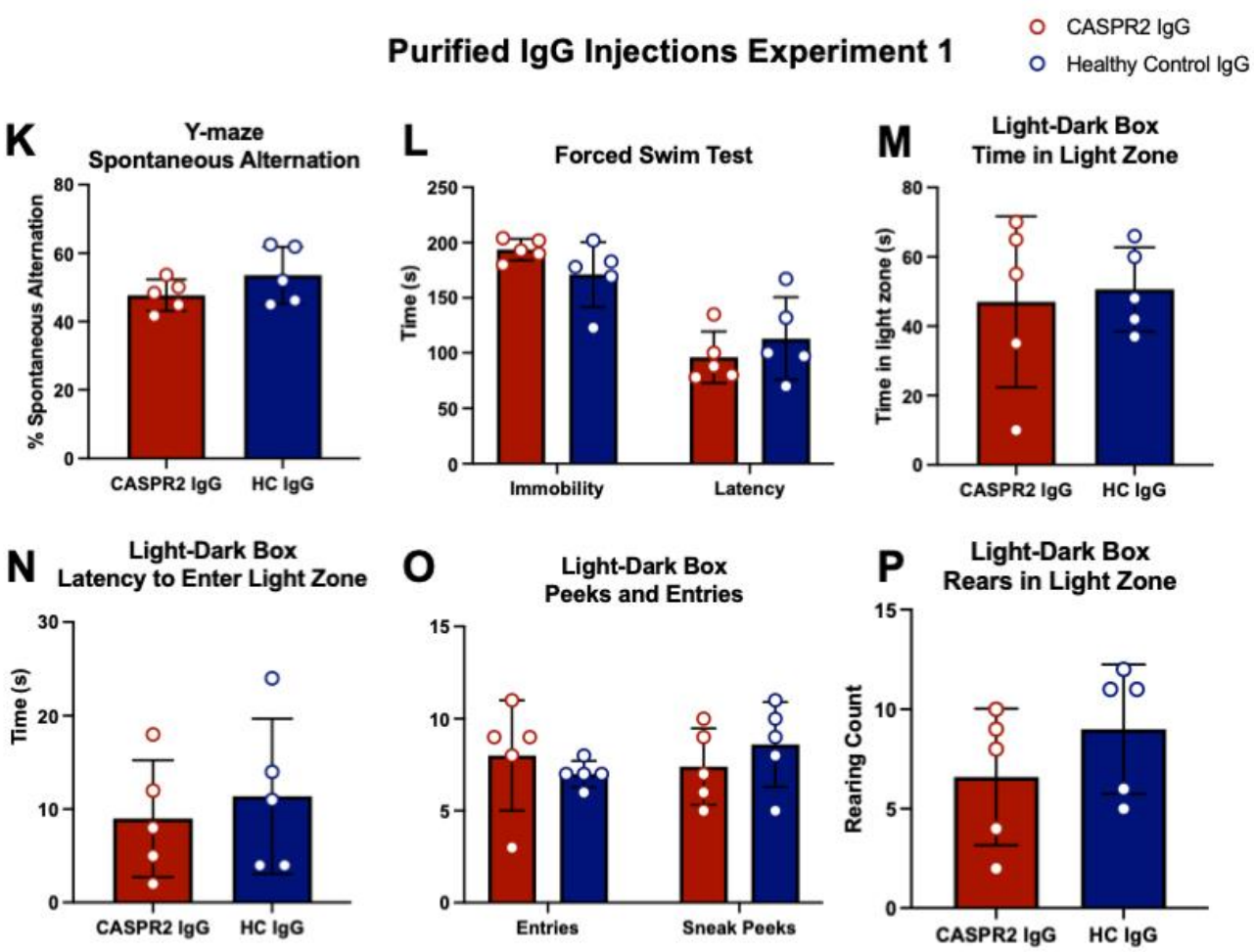


Figure 3.1 (A) Diagram illustrating experimental paradigm for first round of purified immunoglobulin G injections. 0.5ul of IgG purified from patient or healthy control serum was injected bilaterally in the hippocampi of female C57BL/6J mice, followed by a battery of behavioural tests at various time points post-injection. (B) Injections led to no differences in the time spent in the inner zone of the open field at 6 hours, 1 day and 2 days post-injection. (C) CASPR2 IgG-injected mice demonstrated significantly less rearing in the open field at 6 hours post-injection. (D) Caspr2 IgG-injected mice had a significantly higher fecal pellet output (FPO) in the open field at 6 hours and 27 hours post-injection, with a tendency towards a higher FPO at 1 day and 2 days post-injection. (E) When pooled over the whole testing period (6 hours – 48 hours) CASPR2 IgG-injected mice demonstrated a significantly higher total FPO than control mice. (F) There was no difference in the Differentiation index on the Novel Object Recogniton test between CASPR2-IgG-injected mice and control mice. (G) There were no differences in the time taken to start interacting with objects neither at the Familiarization phase nor the Testing phase of the NOR test between the two groups. (H) There were no differences between the two groups in the time spent in the novel arm neither during the first minute nor the first two minutes of testing on the Y-maze Forced Alternation Test. (I and J) There were no differences between the two groups in the (I) time spent in the open zone and (J) the latency to enter the open zone of the elevated zero maze test. (K – P) There were no differences between the CASPR2-IgG-injected mice and the control mice in (K) percentage of Spontaneous Alternation on the Y-maze Spontaneous Alternation Test; (L) Time spent immobile and latency to first immobility episode on the Forced Swim Test; (M) Time spent in the Light Zone; (N) Latency to enter the light zone; (O) Entries and Peeks into the light zone and (P) Rears in the light zone for the Light-Dark Box test. Unpaired student t-tests have been used throughtout, except for graphs B, C, and D, where multiple t-tests were used.

RESULTS

I performed a total of five experiments where purified immunoglobulin G from patient serum was injected into mice, each with slight variations in the injection parameters and behavioural testing paradigms. These are summarised in panel (A) of each of Figures 3.1 – 3.7. Here, I will cover the key findings from each experiment

Experiment 1
For the first experiment, I started with a small group of animals (n = 5), and each group received 0.5µl (the smallest volume normally injected) of IgG at concentration 5mg/ml per hemisphere in the CA3 hippocampal region. Animals received IgG either from patient JR002 or from an age- and sex-matched healthy control (JR740849). Each animal received a total of 5µg of IgG over the course of the experiment. Behavioural testing was performed at 6 hours, 1-day, 2-days, and 3-days post injection, with the following schedule (Figure 3.1(A)):

- 6-hours: Open Field
- 1-day: Open Field, Novel Object Recognition
- 2-days: Open Field, Y-maze Forced Alternation. Elevated Zero Maze
- 3-days: Y-maze Spontaneous Alternation, Forced Swim Test, Light-Dark Box

Despite the small group size and amount of antibody injected, significant results emerged even with this first experiment. Compared to mice that received control-IgG, CASPR2-IgG injected mice reared significantly less in the open field 6-hours post-injection and tended to also do so at 2-days post-injection (3.1(C)), albeit showing no difference in time spent in the inner zone (3.1(B)). In line with lower rearing count suggesting increased anxiety, CASPR2-IgG injected mice also tended to defecate more in the open field across the testing period, with a statistically significant difference from controls at 6-hours and 27-hours post-injection (3.1(D)). When summed up for the entire testing period (20mins of testing), mice injected with CASPR2-IgG had a significantly higher total faecal pellet output (FPO) than controls (3.1(E)). However, this did not reflect on any of the tests for anxiety performed at latter time points, like the EZM (3.1(I, J)) and LDB test (3.1(M-P)). Furthermore, there was no difference between groups in performance on the memory tests (NOR, YmSA, YmFA) or tests for depressive behaviour (FST) (3.1(F-H,K,L)). This allowed me to narrow down the behavioural battery to only assays for anxiety and focus more on the temporal aspects of autoantibody effects. The following IgG experiments will focus on the Open Field, Light-Dark Box and Elevated Zero Maze tests.

Experiment 2
For my second experiment, I attempted to extend the duration of the behavioural effects past the 6-hour time-point. To this end, I did two rounds of bilateral injections on each animal, 24 hours apart, and injected double the volume injected previously (1µl). In CASPR2-IgG injected mice, the first round of injections used IgG from patient JR002, while the second round used IgG from patient JR1284. Control mice received IgG from two different age- and sex-matched healthy controls. Groups were kept small (n = 6), and each mouse received a total of 20µg of IgG over the course of 24h, 4 times the amount received in Experiment 1. Behavioural testing was done at 6-hours after the first injection (Open Field), and then again 6-hours after the second injection (OF, YmSA, EZM), and 12 days later (OF, EZM, LDB), to look at the long-term effects of antibody injection. The YmSA test was used here in addition to all the anxiety assays to assess whether the increase in anxiety could also be affecting cognitive performance. (Figure 3.2(A)) While I expected this experimental strategy to yield more drastic behavioural deficits, this was surprisingly not the case. The differences between groups were in fact milder,

manifesting only in terms of time spent in the inner zone of the open field, where CASPR2-IgG injected mice demonstrated a more anxious phenotype 6-hours and 1-day post-injection (3.2(B)). None of the other assays revealed any significant differences (3.2(C-K)), although some non-significant trends in the data could be observed for certain aspects of the EZM and LDB tests (as seen in Figures 3.2(G) and (K)).

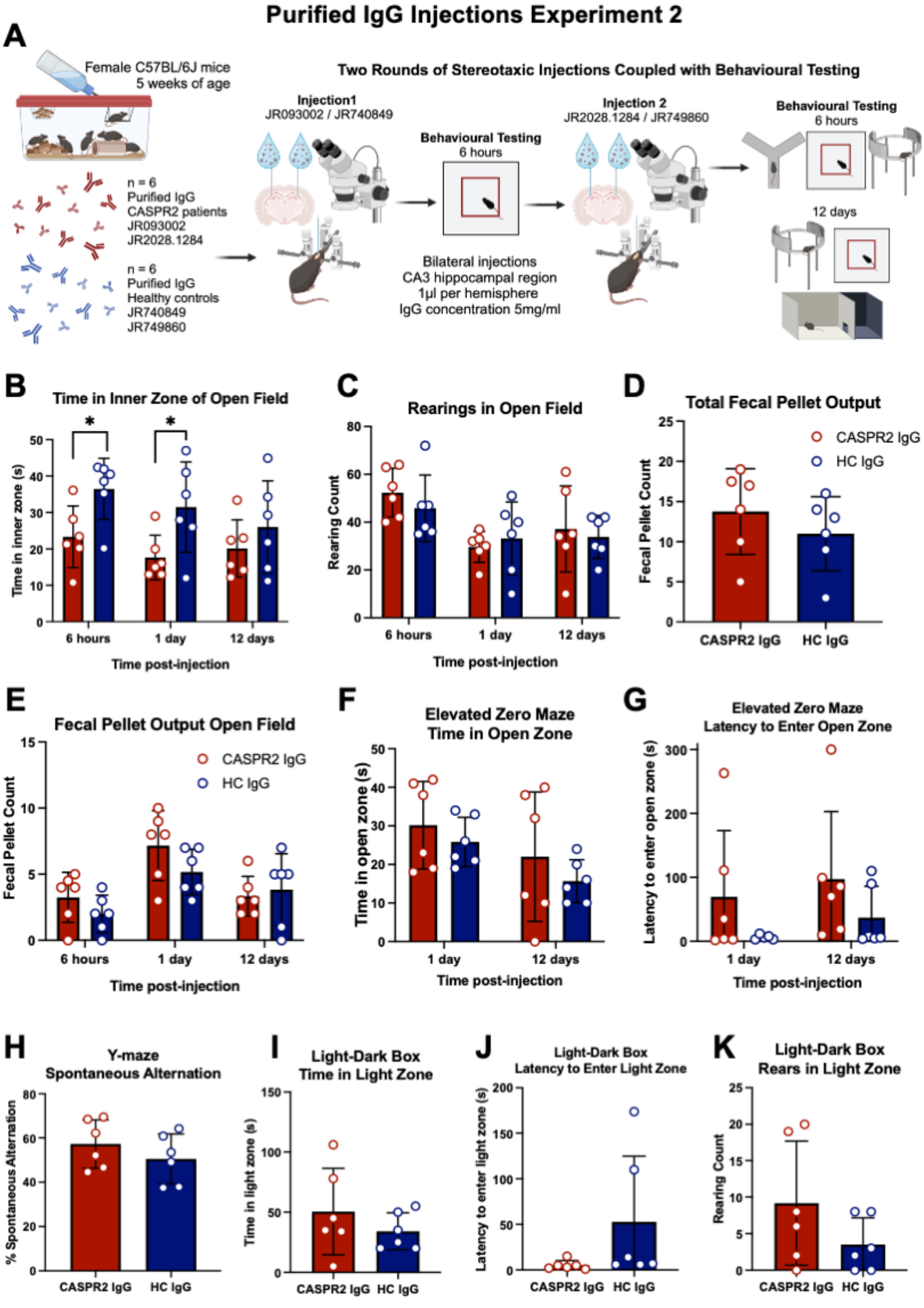
Experiment 3

In my third experiment, I used identical injection parameters as for Experiment 2, albeit with a smaller group size (n = 4), and decided to slightly alter the behavioural testing to focus in on the seemingly important 6-hours post-injection. Thus, Open Field testing was performed at 3-hours and 6-hours after the first round of injections, and again 6-hours after the second round of injections, along with an EZM and YmSA test (Figure 3.3(A)). Once again, the 6-hour time point emerged as significant, as CASPR2-IgG injected mice spent significantly less time in the inner zone of the open field, had a significantly higher faecal pellet output, and tended to rear less than controls at this time point (3.3(B-E)). Additionally, CASPR2-IgG injected mice defecated more than controls 3-hours post-injection (3.3(D)) but showed no other behavioural differences on the YmSA and EZM (3.3(F-H)).

Summary of Experiments 1, 2, and 3

Due to the identical injection strategies used, it is possible to pool results from the same assays performed at the same time points in Experiments 2 and 3, thus allowing a larger sample size (n = 10) and creating a more extensive timeline allowing us a more detailed look at the effects of CASPR2-autoantibodies over time (Figure 3.4(A)). For the Open Field, pooling results revealed significant differences at the 3-hours, 6-hours, and 1-day post-injection time points, with CASPR2-IgG injected mice tending to spend less time in the inner zone and to defecate more, but showing no differences in rearing behaviour (3.4(B-D)). There were no differences in performance on the YmSA and LDB tests (3.4(E), LDB results not shown). On the EZM tests, while there were no statistically significant differences, CASPR2-IgG injected mice tended to take more time to first enter the open zone both at 1 day and 12 days post-injection (3.4(G)). For the 6-hour time point, I also attempted to pool results across the first three experiments (3.5(A)), making note of which mice were given substantially less IgG (grey circles, Figure 3.5(B-D)). Astonishingly, the data points clustered together quite nicely, with the mice that received 4-times less IgG (Experiment 1) not differing too much in performance from those in Experiments 2 and 3. Overall, CASPR2-IgG seems to significantly shift performance on the Open Field test at 6-hours post-injection toward anxiety-related behaviour, with mice that received patient IgG spending significantly less time in the inner zone (3.5(B)) and tending to rear less (3.5(C)). Across the entire testing period, there was a persistent effect of CASPR2-IgG on faecal pellet output, with mice that received injections from patients defecating significantly more than controls (3.5(D))

Figure 3.2 ***(next page)*** (A) Experimental paradigm for second round of purified IgG injections. This time, 1ul of IgG purified from patient or healthy control serum was injected bilaterally in the hippocampi of female C57BL/6J mice, followed by another 1ul injection at the same concentration, but from a different patient or healthy control sample 24 hours later. Behavioural testing was performed 6 hours after the first injection, and then again at 6 hours and at 12 days after the second injection. (B) CASPR2-IgG-injected mice spent significantly less time in the inner zone of the open field at 6 hours and at 1 day after the first injection. (C-E) No differences between groups were seen for (C) rearings in the open field and (E) Fecal pellet output in the open field at the three testing time-points nor for (D) total fecal pellet output over the entire testing period. (F,G) There were no differences between groups in performance on the elevated zero maze in terms of (F) time spent in the open zone or (G) latency to enter the open zone at either the 1 day or the 12 days time points. (H) There was no difference between groups in percentage of spontaneous alternation on the Y-maze sponaneous alternation test. (I-K) There were no differences between groups in performance on the Light-Dark Box Test, neither in terms of (I) time spent in the light zone, (J) latency to enter the light zone, nor (K) rears in the light zone.


Purified IgG Injections Experiment 2
A
Female C57BL/6J mice
5 weeks of age
n = 6
Purified IgG
CASPR2 patients
JR093002
JR2028.1284
n = 6
Purified IgG
Healthy controls
JR740849
JR749860
Two Rounds of Stereotaxic Injections Coupled with Behavioural Testing
Injection1
JR093002 / JR740849
Behavioural Testing
6 hours
Bilateral injections
CA3 hippocampal region
1µl per hemisphere
IgG concentration 5mg/ml
Injection 2
JR2028.1284 / JR749860
Behavioural Testing
6 hours
12 days
B
Time in Inner Zone of Open Field
Time in inner zone (s)
6 hours
1 day
12 days
Time post-injection
C
Rearings in Open Field
Rearing Count
6 hours
1 day
12 days
Time post-injection
D
Total Fecal Pellet Output
Fecal Pellet Count
CASPR2 IgG
HC IgG
E
Fecal Pellet Output Open Field
Fecal Pellet Count
CASPR2 IgG
HC IgG
6 hours
1 day
12 days
Time post-injection
F
Elevated Zero Maze
Time in Open Zone
Time in open zone (s)
1 day
12 days
Time post-injection
G
Elevated Zero Maze
Latency to Enter Open Zone
Latency to enter open zone (s)
1 day
12 days
Time post-injection
H
Y-maze
Spontaneous Alternation
% Spontaneous Alternation
CASPR2 IgG
HC IgG
I
Light-Dark Box
Time in Light Zone
Time in light zone (s)
CASPR2 IgG
HC IgG
J
Light-Dark Box
Latency to Enter Light Zone
Latency to enter light zone (s)
CASPR2 IgG
HC IgG
K
Light-Dark Box
Rears in Light Zone
Rearing Count
CASPR2 IgG
HC IgG

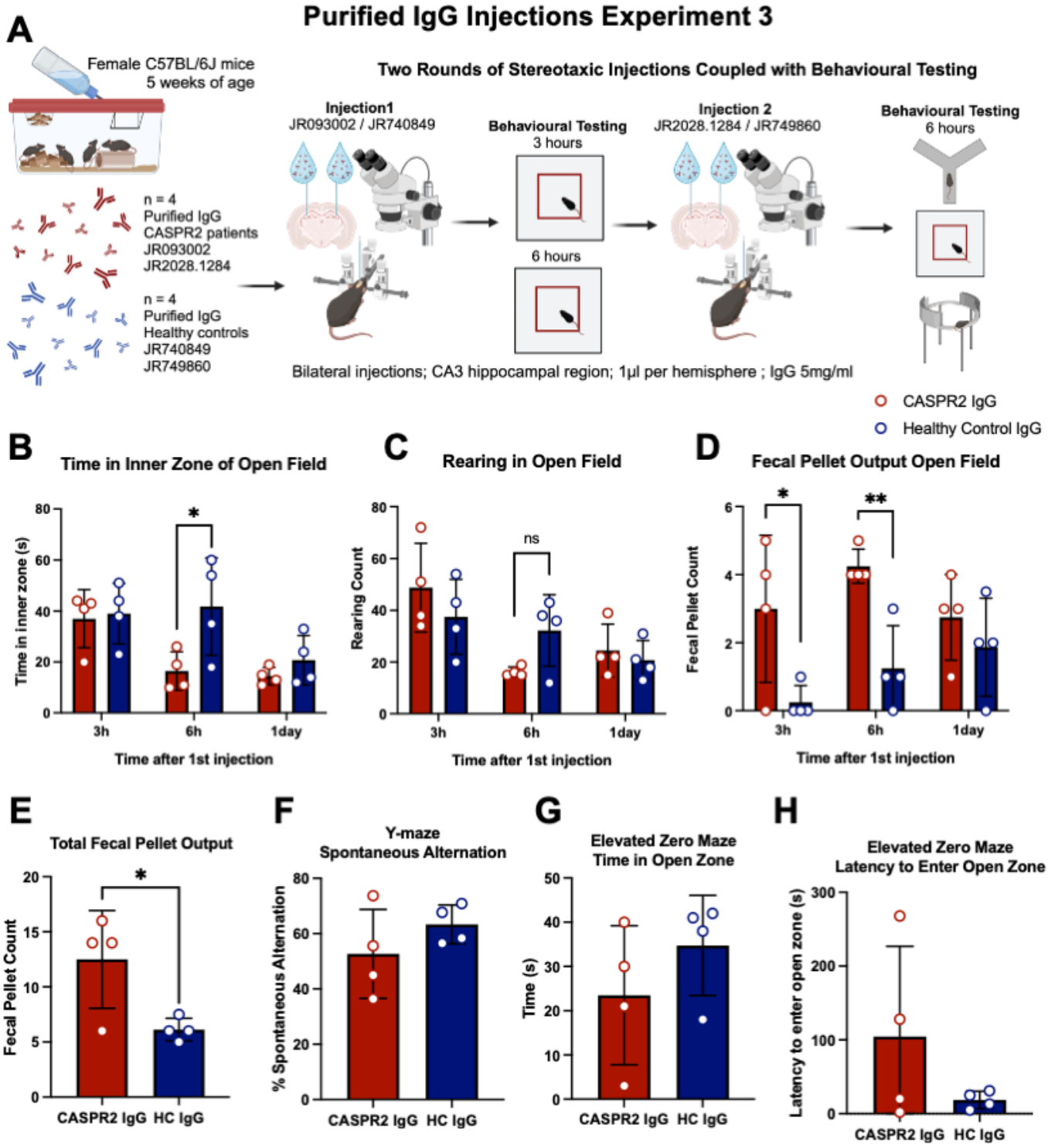


Figure 3.3 (A) Experimental paradigm for third round of purified IgG injections, with injections setup identical as seen for Figure 3.2(A). Here however, behavioural testing was performed at 3 hours and 6 hours after the first injection, and then again at 6 hours after the second injection. (B) CASPR2-IgG-injected mice spent significantly less time in the inner zone of the open field than control mice 6 hours after the first injection and (C) tended to rear less at the same time point, although here the difference was not significant. (D) CASPR2-IgG-injected mice had a higher fecal pellet output in the open field than control mice 3 hours and again 6 hours after the first injection, as well as (E) a higher total faecal pellet output for the entire testing period. (F-H) There was no difference between the two groups in performance on the (F) Y-maze spontaneous alternation test and the (G,H) Elevated Zero Maze test 6 hours after the second injection.

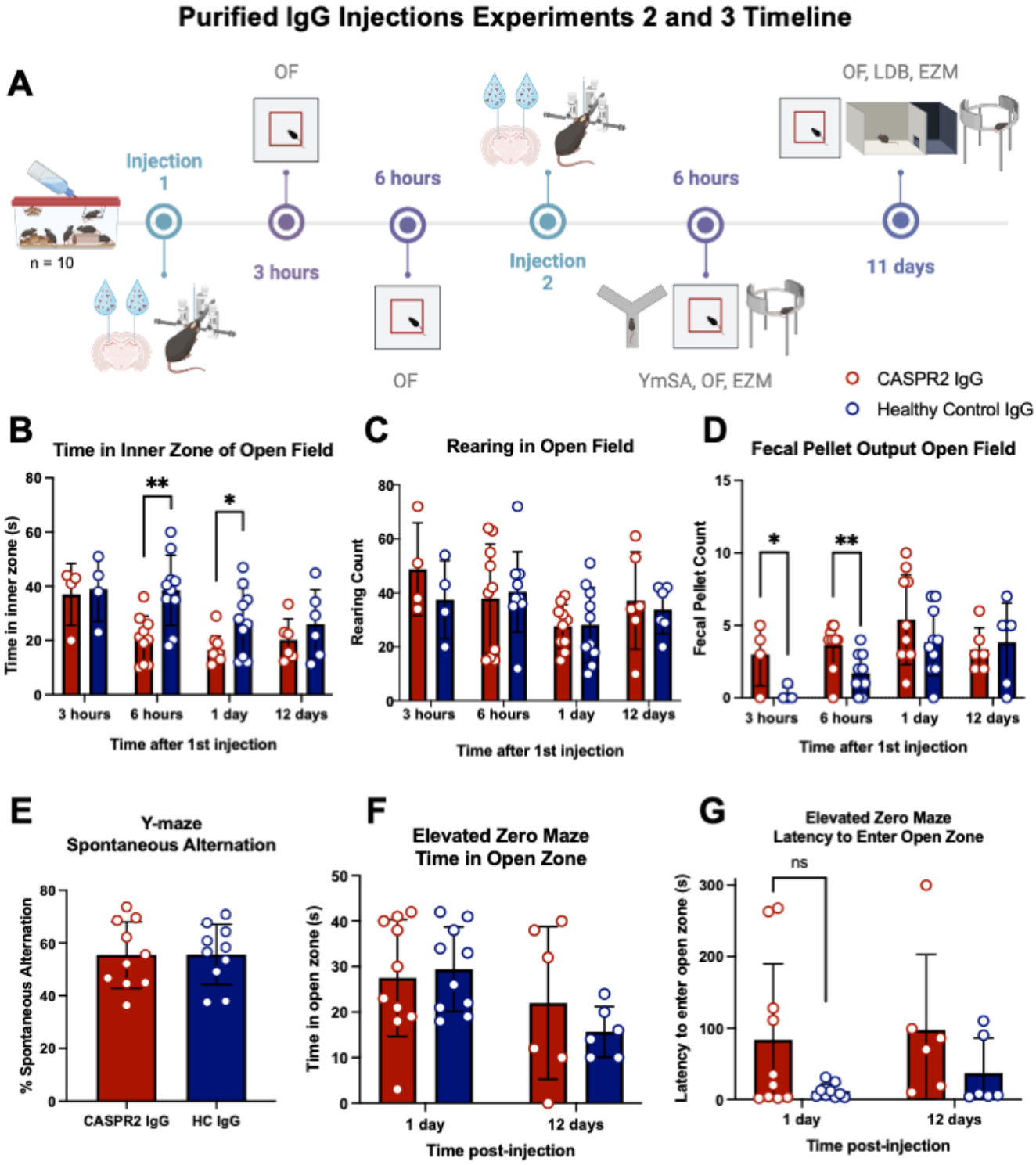


Figure 3.4 (A) Summary of the joint experimental timeline shared by the second and third round of Purified IgG injections. This has allowed for the pooling of results for the two experiments at the 6 hours (post-first injection) and 1 day (post-first injection) time-points, resulting in n=10 per group for these two time points. (B) CASPR2-IgG-injected mice spent less time in the inner zone of the open field than control mice at 6 hours and 1 day after the first injection. (C) there were no significant differences in rearing in the open field between the two groups at any of the testing time points. (D) CASPR2-IgG injected mice had significantly higher fecal pellet outputs in the open field than control mice at 3 hours and 6 hours after the first injection. (E) There were no differences between groups in performance on theY-maze Spontaneous Alternation test at 1 day after the first injection (this was 6 hours after the second injection). (F) There were no differences between groups in the time spent in the open zone of the elevated zero maze at 1 day or 12 days after the first injection, although CASPR2-IgG-injected mice (G) tended to take more time to enter the open zone of the EZM at one day post-1st-injection.

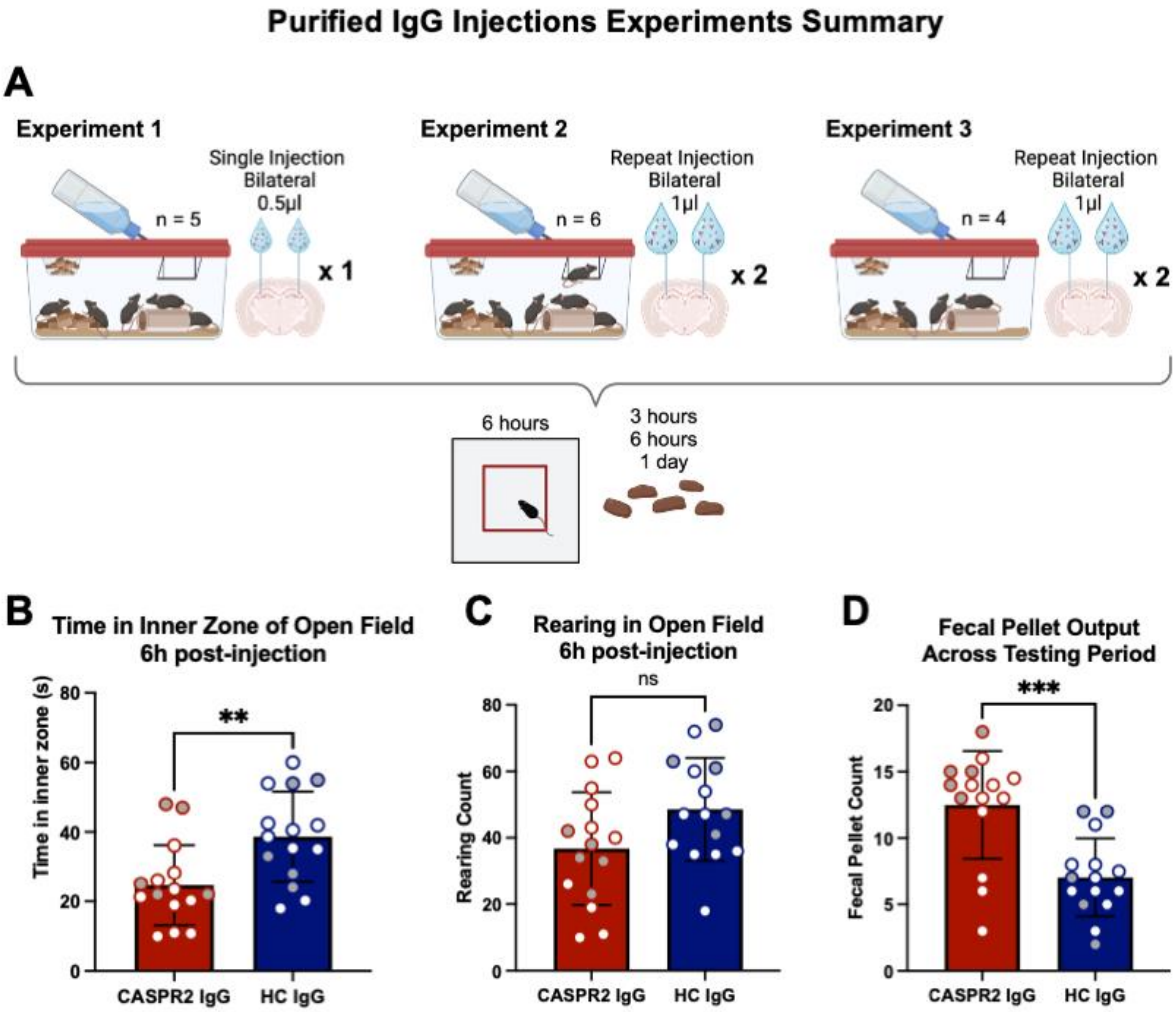


Figure 3.5 (A) Summary of the first three Purified IgG injections experiments. (B,C) Pooling results for Open Field Testing at 6 hours after the first injection showed that (B) CASPR2-IgG-injected mice spent significantly less time than control mice in the inner zone of the open field and (C) also tended to rear less. (D) Summing up the Fecal Pellet Count across the entire testing period revealed that CASPR2-IgG-injected mice had a significantly higher Fecal Pellet Output in the open field than control mice. Grey circles in B, C, and D represent mice from Experiment 1, which received a single injection of half the amount of IgG compared to mice in Experiments 2 and 3.

Experiment 4

With the results from the first three experiments in mind, I next wondered whether the effects of CASPR2-IgG were specific to Open Field performance, or to the 6-hours post-injection time point, as this was the only assay that was always performed at this time point. As performing two rounds of injections did not seem to have an incremental effect on behaviour, I returned to only a single bilateral injection, but retained the 1µl injection volume, as this did not seem to have an adverse effect on the mice. I used a larger group size (n = 10), and this time performed the Light-Dark Box test, which also screens for anxiety, at 6 hours post-injection (Figure 3.6 (A)). CASPR2-IgG injection once again shifted behaviour toward an anxious phenotype, with CASPR2-IgG injected mice spending significantly less time in the light zone, tending to take more time to first enter the light zone, and rearing significantly less than controls (3.6 (B-D))

Experiment 5

For my final experiment featuring purified IgG injections, I aimed to combine the significant findings from the previous four experiments, and test these on a different patient sample that had a similar clinical presentation to patient JR002 but had been treated with immunotherapy at the time of sample collection. Once again, mice received a single bilateral injection of 1µl of IgG at concentration 5mg/ml, and a larger group size (n = 10) was used. Open Field testing was performed at 6-hours, and Light-Dark Box testing at 9-hours post-injection (Figure 3.7 (A)). As seen for patient JR002, IgG from patient JR1284 led to a higher faecal pellet output and less time in the inner zone of the open field, while producing no differences in rearing (3.7(B-D)). Interestingly, a new behavioural observation not witnessed in earlier experiments was that CASPR2-IgG injected mice jumped more in the open field than control mice (3.7(E)). I wondered whether this extended into a more general hyperactivity phenotype, with CASPR2-IgG injected mice moving more in the open field than controls, but analysis using the ANY-Maze software showed no differences in locomotion parameters (distance travelled, average speed, and time immobile) between groups (3.7(F-H)). The behavioural effects of CASPR2-IgG disappeared at 9-hours post-injection, with there being no differences between groups in performance on the Light-Dark Box Test (3.7(I-K))

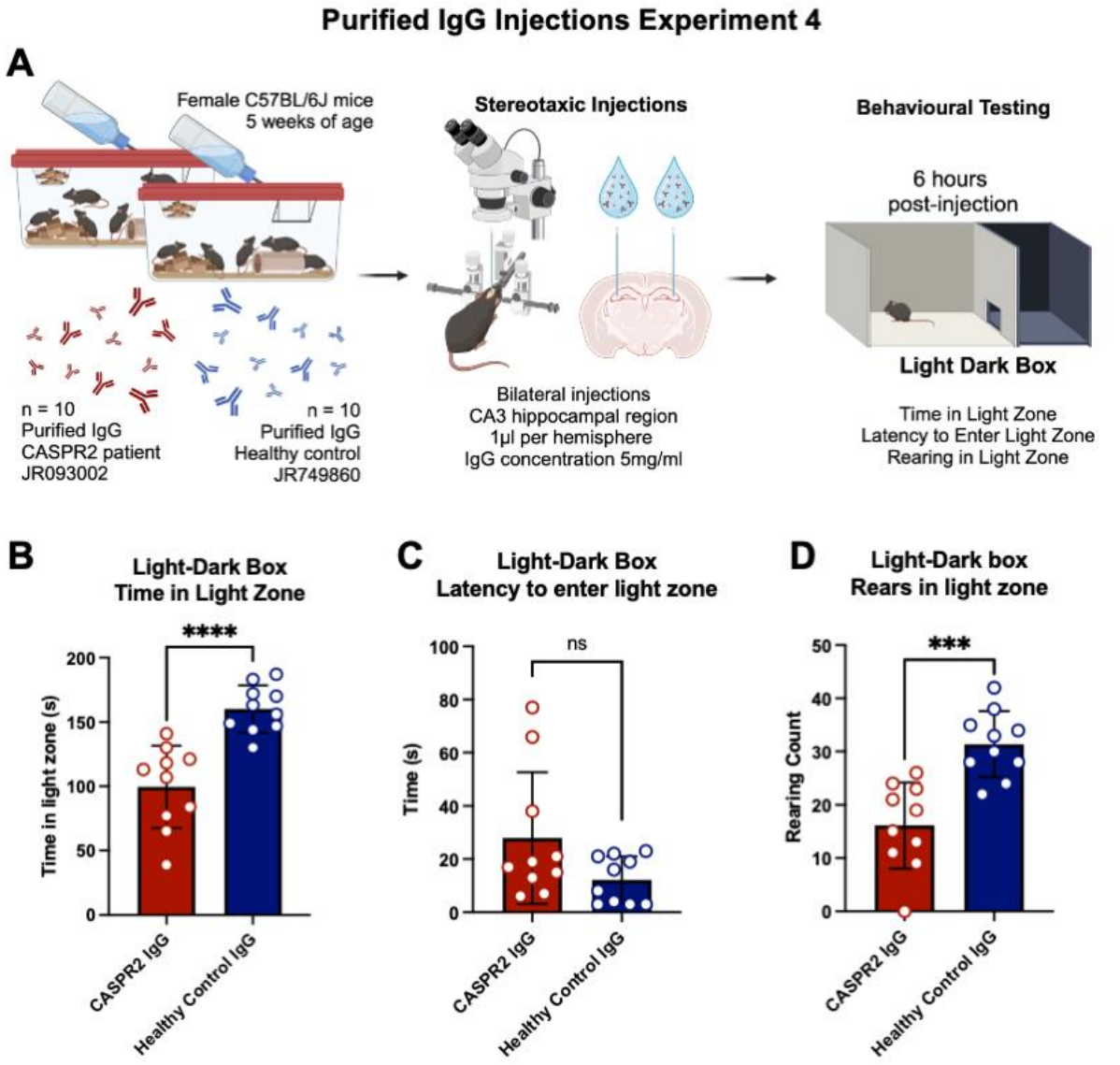
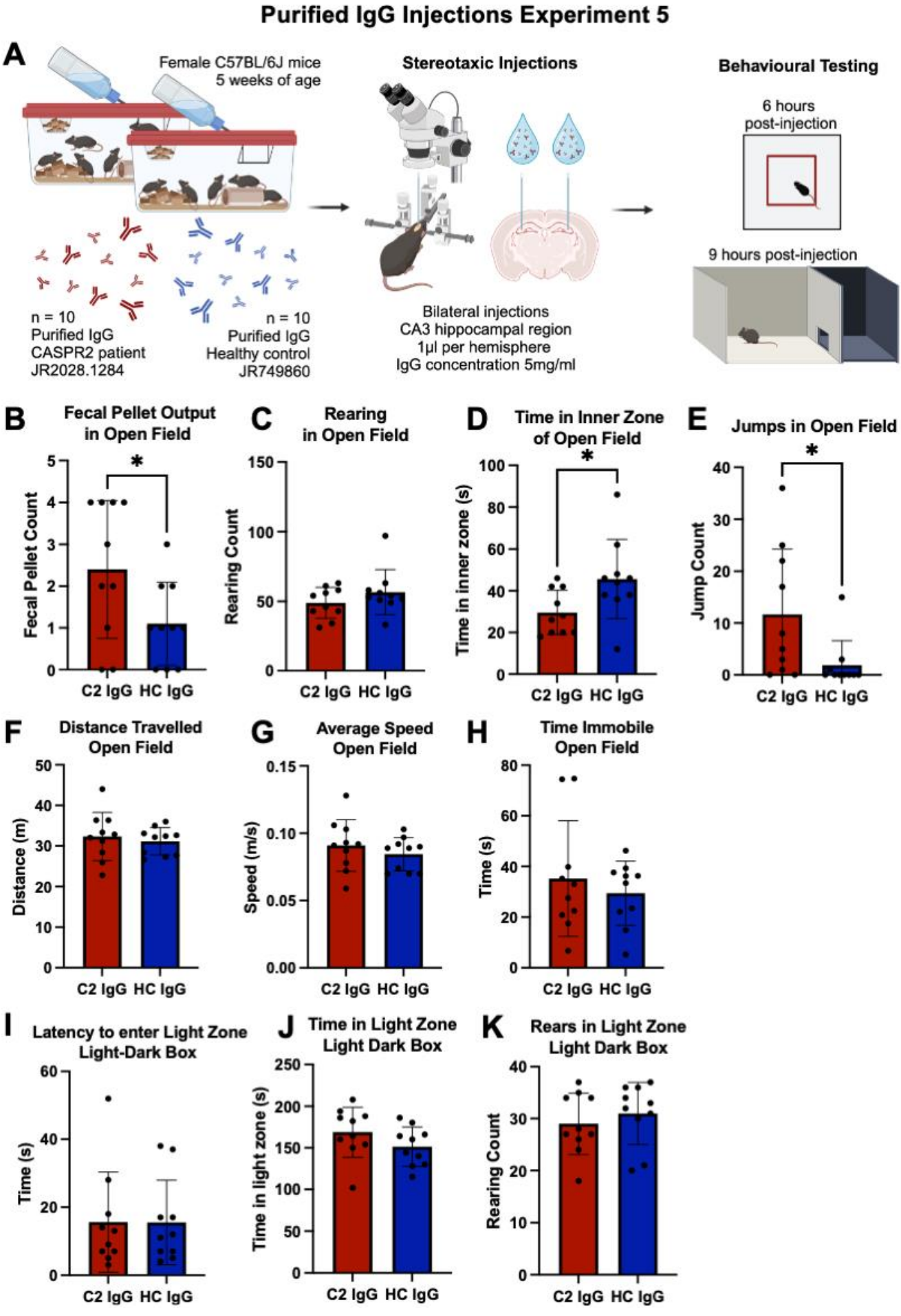


Figure 3.6 (A) Experiment design for the fourth round of Purified IgG injections. Mice received a single round of bilateral IgG injections of 1ul (concentration 5mg/ml), followed by light-dark box testing 6 hours post-injection. On the Light-Dark Box test, CASPR2-IgG-injected mice (B) spent significantly less time in the light zone, (C) tended to take more time to enter the light zone and (D) reared significantly less in the light zone than control mice.

Figure 3.7 ***(next page)*** (A) Experimental design for the fifth and final round of Purified IgG injections. Open field testing was performed at 6 hours and Light-Dark Box testing at 9 hours post-injection. (B-E) Performance on the Open Field Test. CASPR2-IgG-injected mice had a significantly higher fecal pellet output (B), spent significantly less time in the inner zone (D) and jumped significantly more than control mice (E), but there was no difference in rearing between the two groups (C). (F-H) There were no differences in mobility in the open field between the two groups, neither in terms of distance travelled (F), average speed (G), nor time spent immobile (H). (I – K) There were no differences in performance on the Light-Dark Box test 9 hours post-injection between the two groups, neither in terms of latency to enter the light zone (I), time spent in the light zone (J) nor rearing in the light zone (K).


Purified IgG Injections Experiment 5
A
Female C57BL/6J mice
5 weeks of age
n = 10
Purified IgG
CASPR2 patient
JR2028.1284
n = 10
Purified IgG
Healthy control
JR749860
Stereotaxic Injections
Bilateral injections
CA3 hippocampal region
1µl per hemisphere
IgG concentration 5mg/ml
Behavioural Testing
6 hours
post-injection
9 hours post-injection
B
Fecal Pellet Output
in Open Field
Fecal Pellet Count
C2 IgG HC IgG
C
Rearing
in Open Field
Rearing Count
C2 IgG HC IgG
D
Time in Inner Zone
of Open Field
Time in inner zone (s)
C2 IgG HC IgG
E
Jumps in Open Field
Jump Count
C2 IgG HC IgG
F
Distance Travelled
Open Field
Distance (m)
C2 IgG HC IgG
G
Average Speed
Open Field
Speed (m/s)
C2 IgG HC IgG
H
Time Immobile
Open Field
Time (s)
C2 IgG HC IgG
I
Latency to enter Light Zone
Light-Dark Box
Time (s)
C2 IgG HC IgG
J
Time in Light Zone
Light Dark Box
Time in light zone (s)
C2 IgG HC IgG
K
Rears in Light Zone
Light Dark Box
Rearing Count
C2 IgG HC IgG

DISCUSSION

In this chapter, I have investigated the effects of Caspr2-autoantibodies delivered directly into the CNS parenchyma and characterised their time-sensitive nature. Only three prior studies have been published that address pathogenicity of Caspr2-Abs in the CNS, and all used indirect methods to access the parenchyma: *in utero* exposure, intracerebroventricular injection, and peripheral delivery combined with BBB breach.[317, 319, 417] Furthermore, all prior studies have used supraphysiological levels of IgG and delivered the IgG chronically, taking at least 5 days of injections before any effect was observed. The effects observed were either mild, limited to social interaction or, where memory was affected, only manifested once the course of IgG had already been interrupted and disappeared soon thereafter. Finally, the study that produced a significant memory deficit with CASPR2-IgG did so after combining multiple patient samples (a very artificial experimental condition) and demonstrated this by using a test that was not entirely appropriate for the task at hand and was not performed up to the highest behavioural academic standard.

While itself flawed in several aspects which will be discussed later, my work has attempted to circumvent some of these shortcomings by using a much more direct, less intrusive, and simple method of delivery into the CNS parenchyma via stereotactic injection of a small volume (0.5 – 1µl) directly into the CA3 region of the hippocampus. This area was chosen as the focal point of the injection due to its clinical relevance: it exhibits significant T2-hyperintensity on patient MRIs,[421] patients frequently exhibit short-term memory loss and difficulty forming new memories, both deficits indicative of hippocampal impairment,[245] and finally, previous work on Caspr2's 'molecular cousin' LGI1 has shown that LGI1-antibodies injected into this area in mice produced impaired performance on the novel object recognition test.[396, 422] Indeed, the study that produced significant memory deficit with CASPR2-IgG also demonstrated extensive IgG deposition in the hippocampus.[319] Perhaps most surprising then is the finding that my injection method did not produce any memory deficits at all, as observed across multiple tests (NOR, YmSA, and YmFA) and several time-points post-injection. While it was possible to further 'push' the system by transitioning toward a more 'chronic' method of delivery like the osmotic pump, we decided to prioritise animal welfare and thus continue using the direct and 'acute' intracranial injection. Instead of changing the delivery method, I shifted attention toward the interesting and unexpected observation that CASPR2-IgG seemed to induce increased anxiety-related behaviour.

The changes in performance on the open field and light dark box tests, and to some extent the elevated zero maze following CASPR2-IgG administration were surprising as we did not expect to see an effect on anxiety after affecting an area that has primarily been associated with episodic and spatial memory.[423] However, beyond its implication in cognitive functions, the hippocampus plays important roles in the regulation of emotional behaviours, particularly anxiety states, via its interactions with both cortical and subcortical areas that regulate emotion and stress responses, including the prefrontal cortex, amygdala, and hypothalamus. The two distinct roles of the hippocampus have been attributed to differential regulation of cognition and anxiety along the dorso-ventral axis, with ventral hippocampus being heavily implicated in processing anxiety and adjusting its levels in response to stressful conditions.[424-426] The ventral hippocampal area is indeed the focal point of our injection, and the evidence in favour of anxiety-prone behaviour following CASPR2-IgG injection has been demonstrated here across multiple modalities and parameters, including time in the inner zone, rearing and faecal pellet output in the open field, latency to enter, time spent and rearing in the light zone of the light dark box and finally, latency to enter the open zone of the elevated zero maze.

An interesting observation is the significance of the 6-hour time point. Testing earlier than this, at 3 hours post-injection also produced significant results, but we opted to not rely on this one or earlier time points to allow the mice adequate time for recovery post-surgery. The half-life of IgG injected into rodent CSF has been variously estimated to range from several minutes to several hours, with one study estimating it to be around 47mins.[427-429] This is largely due to the highly efficient clearance from the CSF into the systemic circulation via the neonatal Fc receptor.[429, 430] However, as our antibodies are injected directly into the parenchyma rather than the ventricles, making it more likely for them to encounter their target very early on, it is possible that the half-life in our experiments is even shorter. Considering how quickly xenogeneic antibodies are removed from the CNS, it is unsurprising that a second round of injections or doubling of the injected volume did not produce more drastic results, as the first round of IgG was probably cleared by the time the second round was delivered. This could also potentially explain why in the previously published studies massive amounts of IgG had to be delivered to the animals for any meaningful results to be obtained.

Another curious finding in this chapter was the effect of CASPR2-IgG on faecal pellet output in the open field, which was sustained throughout the testing period even past the 6-hour time point. To this date, an *in vivo* pathophysiological effect of this kind has not been described for CASPR2-autoantibodies, nor for any of the other autoantibody targets. We have ascribed this feature to the increased levels of anxiety among the mice injected with CASPR2-IgG, as supported by general performance in the open field, light-dark box, and elevated zero maze. Another possible explanation is that this is mediated by autonomic nervous system dysfunction once the antibodies have left the CNS and entered the systemic circulation. Autonomic dysfunction is a core feature of CASPR2 autoantibody-associated neurological disease, with occasional gastrointestinal manifestations like diarrhoea or constipation[431] and recently published work has even described unique features of the patients' gut microbiota.[432] However, it is worth emphasising that in patients these effects occur after high levels of CASPR2-autoantibodies have been present in the systemic circulation over a prolonged period. In our experiments, we inject a small volume of antibodies into the CNS, a negligible proportion of which might make their way into the periphery. Combined with the acute nature of the effect (visible as early as 3-hours post-injection) it is highly unlikely that the increased defecation is due to autonomic dysfunction and a more succinct explanation is probably the increase in anxiety, known to be tied with higher faecal pellet outputs in rodents.[433]

A shortcoming of this chapter is that the mechanisms via which CASPR2-IgG exerts its effects on mouse anxiety have not been delved into. I have chosen to address this question with the use of monoclonal, rather than polyclonal antibodies due to the former providing the advantage of relating pathogenic effects to a single antibody clone. This will be the topic of Chapter 4, with the use of monoclonal antibodies derived from patient JR002, whose IgG produced the most prominent behavioural effects in the experiments described here. However, this leads to another interesting question of why one sample but not the other induced more profound behavioural deficits, especially considering that the JR002 sample had a lower CASPR2-IgG titre. CASPR2-antibody positive sera are known to bind multiple domains of the protein, so domain-specificity is unlikely to account for this discrepancy.[245] One explanation could be that the sample from patient JR002 was obtained prior to commencing treatment, whereas the sample from JR1284 was collected once treatment was well underway and the neurological symptoms were in remission. While patient JR1284 still had high levels of CASPR2-autoantibodies at this stage, it is possible that immunotherapy had eliminated the antibodies or B-cells most likely to be pathogenic. Of course, this doesn't provide rationale for why mice given IgG from patient JR1284 demonstrated a jumping behaviour not observed in the

experiments using JR002-IgG, especially when the patient did not have any psychotic features at the time of sample collection. Perhaps this reflects that different litters of mice could respond differently to the same insult (Experiment 1 and 5 were performed over a year apart), or perhaps this gives another argument against pooling samples from different patients in animal experiments, as each sample might have unique *in vivo* behavioural effects that could be otherwise masked by pooling.

Finally, one key conundrum that is to be addressed is the demonstration of a phenotype which is not necessarily representative of patients with CASPR2-autoantibodies. While patients are known to experience cognitive deficits, psychiatric disturbances, and personality changes, anxiety is hardly a cardinal symptom of CASPR2-autoantibody associated limbic encephalitis. It is in fact memory deficits, notably *not* observed in the assays used here that are one of the defining features. In patients however, the memory disturbances tend to emerge after what is probably a longer course of 'chronic' autoantibody presence in the CNS. Indeed, a recent study focusing on initial presentation of CASPR2-antibody encephalitis found memory disturbances to be quite infrequent at initial presentation, with mood disorders being far more prevalent.[434] In the disease progression course, acute confusion, behavioural changes, and psychosis tend to precede memory deficits.[406] Experimentally, memory deficits with CASPR2-autoantibodies were only produced following long-term high-level exposure. It is therefore plausible that within our 'acute' model of CASPR2-autoantibody pathology, we are simply observing the quick and immediate effects of the antibodies that produce mood changes, and the perturbations in memory only come later, after long-term exposure of the hippocampus to the offence.

In this Chapter, I have demonstrated acute behavioural changes following administration of a small amount of CASPR2-specific immunoglobulin G using a direct intracranial injection. While the reproducibility of these results can be called into question as the exact same paradigm was never tested more than once, there is an argument to be made for the robustness of the system, for the behavioural observations were consistent with an anxious phenotype despite changes in the experimental strategy. This was confirmed via performance parameters across the open field, light-dark box, and elevated zero maze tests. Despite their prominence in patients, memory-related and locomotory deficits were not observed in my animal model. Instead, the injections produced perturbances more prevalent during the initial stages of disease consistent with an 'acute' autoantibody effect. If we consider that an animal received between 5 – 20μg of IgG, a very small proportion of which is likely to be CASPR2-reactive, the size of the effect becomes even more astounding. Perhaps most valuable with the aims of this thesis in mind, Chapter 3 has provided a passive transfer animal model of Caspr2-autoantibody pathogenicity that emulates the early stages of human disease and therefore meets one of the three Rose-Witebsky-Bona criteria laid out in the thesis introduction. In the next chapter, I will turn my attention towards the molecular mechanisms underlying this pathogenic effect, which constitute another aspect of the Rose-Witebsky-Bona criteria. The consequences of injecting anti-Caspr2 monoclonal antibodies will be investigated with the use of mAbs derived from patient JR002, whose serum IgG produced behavioural changes.

CHAPTER 4

IN VITRO AND *IN VIVO* CHARACTERISATION OF PATIENT-DERIVED CASPR2 MONOCLONAL ANTIBODIES

Preface

The previous chapter featured injections of polyclonal immunoglobulin G purified from patient sera positive for Caspr2-autoantibodies into mouse hippocampi. While extensive behavioural experiments were performed on the injected mice, the molecular mechanisms via which Caspr2-autoantibodies exert their effects were not explored. This question is instead addressed here with the use of monoclonal antibodies (mAbs) derived from peripheral B-cells of patients with Caspr2-antibody encephalitis. Attempts by myself and others to characterise the *in vitro* and *in vivo* pathogenicity of Caspr2-mAbs will be presented. These findings will be related to features like antibody subclass, affinity, and domain specificity. Five mAbs used in Chapter 4 were derived from a patient whose serum sample was used as a source of polyclonal IgG in Chapter 3, creating the opportunity to draw direct comparisons between the pathogenic potentials of a polyclonal versus a monoclonal antibody insult to the nervous system.

INTRODUCTION

A growing trend within the autoimmune neurology field has been to use monoclonal antibodies (mAbs) rather than polyclonal sera to obtain higher-resolution data on what specific antibody attributes contribute to pathogenicity. Given the heterogeneity of patient samples, the contributions of a single antibody to pathogenicity cannot be definitively assigned. In this respect, single-cell technologies have led to significant advancements in the study of antibody-mediated neurological disorders, providing deeper insight into the B-cells responsible for autoantibody production, and revealing the characteristics, including epitope specificity and binding affinity, that regulate the pathogenic potential of an antibody.[435-437]

There are no publications on patient-derived mAbs in the Caspr2 field, in contrast to work done on other autoantibody targets in the CNS.[396, 399, 400, 438-440] This discrepancy could be partly ascribed to the rarity of Caspr2-autoantibody positive patients, or it could be that the previous publications on polyclonal Caspr2-reactive sera provided little promise that going through the effort of generating patient-derived mAbs could yield any fruitful results. Regardless of the reason for the non-existent prior work, in this chapter I will present an effort to generate patient-derived Caspr2-mAbs, and their subsequent use as tools to study the pathophysiology of Caspr2-antibody encephalitis.

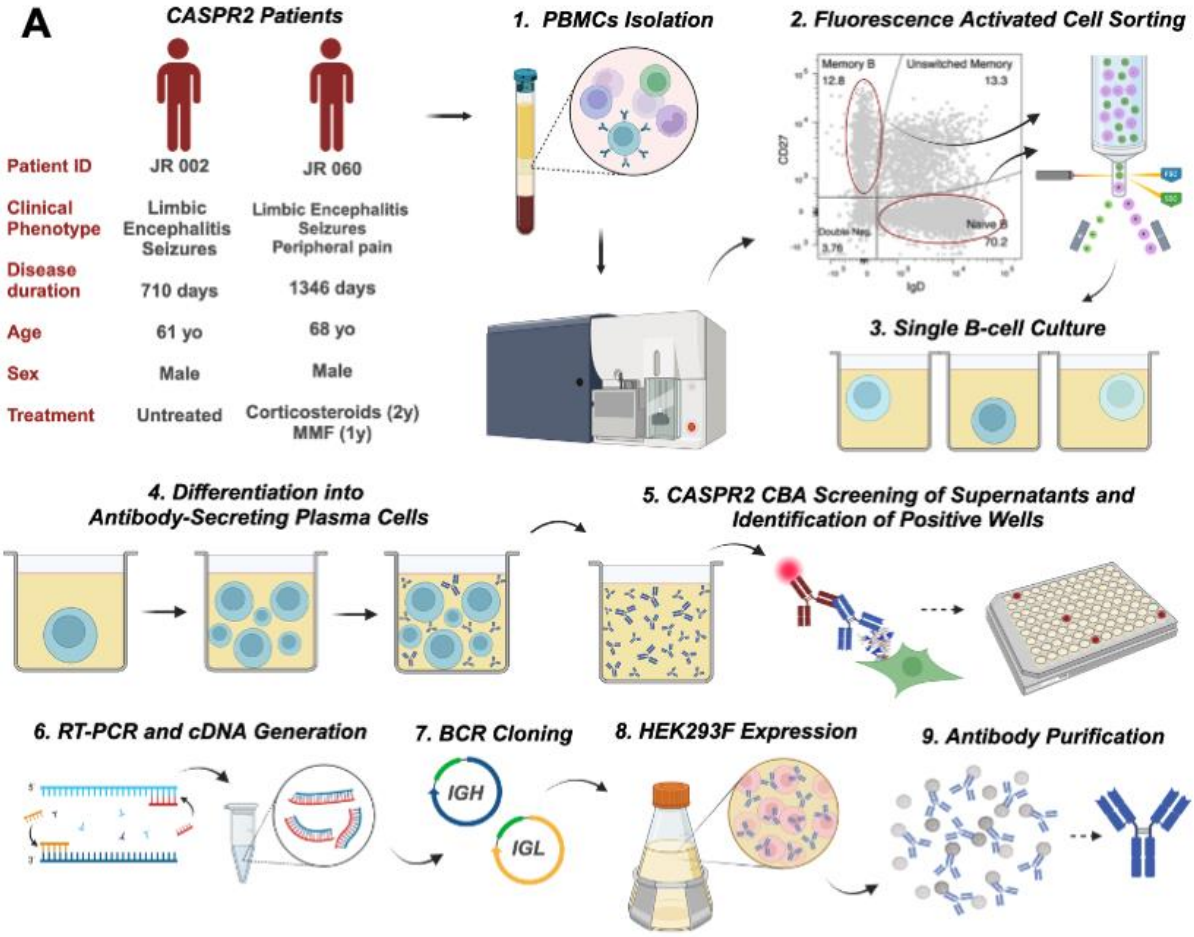


Figure 4.1 Detailed illustrated protocold depicting the path to monoclonal antibody generation including (A) CASPR2 patient details; (1) PBMC isolation; (2) Fluorescence-activated cell sorting; (3) Single B-cell culture (4) Differentiation into antibody-secreting plasma cells; (5) CBA Screening of supernatants for CASPR2 reactivity and identification of positive wells; (6) reverse transcriptase-PCR and generation of cDNA; (7) Cloning of B-cell receptors; (8) Expression of IGH and IGL/IGK genes in HEK293F cells in suspension; (9) mAb purification, to be later followed by downstream functional characterisation.

METHODS

Study approval, participants, and sample collection
Blood samples from two patients referred to the Oxford Autoimmune Neurology clinic were collected at the John Radcliffe Hospital, Department of Neurology after informed consent was obtained under research ethics approval committee (REC reference 16/YH/0013, IRAS Project ID: 195152). De-identified blood samples were collected into 9ml sodium-heparin tubes (Greiner Bio-One) and processed for peripheral blood mononuclear cells (PBMCs) isolation.

Isolation of PBMCs
Bloods were centrifuged at 500g for 10mins at room temperature and plasma supernatants were aliquoted into separate tubes without disturbing the red blood cell (RBC) pellet. RBCs for the same patient were pooled into 50ml Falcon tubes and supplemented with PBS + 1% BSA to a final volume of 35ml. Diluted RBC pellets were transferred to a new 50ml tube containing 15ml Ficoll-Paque (Sigma-Aldrich), while paying attention the two solutions don't mix. Tubes were centrifuged at 400g for 30min at room temperature (acc 3, dec 0). After centrifugation, a 3ml sterile plastic Pasteur pipette was used to carefully transfer the PBMC layer into a new 50ml Falcon tube, transferring as little Ficoll as possible in the process. PBMCs were topped up with PBS + 1% BSA at a minimal ratio of 1:1 and washed twice by spinning at 200g for 10mins at room temperature (acc 9, dec 7) and resuspending in PBS + 1% BSA. Cells were counted using a Türk Counting Solution for Leucocytes. Post-washes, cell supernatant was carefully removed, and cells were first resuspended in 0.5ml of 'Freezing Medium I' (RPMI 1640, foetal calf serum, Penicillin/Streptomycin, Glutamax, β-Mercaptoethanol, IgG-depleted transferrin), transferred to cryovials, and then slowly supplemented with the same volume of 'Freezing Medium II' (20% DMSO in FCS) added in whilst agitating the mixture over 30-40s. Cryovials were immediately placed on wet ice, and then transferred into Corning® Cool Cell® Freezing Containers for steady cryopreservation at -80°C. The next day, PBMCs cryovials were transferred to a liquid nitrogen storage tank where they were kept until use.

Cell Labelling, Fluorescence Activated Cell Sorting, and Cell Culture (OANG)
Whole PBMCs were thawed at 37°C in a water bath and transferred to complete B-cell media: RPMI supplemented with 5% IgG-depleted FCS, Pen-Strep, L-Glutamine, IgG-depleted transferrin, and β-Mercaptoethanol. Samples were washed 3-times in PBS + 1% BSA and labelled at 4°C for 30mins with antibodies against the following cell markers:

- CD3 (clone UCHT1, Pacific Blue, BioLegend)
- CD14 (clone HCD14, Pacific Blue, BioLegend)
- CD19 (clone SJ25C1, APC-Cy7, BD Biosciences)
- CD27 (clone O323, BV605, BioLegend)
- CD20 (clone 2H7, FITC, BD Biosciences)
- IgD (clone IA6-2, PE-CF594, BD Biosciences)
- CD38 (clone HB7, PE-Cy7, BD Biosciences)

Post-labelling, cells were washed 3-times in PBS + 1% BSA and DAPI (ThermoFisher) was added prior to sorting with a FACS Aria III. Dead cells and unwanted cell populations were removed by gating on CD3-CD14-DAPI prior to cell sorting, after which B-cells within gates corresponding to naïve B-cells ($CD19^{+}IgD^{+}IgM^{+}CD27^{-}$) and class-switched memory B-cells ($CD19^{+}IgD^{-}IgM^{-}CD27^{+}$) were positively sorted into RPMI and MS40L-expressing cells with cytokines IL-2, IL-21, IL-4 and BAFF.

Identification of CASPR2-reactive Immunoglobulin (OANG)

Following 26 days of in vitro culture, 50µl of culture supernatant was used to determine the presence of CASPR2-reactive IgM/IgG using a live cell-based assay on HEK293T cells, as described in Chapter 2, the difference being that the secondary antibody here was an AF594-conjugated goat anti-human IgM (Invitrogen) or IgG (Invitrogen). Wells positive on the live CBA were lysed with RNAsin plus (12.8µl/ml, Promega) in TE buffer, pH 8 (Bioultra), flash frozen on dry ice and stored at -80°C for downstream RT-PCR.

cDNA Synthesis and Amplification of VH and VL Sequences (OANG)

Post-culture RT-PCR was performed as described previously.[437, 441] In brief, 15.5µl of RT-PCR mixture consisting of 150ng random primers $dT_{23}VN$ (New England Biolabs), 10µl of 5X Superscript IV buffer (Invitrogen), 2.5µl of 10mM dNTP mix (NEB), 0.5µl of 25% IPEGAL (Sigma-Aldrich), 1µl SuperScript Enzyme IV (SSIV, Invitrogen) and 0.1M DTT (Invitrogen) was used per sample. Once cDNA was generated, the variable immunoglobulin genes of the heavy chain (IGH) and the two possible light chains (IGK and IGL) were amplified using a nested PCR strategy in two steps,[437] further summarised in Chapter 5.

Monoclonal Antibody Production (OANG)

1. *Cloning*

Cloning was performed as previously described.[437] Restriction sites were introduced into the heavy chain (AgeI and SaII) and light chains (AgeI and BsiWI for IGK; AgeI and XhoI for IGL) using individual immunoglobulin gene-specific primers. Restriction digest was performed at 37°C for 1h in 20µl containing 10ng of purified specific PCR product and 1U each of enzymes AgeI, SaII, BsiWI and XhoI (NEB). Purified products were then ligated into expression vectors containing human IGHG1, IGHG4, IGHM and IGK or IGL constant regions with a cytomegalovirus (CMV) promoter and an ampicillin resistance gene. 5µl of ligation product was transformed into 20µl of high-efficiency 5-alpha competent *E. coli* bacteria (NEB) and positive colonies were sequenced for inserts. Products with 100% identity to the original PCR sequence were isolated and expanded in lysogeny broth (Sigma-Aldrich) for downstream purification using a QIAGEN MaxiPrep® Kit.

2. *Expression*

HEK293F cells were cultured in 60ml FreeStyle expression medium (Thermofisher) in 250ml Erlenmeyer flasks rotating at 120rpm, 37°C, 8% CO_2. Cells were transiently transfected using a transfection solution of 4ml phenol red-free DMEM (Gibco), 30µg heavy-chain vector, 30µg light-chain vector, and 90µl branched polyethylenimine solution at a concentration 1mg/ml (Sigma-Aldrich). Supernatant with expressed antibody was harvested 5-days post-transfection. Where suspended HEK293F cells were not available, adherent HEK293T cells were grown in T-175 Cell Culture flasks (Thermo Fisher) and once 70% confluent, were transfected overnight with 22.5µg heavy-chain vector DNA, 22.5µg light-chain vector DNA, and 135µl linear PEI in 4ml non-supplemented DMEM. The following day cell medium was changed to serum-free DMEM with Nutridoma and Antibiotic-Antimitotic, and cells were cultured 4-5 days before harvesting supernatant. Supernatant was centrifuged to pellet down dead cells, then filtered through a 0.2µm filter using a pressure syringe

3. *Purification*

Desalting and size exclusion automated protein-A affinity chromatography (5ml HiTrap™ mAbSelect™ columns) was performed on a single AKTA™ pure 25M, with the instrument operated at 4°C. Where size exclusion chromatography was not available Dynabeads™ Protein G for Immunoprecipitation (Thermo Fisher) were used as per the manufacturer's instructions.

Chromogenic Endotoxin Quantification
Pierce™ Chromogenic Endotoxin Quantification Kit (ThermoScientific) was used to measure levels of endotoxin lipopolysaccharide (ET-LPS) in purified mAbs. This quantitative end-point assay uses amoebocyte lysates derived from blood of the horseshoe crab to determine levels of ET-LPS, a membrane component of Gram-negative bacteria. When amoebocyte lysate comes into contact with ET, a series of enzymatic reactions results in the release of p-nitroaniline (pNa) from a colourless chromogenic substrate, producing a yellow colour. After stopping the reaction, the released pNa is measured photometrically at 405 nm using a plate reader. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0 EU/ml range, and the developed colour intensity is proportional to the amount of endotoxin present in the sample, which can then be calculated using a standard curve.

Domain Specificity Mapping (Dr Bo Sun)
Using a full-length human CASPR2-EGFP construct as template, CASPR2-CASPR4 domain swapped constructs were generated by Dr Bo Sun in the following way. First, gene fragments corresponding to CASPR4 protein domains Discoidin, Laminin G-like 1-4, and Fibrinogen C were synthesised by IDT with complementary overhangs matched to the 5' and 3' regions of the respective CASPR2 domains. Alignment of respective domains was defined by UniProt numbering and MAFFT multiple sequence alignment. Then, each individual domain was sequentially swapped by overlap extension PCR using an insert-to-donor vector ratio of 3:1 and Q5® High Fidelity 2X Master Mix (NEB). Insert and vector underwent denaturation (98°C for 30s), annealing (60°C for 30s) and polymerase-catalysed extension for 30s per kb for a total of 34 cycles. PCR products were then digested with DpnI at 37°C for 1h to remove template plasmids prior to transformation of competent *E. coli* cells. Colonies were screened and validated by Sanger sequencing performed by Source Bioscience. Live cell-based assays using the CASPR2-CASPR4 domain-swapped constructs were performed as described in Chapter 2. A mAb was known to bind a specific domain if it bound all constructs apart from the one where that CASPR2 domain was swapped for a CASPR4 one.

Primary Hippocampal Neuronal Cultures
Pregnant Wistar Han time-mated female rats were supplied by Envigo and at day E18, pups were dissected from the dam, their brains removed and placed into Hanks balanced salt solution (HBSS) with 10mM HEPES buffer (pH 7.3). Hippocampi were dissected under microscopic guidance following standard sterile surgical procedure and incubated with 2.5% trypsin at 37°C, 5% CO_2 for 15mins, washed three times in HBSS and gently triturated in 1ml Hibernate-A supplemented with B27. Dissociated cells were plated at a density of 70,000 cells/well in neurobasal media supplemented with glutamax, penicillin/streptomycin and B27 on coverslips coated with 10µg/ml PLL and 0.1% gelatine. On culture day 4, neurons were supplemented with fresh neurobasal media and cultured for a maximum of 21 days before being used for staining. Staining was done similar to a live cell-based assay, with washing and fixing done in PBS with 4% sucrose.

Immunohistochemistry
Mice were sacrificed and perfused with heparinised saline, with brains dissected and frozen by holding over liquid nitrogen fumes. Tissues were cryosectioned at -18ºC into 12µm sections on gelatinised glass slides (VWR). For staining, samples were fixed in 4% PFA for 10 mins, dried for 20mins in a 37ºC incubator, washed in PBS and placed in 1% H_2O_2 in methanol for 10mins to quench endogenous peroxidase. Following two more PBS washes, slides were placed in a *Sequenza™ Slide Rack and Coverplate™* and incubated with 10% normal goat serum (NGS) for 1h. Antibodies at a concentration of 20µg/ml in PBS + 1% NGS, were added

to the slides and left to incubate overnight at 4ºC. The following day, slides were washed with PBS and incubated for 2h at room temperature with secondary goat-anti-human IgG $(H+L)^{BIO}$ antibody (Vector, BA-3000) in 1% NGS. Staining was developed using ABC Vectastain Solution (Vector Laboratories Inc., Elite Standard PK-6100) and incubation with 3,3'-diaminobenzidine (DAB) diluted in phosphate buffer and H_2O_2. For neutrophil staining, an anti-mouse neutrophil antibody (produced in Experimental Neuropathology Lab) was used. When staining for injected mAbs, the primary antibody incubation step was skipped. After development using DAB, slides were stained with cresyl violet followed by dehydration in alcohols of ascending concentration, immersion in xylene, and mounting using DPX.

Field Recordings from Acute Hippocampal Slices

Slice preparation and field recordings were performed as previously described.[442] Mice were sacrificed by cervical dislocation and decapitation, and 350μm thick coronal slices were cut using a vibratome (HM650V, ThermoScientific). Borosilicate glass capillaries (Harvard Apparatus, 30-0044,) were made into recording electrodes using a pipette puller (Sutter Instrument Co., P-97) to obtain a tip resistance of 1-3 MΩ. A cluster microelectrode (FHC, CE2C75) was used as the bipolar stimulating electrode. For data acquisition, WinWCP electrophysiology recording software (V5.1.1) was used. To record fEPSPs, the stimulating electrode was placed in the Schaffer collaterals of CA3 neurons to deliver stimuli, and the recording electrode was placed in the stratum radiatum of CA1 neurons. Neurons were stimulated once every 14.5 seconds. Once a stable field was observed, a baseline recording was made for 15-20 mins. Long-term potentiation (LTP) was induced using a theta burst stimulation consisting of 4 bursts of 100Hz repeated 20 times, each burst 200ms apart. Recording was then resumed for 1h. The magnitude of fEPSP was determined as the gradient of the rising slope using ClampFit software (Molecular Devices).

Electrophysiology on Cortical Neuronal Cultures (Dr Dominique Fernandes)

Electrophysiological recording from cultured cortical neurons was performed by collaborators at University of Coimbra as previously described.[318] Primary cortical neurons were harvested from Wistar rat pups at postnatal day 0 (P0), and mAb effects were assessed on DIV15 at a concentration of 20μg/ml following a 2h incubation. AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were pharmacologically isolated by adding 1μM TTX, 20μM Bicuculline and 100μM APV to aCSF and measured in a whole-cell patch-clamp configuration. Cells were held at -80mV and mEPSC data was digitized at 10kHz with a data acquisition board (National Instruments, TX, USA) and acquired using the Igor Pro software (WaveMetrics). For each cell at least 200 consecutive mEPSCs were analysed using the MiniAnalysis program (Synaptosoft), with the detection threshold set at three times the root mean square (RMS) noise level. Staining and quantification for the intensity of surface AMPAR puncta was performed using standard immunocytochemical means.[318]

Stereotactic Injections and Behavioural Experiments

Stereotactic injections and behavioural experiments were performed as outlined in Chapter 3, with minor differences in the pilot experiment. In the pilot, female CD1 mice aged 7-12 weeks received a single unilateral injection of 0.5μl of monoclonal antibody, all other conditions being the same as in Chapter 3. Chapter 3 also featured all final versions of the behavioural protocols used. The protocol that underwent the biggest changes was for the Novel Object Recognition test. This initially included two habituation sessions, each 10mins in duration, a training phase of 15mins, and testing lasting 10mins performed 2h after the training phase, which eventually evolved into the protocol described in Chapter 3. For further details of the evolution of the behavioural testing protocols, please refer to the Results section of Chapter 4.

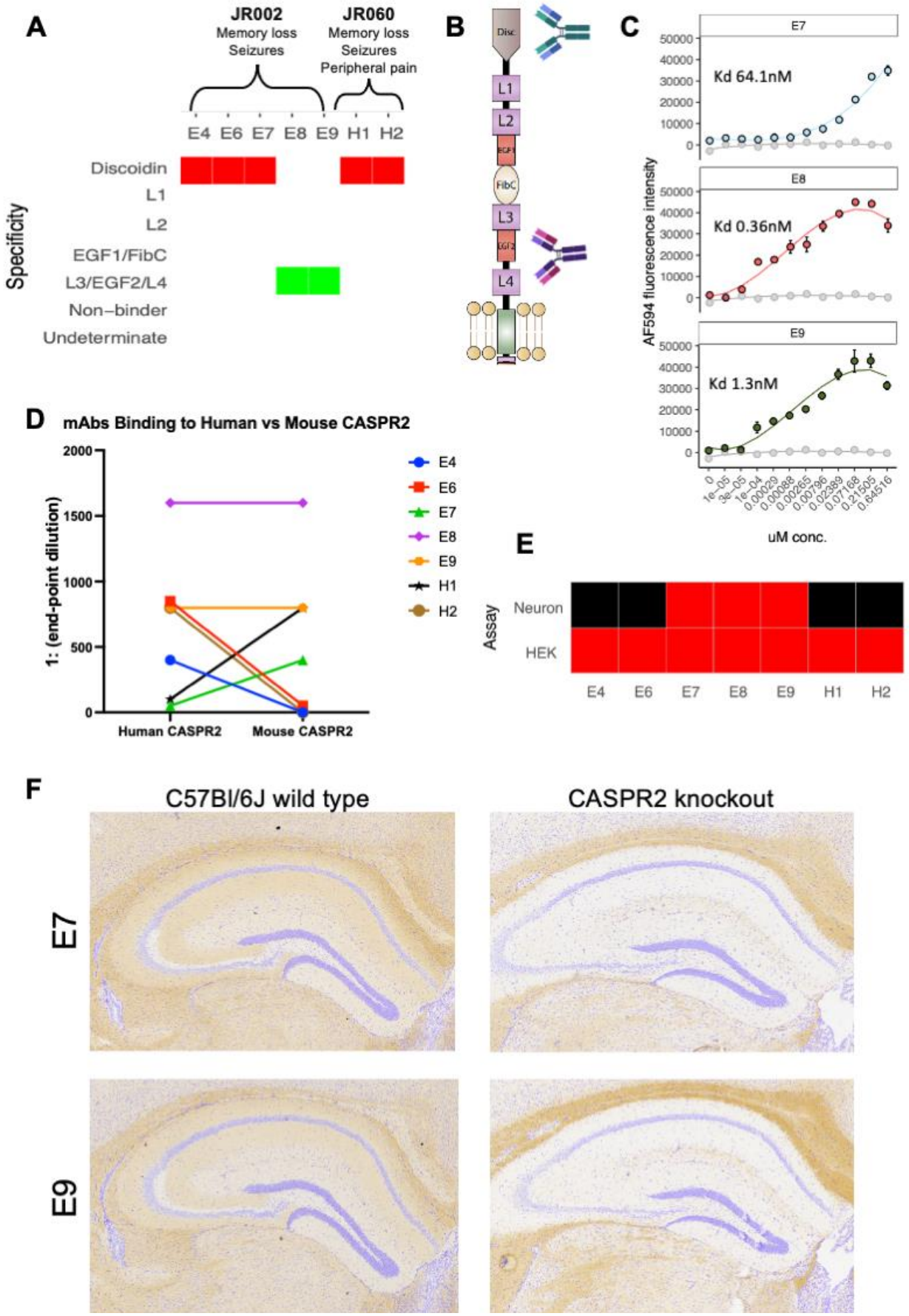


Figure 4.2 (A) Summary of domain specificities of the seven CASPR2-mAbs dervied from memory B-cells across patients JR002 and JR060. Five mAbs bound the discoidin domain and two the L3 domain; (B) Schematic depicting the two domains principally targeted by the mAbs. (C) Binding curves for mAbs E07, E08, and E09, obtained from a live CBA using a BMG OMEGA FLUOSTAR plate reader. Kd values were extracted from fitted curves using the drc package in R (Dr Bo Sun) (D) Differential binding of mAbs to human versus mouse CASPR2, as determined via end-point titration on a live CBA and plotted as 1 : (end-point dilution); (E) Summary of mAb binding across various screening modalities; (F) Illustrative mAb binding on wild-type versus CASPR2-knockout brain tissue for mAbs E07 and E09, selected for further in vivo studies.

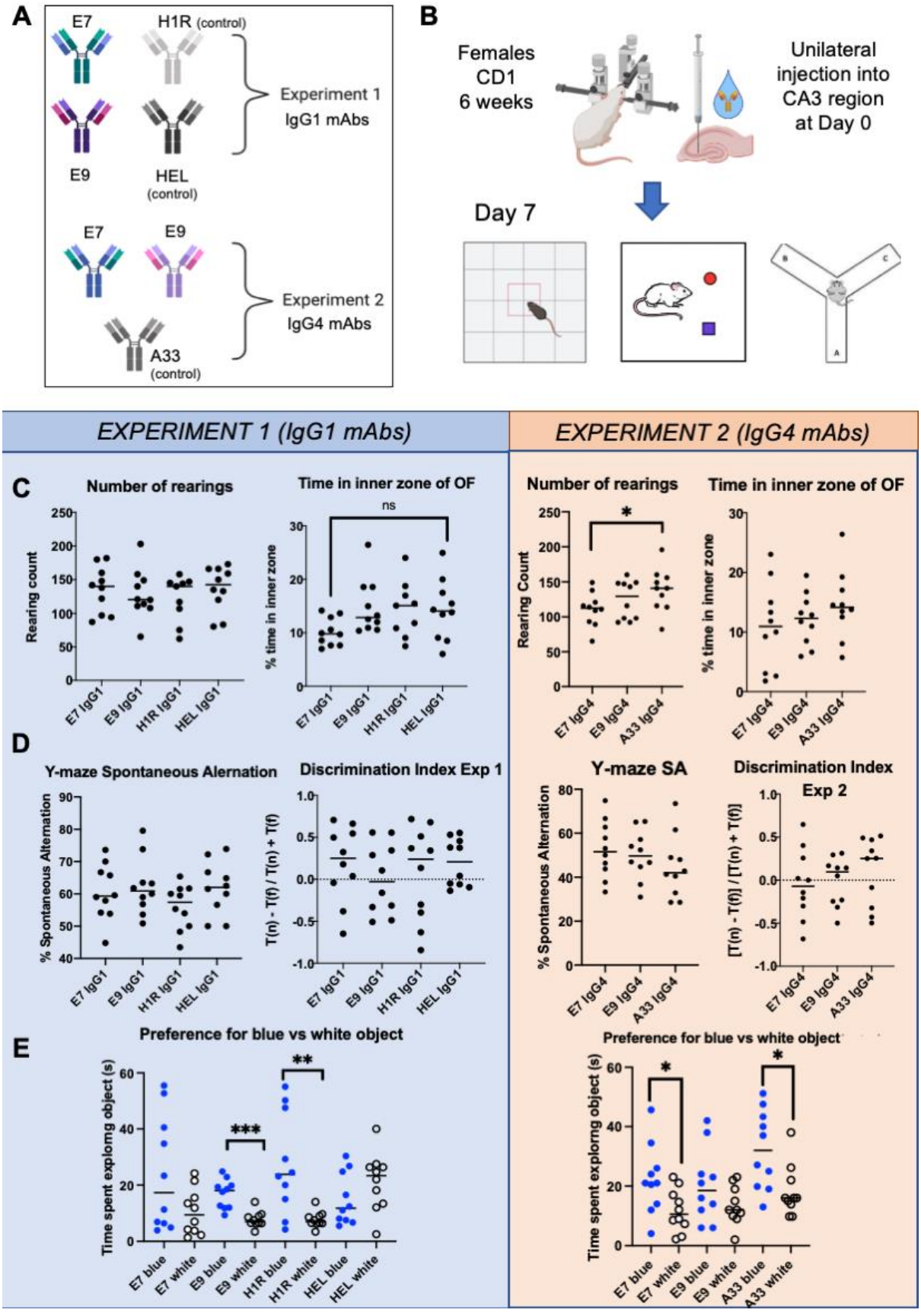


Figure 4.3 Pilot experiment injecting CASPR2-mAbs in mice. (A) Summary of mAbs available for pilot experiment; (B) Experiment design: female CD1 mice, 6 weeks of age (n = 10) received a unilateral injection of 0.5ul of CASPR2-mAb or isotype control into the CA3 region of the hippocampus, followed by Open Field, Novel Object Recognitio, and Y-maze Spontaneous Alternation testing 7 days later. (C) Results from Open Field performance across the IgG1-mAb injected mice (left-side, blue background) and IgG4-mAb injected mice (right-side, orange background), showing rearing count and time in the inner zone of the open field; (D) Performance on the memory tests, Y-maze SA and Novel Object Recognition (shown as Discrimination Index) for the two experiments; (E) Quantifying time spent exploring each object in the NOR test, regardless of whether it was novel or not, revealed a clear preference for one of the two objects used across both experiments, leading to further changes in the novel object protocols

RESULTS

Characterising patient-derived CASPR2-mAbs in vitro

Prior to the start of my project, 7 CASPR2-mAbs were cloned from memory B-cells derived from patients JR002 and JR060, who were also featured in Chapter 2 and 3 (Figure 4.1). All mAbs were cloned on an IgG1 and IgG4 backbone, reflecting the presence of both subclasses among CASPR2-reactive antibodies (Chapter 2). Screening mAbs for reactivity on CASPR2-CASPR4 domain-swapped constructs revealed that two mAbs (E08 and E09) bound the Laminin G-like 3 (L3) domain of CASPR2, whereas the remaining five (E04, E06, E07, H01, and H02) bound the discoidin domain (Figure 4.2A). An epitope-binning cross-competition assay was performed (Dr Bo Sun, not shown) to assess whether the mAbs converge towards shared or overlapping epitopes. Among the discoidin-binding mAbs, E07 and H01 competed with E04, likely suggesting a shared epitope despite origins from different patients, whereas H02 and E06 competed for the same epitope. Forming their own cluster, mAbs E08 and E09, both specific for the L3 domain, were highly cross-competitive, suggesting overlapping epitopes. While all seven mAbs bound CASPR2-transfected HEK293T cells, only three (E07, E08, E09) bound live primary cultured hippocampal neurons and rodent brain sections (Figure 4.2E). Furthermore, all mAbs bound human iPSC-derived sensory neurons, but only H01, E07, E08 and E09 bound mouse dorsal root ganglia (DRG). Of the three cross-species neural binders, E08 had lowest K_D value and highest affinity for CASPR2, followed by E09, and then E07 (Figure 4.2C). To address the cross-species discrepancies in binding, mAbs were titrated on HEK293T cells transfected with either mouse or human CASPR2. Only mAbs E08 and E09 bound with equal end-point dilutions to both human and mouse CASPR2, with E04, E06, and H02 losing their binding on mouse CASPR2, and E07 and H01 binding more strongly to mouse than human CASPR2. (Figure 4.2D). mAbs E07 and E09 were selected for further functional experiments in vivo, due to their consistent cross-species reactivities, distinct domain specificities, shared patient origin, and demonstrated binding to mouse CASPR2. Both mAbs also showed strong binding to the hippocampi of wild-type C57BL/6J mice, abrogated in sections from CASPR2-knockout mice (Figure 4.2F)

Pilot in vivo experiment

As no studies had been done with patient-derived CASPR2-mAbs prior to this project, there was very limited existing literature to base my preliminary experiments on. I relied instead on published work on LGI1-mAbs, which I assumed to be relatively close to the CASPR2 field to be used as a foundation.[396] My first two *in vivo* experiments aimed to study how the domain-specificity, CASPR2-affinity and antibody subclass could influence pathogenicity. Experiment 1 featured mAbs E07 and E09 as IgG1, while Experiment 2 featured the same mAbs as IgG4. In both experiments, isotype control antibodies that didn't bind to CASPR2-transfected HEK cells, hippocampal neurons or brain tissue were used: HEL IgG1 and H1R IgG1 for Experiment 1, and A33 IgG4 for Experiment 2 (Figure 4.3A). Adult female CD1 mice (n = 10 per group) received a unilateral injection of 0.5µl mAb (2mg/ml) into the CA3 region of the hippocampus. A week later, they were tested for anxiety, long-term object-based memory and spatial working memory using Open Field (OF), Novel Object Recognition (NOR), and Y-maze Spontaneous Alternation (YmSA) behavioural assays, respectively (Figure 4.3B). Injection of E07 IgG4, but not E07 IgG1 led to a reduced rearing count in the open field, suggesting a negative effect on exploratory behaviour. Both E07 IgG1 and E07 IgG4 produced a downward trend in time spent in the inner zone of the open field which didn't reach statistical significance. (Figure 4.3C) None of the injected antibodies had a significant effect on memory in the Y-maze and NOR tests. (Figure 4.3D) While on the NOR test in Experiment 2, E07 IgG4 and E09 IgG4 did seem to lead to a downward trend in the Object Discrimination Ratio, this data was difficult to

interpret as it showed a dichotomous split between positive and negative values, suggesting a possible bias of the animals towards one of the objects used. Indeed, when time spent exploring each object regardless of whether it was novel or familiar was plotted for each group, this revealed a statistically significant preference for one of the two objects (Figure 4.3E) which in turn led me to make some improvements in the NOR protocol

Evolution of Behavioural Testing Protocols

Changes were made to the NOR protocol to eliminate object bias, increase object exploration time, and reach Discrimination Ratio values that reflect literature standards. (Figure 4.4A) Firstly, I began using C57BL/6J mice instead of CD1 mice for behavioural testing, as the former tend to outperform the latter on memory tasks and are considered well-suited for object discrimination tests.[443, 444] All protocol optimisation experiments were performed using adult female C57BL/6J mice that had not undergone any procedures ('naïve wildtypes'). The objects used initially where a bias had been demonstrated were first replaced with more colourful Lego blocks, but once these produced very low object interaction times, I opted for objects of various textures, materials, and colours, to encourage more exploration (Figure 4.4B). Along with more extensive habituation to the testing conditions, this improved DR values. Narrowing the gap between training and testing from 120mins to 5mins, resulted in DR values close to 0.5 which reflected literature standards (Figure 4.4A, pink). To decide on optimal testing time, time-binned DR values were plotted for each mouse across the testing period, which revealed most mice reached the coveted DR = 0.5 values within the first 6mins of testing and led to subsequent shortening of the testing times used for analysis. (Figure 4.4C) The Y-maze Forced Alternation (YmFA) test was included to replace or accompany the YmSA test (Figure 4.4D). The former was used to test for spatial working memory and the latter for reference memory.[445]

Effect of CASPR2-mAbs on Memory and Anxiety Following Protocol Optimisation

With the changes in the NOR and Y-maze testing described in the previous section, I made another attempt at injecting CASPR2-mAbs. This time, female C57BL/6J mice received a bilateral injection of 1µl of mAb (2mg/ml) in CA3, and behavioural testing was performed the day after injection. As the only mAb with some effect shown in the pilot experiment, E07 IgG4 was used, and its effect compared to that of an isotype control (A33 IgG4), as well as mice that had no procedures performed on them (naïve WT). Disappointingly, E07 IgG4 injection did not produce any behavioural deficits on any of the tests attempted. (Figure 4.5A-C)

Co-injection of mAbs E07 and E09 to Model Polyclonality

Once injection of mAb E07 failed to produce any notable behavioural deficits, I hypothesised that perhaps multiple domains of CASPR2 need to be targeted for a pathogenic effect to be observed. Indeed, antibodies in patients have been demonstrated to target multiple domains[314, 338, 446] and Chapter 3 showed that polyclonal sera produce observable behavioural changes. In an attempt to reproduce this polyclonality, I co-injected mAbs E07 and E09, with mAb A33 used as an isotype control. Adult female C57BL/6J mice, received bilateral injections of 1µl total mAb in the CA3 region (1mg/ml) and were tested in the open field 6h and the light-dark box 7h post-injection. (Figure 4.6A) Combining mAbs E07 and E09 did not enhance their pathogenicity in the open field (Figure 4.6B-G) and in the light-dark box (Figure 4.6H-J). This failure to produce behavioural deficits across multiple experimental modalities led us to shift attention toward the histological and electrophysiological effects of the antibodies.

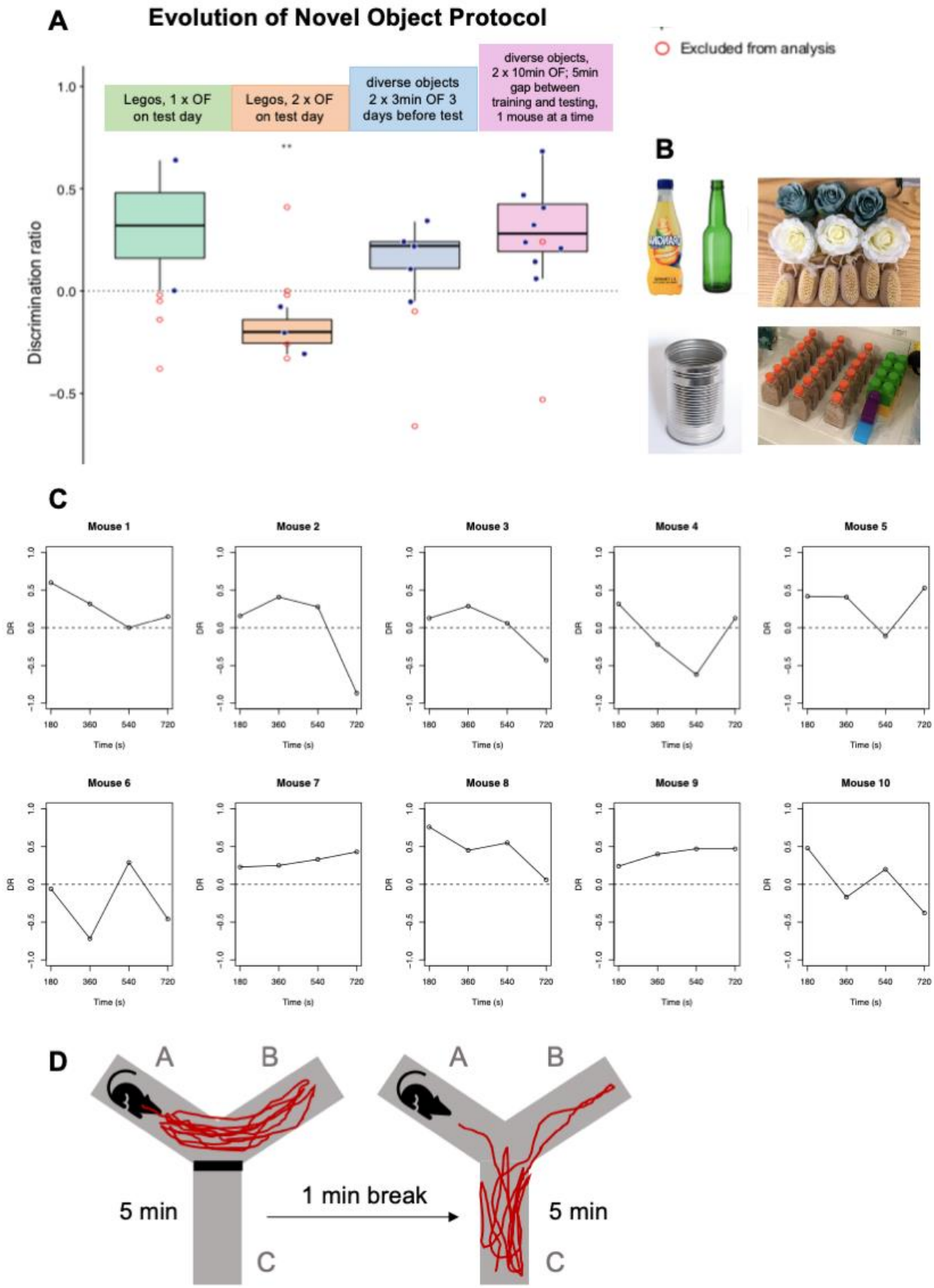


Figure 4.4 (A) Gradual changes in the novel object protocol, tested on naïve mice, led to an improvement in the Discrimination Ratio scores, as well as more mice in the experiment spending the required time interacting with the objects and therefore not being excluded from the analysis; (B) Examples of types of objects used in the test; (C) Time-binned Discrimination Ratios for ten mice used when optimising the protocol, showing the majority of mice reach a DR value of 0.5 within the first 6 mins. This led to a reduction in the testing time thereafter from 10 to 6 minutes. (D) Illustration of the Y-maze Forced Alternation protocol, which was adopted later on either in lieu of or alongside the YmSA protocol.

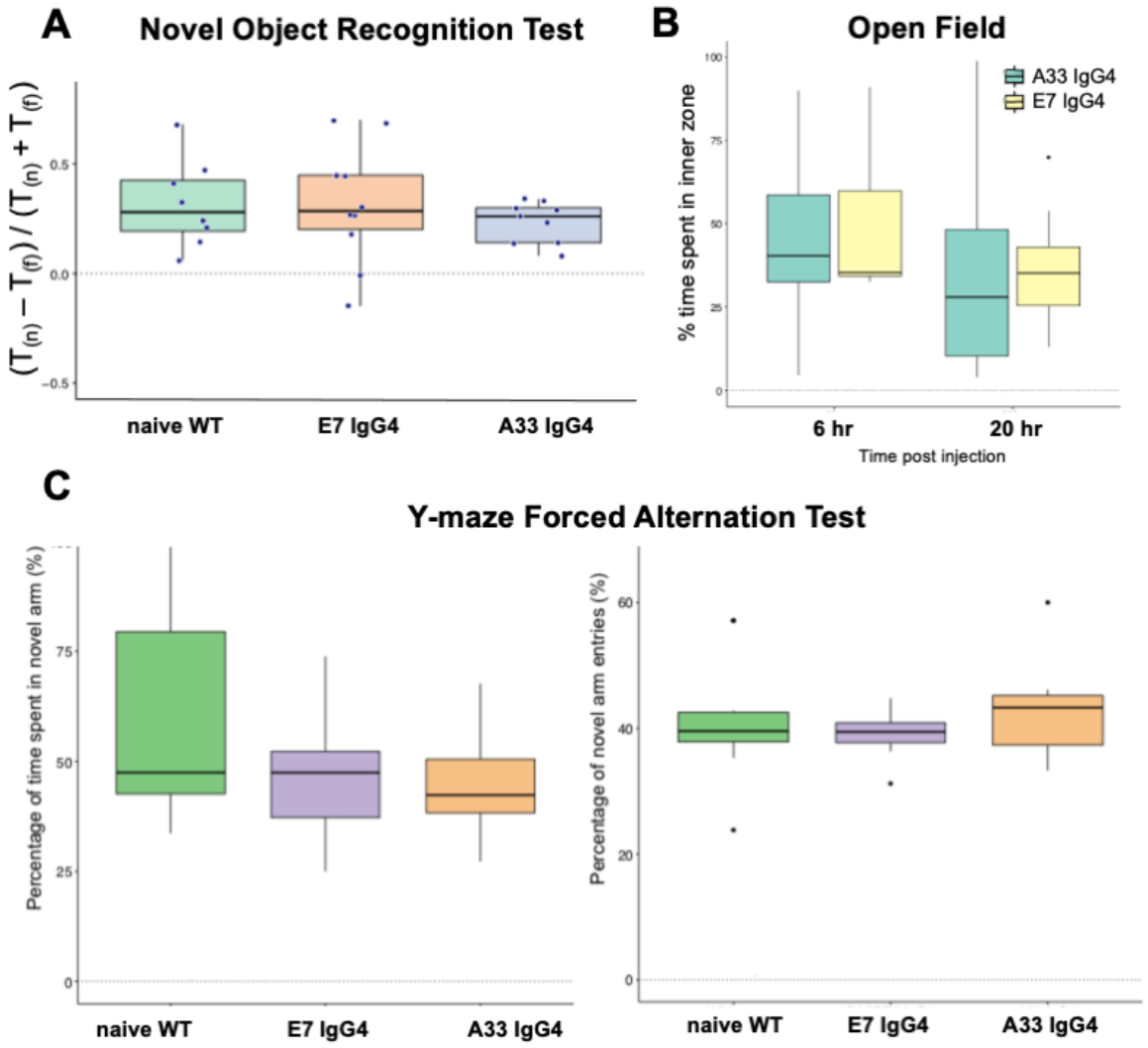


Figure 4.5 Second attempt at performing injections once changes in the behavioural protocols had been adopted. (A) No difference in NOR test performance between naïve mice, mice injected with E7 IgG4 (anti-CASPR2) mAb, and mice injected with an isotype control (A33 IgG4); (B) No differences in performance were seen on the open field test at either 6h or 20h post-injection; (C) No differences in performance between groups were seen for the Y-maze forced alternation test. (One-way ANOVA used throughout)

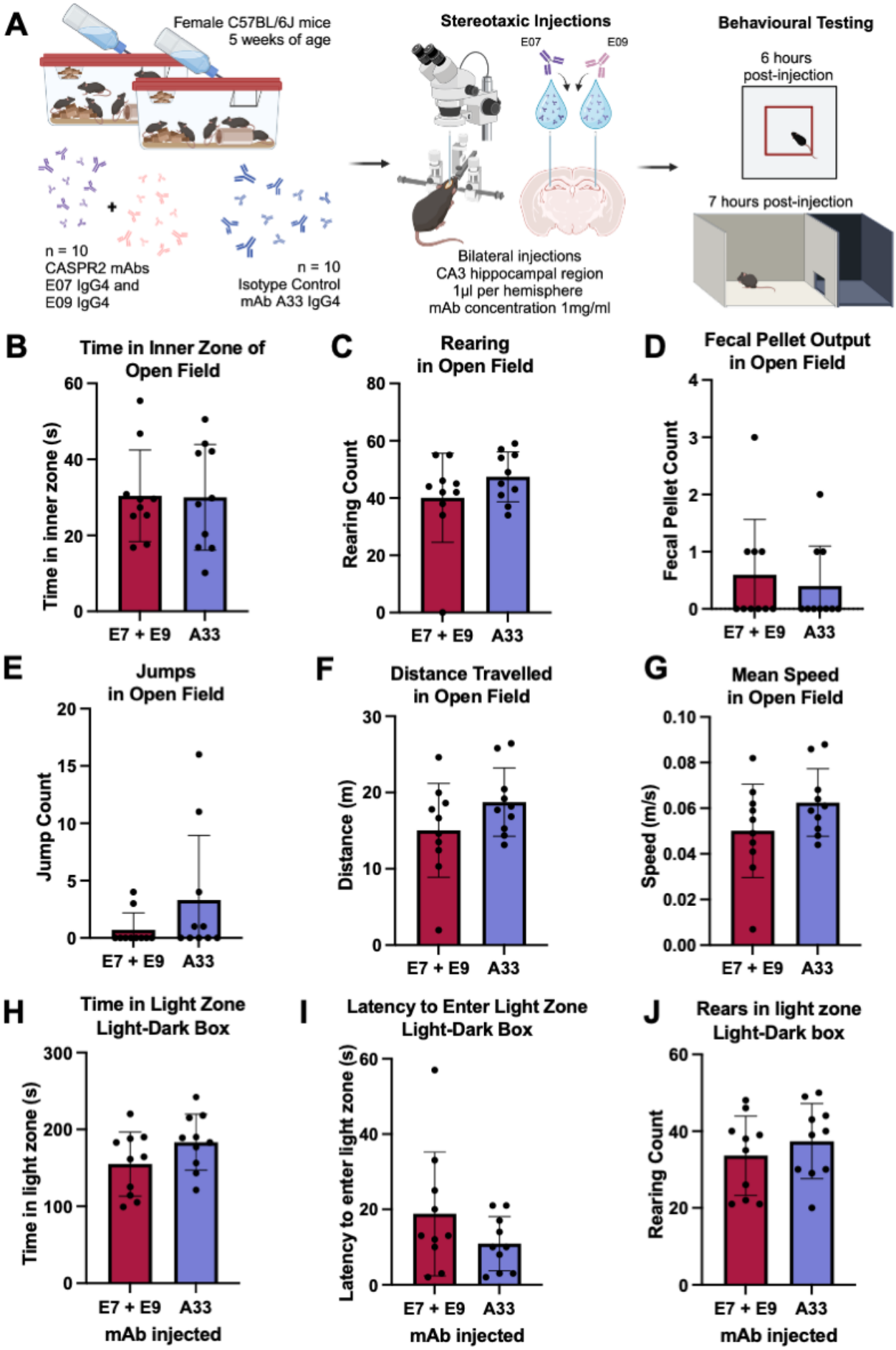


Figure 4.6 Co-injecting mAbs E7 and E9 (A) Experiment design: female mice, 5 weeks of age, received a bilateral intrahippocampal injection of either an isotype control mAb (A33 IgG4) or a combination of two CASPR2-mAbs (E7 and E9) to model the polyclonality observed in sera. Behavioural testing was performed at 6 hours (Open Field) and 7 hours (Light-Dark Box) post-injection. (B-G) No differences in any performance parameters in the Open Field were seen between the two groups; (H-J) No differences in performance on the Light-Dark Box test were seen between groups.

Injection of CASPR2 mAbs Leads to Neutrophil Recruitment in Limited Samples
Surprisingly, when staining the brains of mice injected with mAb E07 6h post-injection, we observed prominent neutrophil infiltration in the hippocampi of mice injected with mAbs E07 IgG1 and E07 IgG4 (Figure 4.7A(A,B)), but not those injected with an isotype control (HEL IgG1 and A33 IgG4), LGI1-mAb, or vehicle control (Figure 4.7A(C-F)). This suggested a CASPR2-specific effect. Neutrophil distribution reflected distribution of the antibody injected for E07 IgG1 and E07 IgG4 (Figure 4.7A(A,B)). A major concern raised by this finding was whether it was potential endotoxin contamination, rather than the mAb, that was driving the immune cell response. Measuring endotoxin levels in the mAb solutions revealed no significant differences between those that did and didn't produce a neutrophil response (Figure 4.7C). Spin-concentrating the mAbs, often done before injection to reach the desired concentration, did not seem to alter endotoxin levels significantly (Figure 4.7D). Endotoxin levels ranged between 1.0 and 4.0 EU/ml. Considering we are injecting a total of 2µl of mAb per mouse, this equates to a mouse receiving 0.002-0.008 EU, or less than 0.001ng of endotoxin, an amount highly unlikely to drive the neutrophil response considering the levels of LPS injected to elicit this are normally on the scale of milligrams.[447, 448] While this finding was indeed interesting, it was not the preliminary intent of the experiment, which was designed to simply help visualise the distribution of the injected antibody (Figure 4.7(B)). Hence, only one animal was culled per condition, and only one slide stained and image obtained per animal, making it difficult to draw any solid conclusions about neutrophil infiltration and impossible to perform any statistical analyses regarding the extent of neutrophil recruitment.

Field Recordings from Acute Hippocampal Slices
To look at the effects of CASPR2-mAbs on long-term potentiation (LTP), female C57BL/6J mice received a bilateral injection of 1µl of mAb E07 or an isotype control (concentration 2mg/ml) and were culled 12h post-injection, after which acute hippocampal slices were prepared and field recordings were performed. (Figure 4.8A) Naïve WT mice that had not received any injections were used as an additional control. Preliminary field recordings were limited in success and showed little promise in terms of the data obtained (Figure 4.8B-C), which is why they were soon abandoned in favour of patch-clamp recordings performed on cortical neurons in culture by an external collaborator, discussed below.

Effect of CASPR2-mAbs on neuronal AMPA Receptors (Dr Dominique Fernandes)
Primary rat cortical neurons in culture were incubated for two hours with each of mAbs H01 and E08 expressed as IgG4. While mAb H01 induced downregulation of both total and synapse restricted AMPARs (Figure 4.9A-B), E08 had an opposite effect, increasing both total and synaptic AMPAR levels (Figure 4.9G-H). Despite their contrasting effects on expression of AMPAR, both mAbs decreased the amplitude of AMPAR-mediated mEPSCs, suggesting an impact to reduce synaptic firing (Figure 4.9C-F and I-L).

Injection of mAb E08 Affects Anxiety-Related Behaviours
Following the results reported in the previous section, I decided to have another go at in vivo injections, this time focusing on mAb E08, shown to significantly affect AMPAR physiology. I used an experimental strategy similar to that in Experiment 5, Chapter 3. Female C57BL/6J mice (n = 10 per group) received a bilateral injection of 1µl of E08 IgG4 or an isotype control (1mg/ml) followed by open field testing at 6h and light-dark box testing at 9h post-injection (Figure 4.10A). This showed mAb E08 had a pro-anxiety effect on the Open Field test 6h post-injection, leading to a higher faecal pellet output, a trend towards less rearing, and a tendency to exhibit more jumping, (Figure 4.10B-E) whilst not affecting any mobility parameters (Figure 4.10F-H). Surprisingly, this effect was reversed at 9h post-injection, with E08-injected mice

exhibiting less anxiety-related behaviour than controls in the LDB, spending more time and rearing more in the light zone of the light-dark box (Figure 4.10I-K)

Comparison of mAb-injected Mice to Un-injected Naïve Controls

To verify whether the behavioural change described in the previous section (Figure 4.10) was a consequence of the Caspr2-mAb or reflected a broader effect of the intracranial injection on the anxiety levels of the animals, I further compared the results in Figure 4.10 to data from mice who had not had any surgical procedures performed on them (naïve mice). This data had been obtained from the experiments where the Novel Object Protocol was being optimised on untreated mice (Figure 4.4(A)), notably the final trial (presented in pink), where the best results on the object discrimination task were obtained. As this protocol featured a 10-minute open field task on the first day of training, the first 5 minutes of the task were used to quantify the time in the inner zone, rearing, jumping, and faecal pellet output of the mice. One-way parametric and non-parametric ANOVA with a multiple comparisons test were used to compare the three groups. This revealed either the E08- or the A33-injected group, or both, to be significantly different from the naïve group on all parameters, but on no parameters were the two mAb-injected groups different from each other. Hence, both the E08- and the A33-injected mice spent less time in the inner zone of the open field than naïve mice, but only the E08 group exhibited less rearing than naïve animals. Furthermore, the E08 group had a significantly higher faecal pellet output in the open field than naïve animals, but did not differ from naïve controls in terms of jumping behaviours, whereas the A33 mAb-injected group jumped significantly less than naïve animals. The results shown in Figure 4.11 thus caution against being overly zealous in the interpretation of the results from Figure 4.10 and argue in favour of the use of a non-injected control when conducting antibody injections. It is possible that Figure 4.11(A) points towards increased levels of anxiety in the mAb-injected groups, while Figures 4.11(B and C) suggest that the E08-injected animals might be slightly less anxious than A33-injected ones, although this is contradicted in Figure 5.11(D), where it is the A33-injected mice that exhibit significantly less jumping than controls. Unfortunately, data on performance of naïve mice in the light-dark box test was not available as this test never required optimisation in untreated animals.

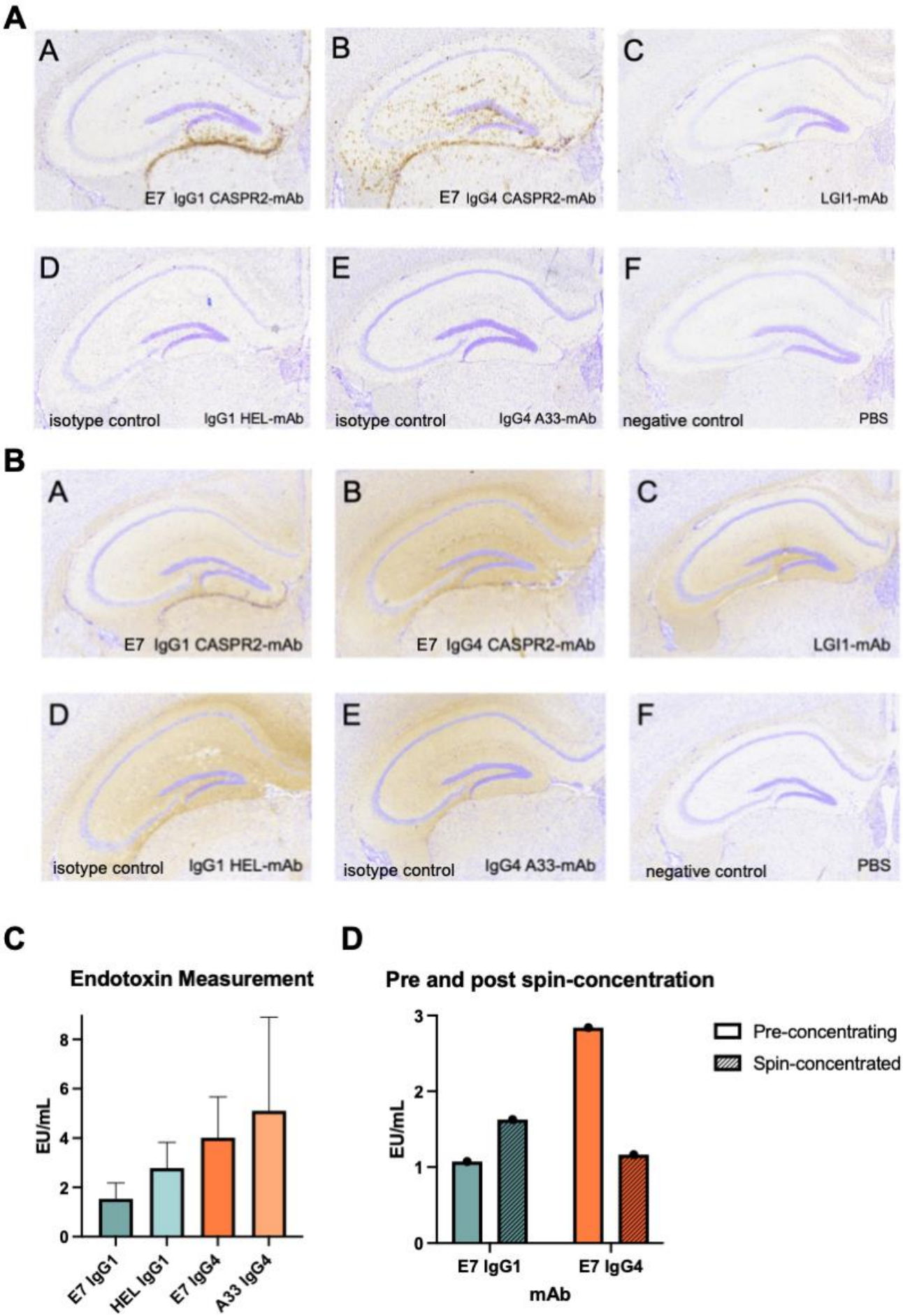


Figure 4.7 (A) Staining 6 hours post injection reveals profound neutrophil infiltration in the injection site for animals injected with E7 IgG1 (A.A) and E7 IgG4 (A.B), but not for mice that received an LGI1-specific mAb, isotype controls or just PBS (A.C-A.F); (B) IgG staining with a goat anti-human IgG secondary on the same brains revealed the antibody depositon in all brains where a mAb was injected (B.A-B.E). For E7 IgG1 and E7 IgG4 (B.A and B.B), the distribution of the antibody reflected the distribution of the neutrophils in the upper panel (C) No significant differences in endotoxin levels were seen between the CASPR2-mAbs and isotype controls and (D) spin-concentrating mAbs did not seem to significantly alter endotoxin levels. EU = endotoxin unit; 1 EU/ml = 0.1 ng/ml

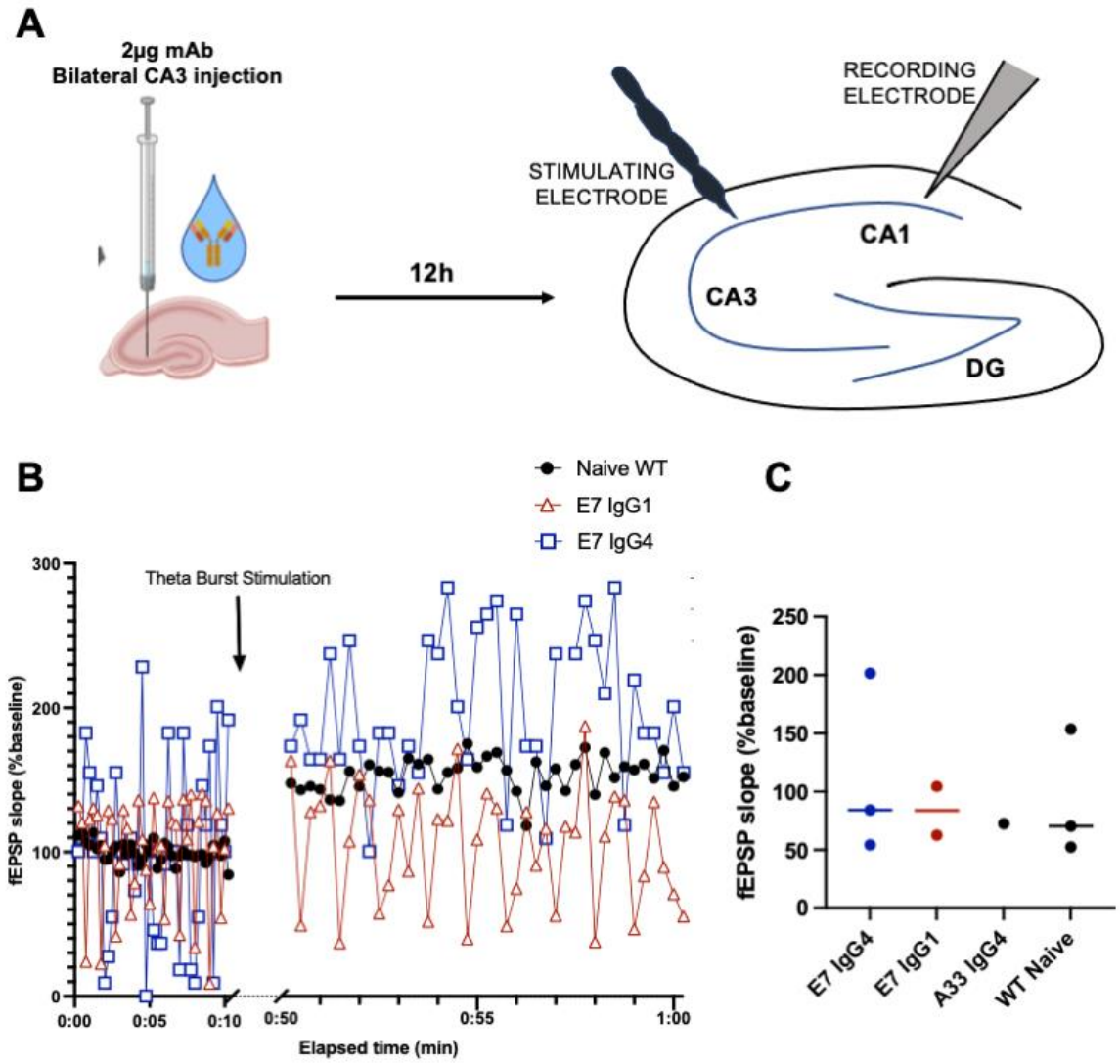
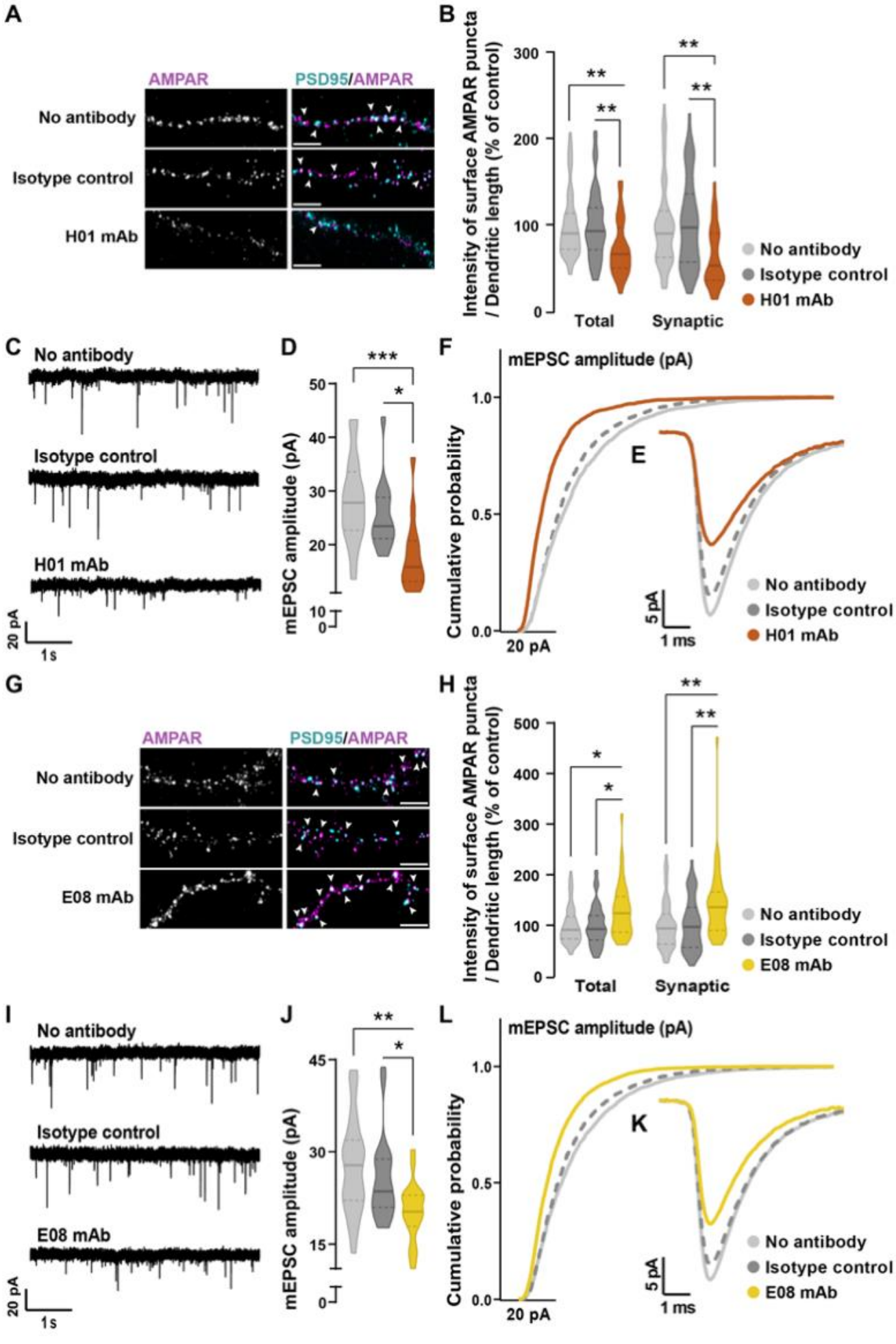


Figure 4.8 (A) Schematc showing experimental design of preliminary electrophysiological experiments in female C57BL/6J mice that had received stereotactic injections, as well as electrode placement when recording from acute hippocampal slices; (B) Single traces showing fEPSP slope 50mins following theta burst stimulation (TBS, arrow). Baseline is calculated as the average fEPSP slope in the 10mins prior to TBS and all measurements are expressed as a % of this value. Graph shows mild LTP induction for naïve animals, and a slight upward trend post-TBS for those mice injected with E7 IgG4. (C) Mean fEPSP slope in the 50-60mins following TBS from several recording attempts, with Kruskal-Wallis test showing no differences between groups.

Figure 4.9 (next page) Data generated by Dr Dominique Fernandes (external collaborator, University of Coimbra), studying the effects of select CASPR2 mAbs on AMPA receptors (AMPARs). (A-B) mAb H01 induced downregulation of both total and synaptic AMPARs, the latter colocalised with the excitatory post-synaptic marker PSD-95. (C-F) Furthermore, H01 decreased the amplitude of AMPAR-mediated miniature excitatory post-synaptic currents (mEPSCs) recorded from cortical neurons in culture. By contrast, mAb E08 increased total and synaptic AMPAR levels (G-H), while simultaneously reducing AMPAR-mediated mEPSC amplitude (I-K) when compared to either a 'no antibody' condition or an isotype control.


A
AMPAR
PSD95/AMPAR
No antibody
Isotype control
H01 mAb
B
Intensity of surface AMPAR puncta / Dendritic length (% of control)
300
200
100
0
**
**
**
**
Total
Synaptic
No antibody
Isotype control
H01 mAb
C
No antibody
Isotype control
H01 mAb
20 pA
1s
D
mEPSC amplitude (pA)
50
40
30
20
10
0
***
*
F
mEPSC amplitude (pA)
Cumulative probability
1.0
0.5
0.0
20 pA
E
5 pA
1 ms
No antibody
Isotype control
H01 mAb
G
AMPAR
PSD95/AMPAR
No antibody
Isotype control
E08 mAb
H
Intensity of surface AMPAR puncta / Dendritic length (% of control)
500
400
300
200
100
0
*
*
**
**
Total
Synaptic
No antibody
Isotype control
E08 mAb
I
No antibody
Isotype control
E08 mAb
20 pA
1s
J
mEPSC amplitude (pA)
45
30
15
0
**
*
L
mEPSC amplitude (pA)
Cumulative probability
1.0
0.5
0.0
20 pA
K
5 pA
1 ms
No antibody
Isotype control
E08 mAb

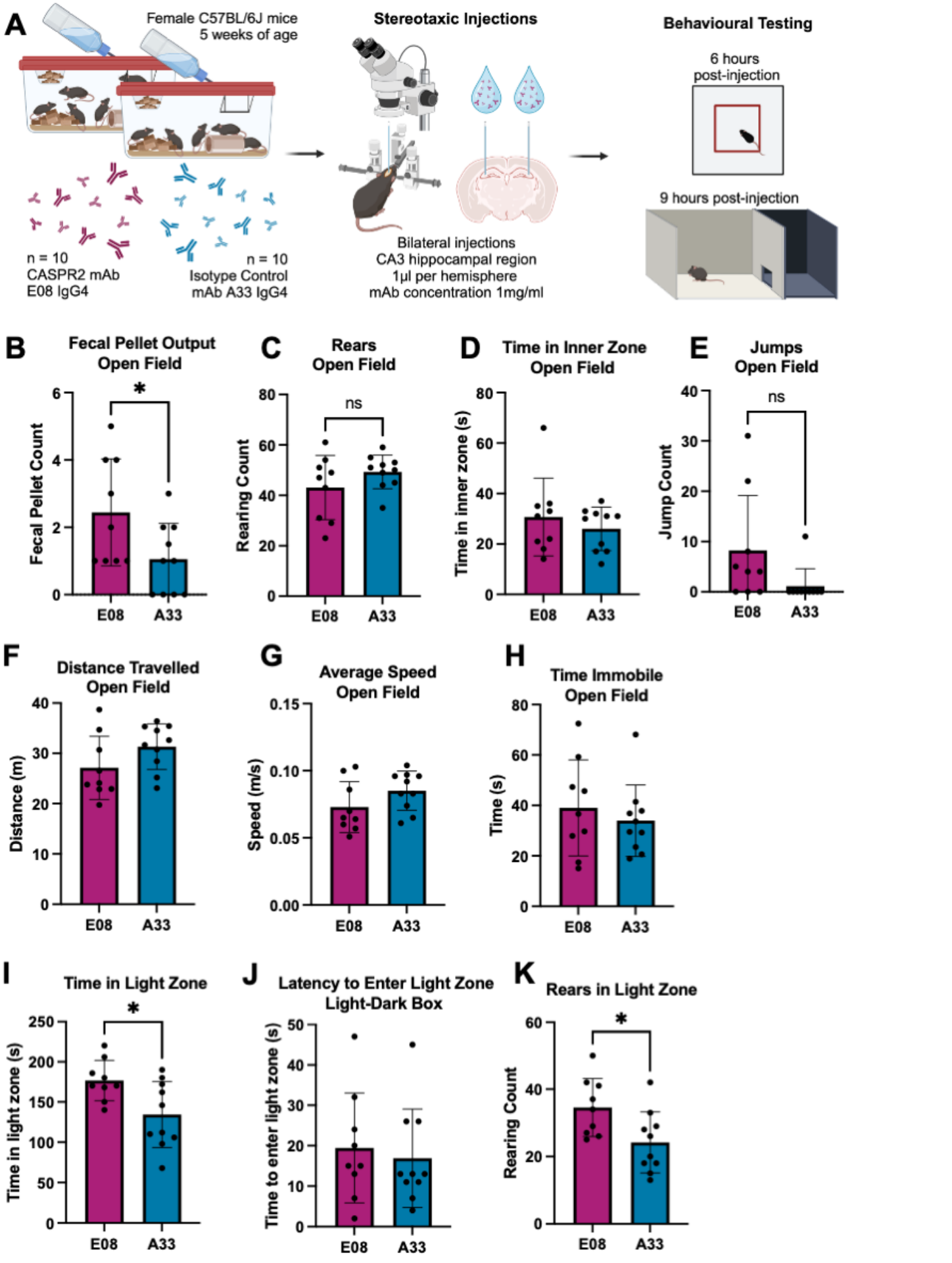


Figure 4.10 Final behavioural experiment involving CASPR2-mAbs, performed after obtaining the results presented in Figure 4.9. (A) Diagram illustrating experimental design; (B-E) mAb E08 led to a higher faecal pellet output in the open field, while also mildly reducing rearing, and tending to produce more jumping behaviours (F-H) No differences between groups were observed in any mobility parameters in the open field; (I-K) 9 hours post-injection, mice injected with E08 exhibited a more 'disinhibited' behaviour on the Light-Dark Box test compared to controls, with a significant increase in the time spent and rearing count in the light zone.

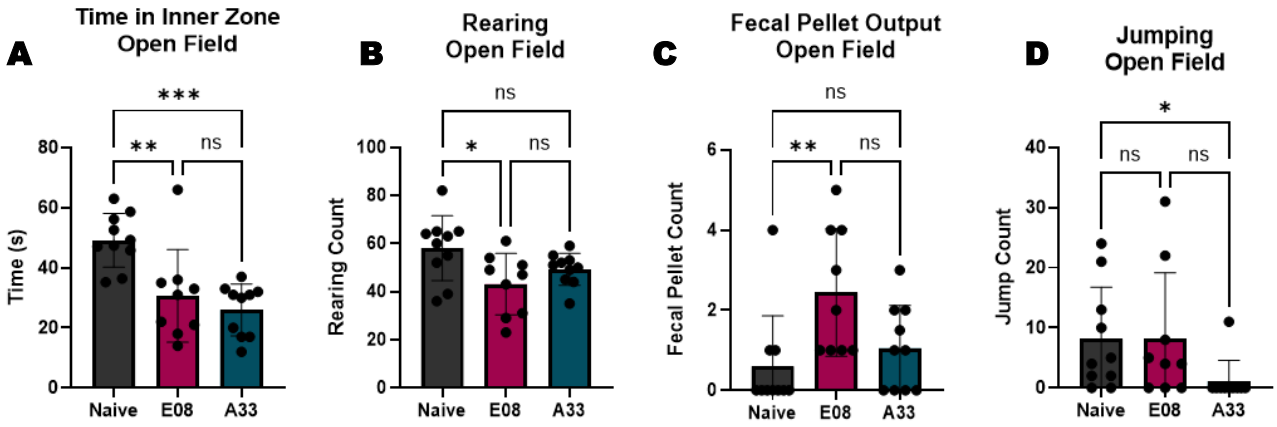


Figure 4.11 Comparison of Open Field measurements presented in Figure 4.10 (B-E) to performance of naïve un-injected C57BL/6J mice on the same behavioural task. Ordinary one-way ANOVA with Tukey multiple comparisons test was used for Time in inner zone and Rearing (A, B). Non-parametric ANOVA with Dunn's method for multiple comparisons used for Faecal Pellet Output and Jump Count (C, D). Significance levels reported as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

DISCUSSION

In this chapter, I have presented a comprehensive attempt by myself and others to generate and functionally characterise CASPR2-specific mAbs from peripheral memory B-cells donated by patients with CASPR2-antibody encephalitis. The work described here, while not without its limitations, is the first of its kind in the field, aiming to resolve the specific antibody characteristics that could contribute to pathogenicity using both *in vitro* and *in vivo* platforms. That said, the results are far from conclusive and oftentimes even conflicting.

Across the experiments described here, three mAbs, E07, E08, and E09, were trialled both *in vitro* and *in vivo*, out of which only E08 managed to induce any behavioural changes, albeit this was called into question when the results were reinterpreted in light of the behaviour of untreated naïve control animals. It was shown that E08 had the highest affinity for human CASPR2 and comparably high binding to mouse CASPR2, as well as strong reactivity across multiple assays including live HEK293T CBA, hippocampal and DRG neurons, human iPSCs-derived sensory neurons, and rodent tissue sections. Furthermore, E08 was one of the two mAbs where collaborators had managed to show a significant effect on AMPARs expression levels and mEPSC amplitude. The other mAb that was demonstrated to have an effect in this *in vitro* cortical neuron paradigm, H01, had not been trialled in any of my *in vivo* injections due to its unconvincing binding to cultures and tissue sections. Instead, mAb E07, another discoidin binder, was used, but showed only mild effects in the pilot experiment and no effects in later behavioural experiments.

Early in the design of these studies, my starting hypothesis was that it would be the discoidin domain-specific, rather than the L3-specific mAb, that would produce behavioural deficits *in vivo*, due to the seemingly obligatory binding of patient sera to this domain.[314, 338, 446] However, while both a discoidin-specific (H01) and an L3-specifc (E08) mAb disrupted AMPAR physiology in cortical neuronal cultures, only an L3-specifc mAb had its effects carried forward *in vivo*. This mAb was shown to produce a slightly over-active disinhibited phenotype, which I would be cautious to interpret as 'psychotic' or indicative of 'confusion'. However, a few questions and caveats do arise from this result, which will be discussed below.

Firstly, one could compare the results from mAb E08 IgG4 injection to those from Experiment 5 in Chapter 3, due to similar experimental design. It is apparent that at the 6 hours mark, the effects of the mAb and the polyclonal IgG derived from serum are nearly identical, leading to higher faecal pellet output, notable jumping behaviour, and a tendency to rear less or spend less time in the inner zone of the open field. However, with the purified IgG these effects wane off by the 9-hour mark, when no differences are observed from controls in the light-dark box, while for E08 the behavioural effects persist, albeit heading in the opposite direction. Notably, the 6-hour time point coincides with the histological findings, where deposition of the antibody and infiltration of neutrophils in the parenchyma was seen. While the credibility of the neutrophil finding is questionable, it could indicate that a neuroinflammatory process underlies the increased anxiety observed in CASPR2 antibody-injected mice 6-hours post-injection.

It is paramount that all of these results are interpreted with a healthy dose of scepticism, for the behavioural changes produced with E08 are the results or a surgical procedure which on its own could cause distress to an animal and henceforth affect performance on a task designed to test anxiety. As highlighted in Figure 5.11, both animals injected with E08 and A33 (a control mAb) spent less time in the inner zone of the open field than un-injected naïve mice, and mice who received A33 jumped significantly less than naïve controls, suggesting perhaps that the jumping behaviour produced by E08 is not indicative of pathology after all. This is however contradicted by the demonstration that E08-injected mice do rear significantly less and defecate significantly more than naïve animals, although this did not hold when they were compared to A33-controls in the ANOVA. While these results may be confusing, what they do point to is that inclusion of untreated controls, not just non-binding antibody controls, when studying the effects of mAbs which have not been tested *in vivo* is essential.

As per the *in vitro* studies, mAb E08 could modulate AMPAR function *in vivo*, albeit it is unclear whether this is in a gain-of-function or loss-of-function fashion. Previous work has shown that genetic ablation of the GluR1 subunit of the AMPA receptor can lead to locomotor hyperactivity in the open field as well as deficits in pre-pulse inhibition, collectively interpreted as 'schizophrenia-related' behaviours in mice.[449] While not identical to the behaviour observed in my experiments, this had led me to postulate that it is perhaps dysfunctional glutamatergic neurotransmission, mediated by reduced AMPAR currents and/or changes in AMPAR expression that produce the behavioural effects observed. Combined with the infiltration of polymorphonuclear cells, likely indicating a neuroinflammatory process, this could explain the seemingly discordant phenotypes observed within the same mice on different behavioural tests following E08 injection. An alternative interpretation is taking the two sets of results across the two time-points as jointly indicating a tendency for mice injected with a CASPR2-mAb to be hyperactive and ascribing this to AMPAR dysfunction, although the mobility parameters tested for in the open field would not support this.

The relationship between CASPR2 expression and AMPA receptor function has been studied previously and is discussed in the thesis introduction. Caspr2 is expressed in excitatory synapses in the cortex, and its silencing *in vitro* or *in vivo* decreases synaptic expression of AMPA receptors as well as the amplitude of AMPAR-mediated currents.[318, 339] These findings have been reinforced using purified IgG form CASPR2 patients, which decreased dendritic levels of CASPR2 and synaptic AMPAR trafficking, as well as perturbing excitatory synaptic transmission in the visual cortex.[318] An important caveat is that so far, all effects of CASPR2 antibodies on AMPA receptors have been studied in cortical neurons, the experiments discussed here included, whereas for our *in vivo* injections the focus of the lesion was always

the hippocampus. Whether effects observed in cortical neurons would extend to hippocampal ones remains to be seen, and the possible discrepancies between the two cell types might undermine the pathophysiological mechanism I proposed in the previous paragraph.

Another question raised in this chapter is why mAbs with seemingly identical characteristics fail to produce similar functional effects, as most notable in the case of E08 and E09, but also E07 and H01. Due to consistent evolution in the experimental protocols, the effects of these mAbs were never systematically compared using identical behavioural paradigms. However, even with the mEPSC recordings, which used an identical experimental setup for all antibodies, mAbs E08 and H01 produced effects that mAbs E09 and E07 did not recapitulate, despite binding the same domains, having overlapping epitopes, and comparable binding affinities and assay reactivities. Perhaps this suggests that our methods have not been sufficiently advanced to finely resolve antibody epitopes, hence obscuring any functionally relevant discrepancies, or that there are biophysical properties beyond K_D values and the simple 'yes or no' output of our screening assays that give insight into antibody potency. Of course, the role of isotype and subclass can't be ignored, but these are largely controlled for in my experiments by relying on either only IgG1 or only IgG4 antibodies. It is perhaps encouraging however, that the only mAbs that seemed to produce an effect *in vitro*: E07 (neutrophil recruitment in limited samples) and E08 (AMPAR levels and mEPSCs) were also the ones that led to any sort of behavioural change upon injection. What remains as a conundrum was why co-injection of two mAbs intended to model polyclonality observed in patient sera failed to produce a behavioural deficit. Perhaps this suggests that multiple domains across the CASPR2 protein, not just two, need to be targeted in disease, or perhaps it reminds us that antibody pathogenicity cannot be predicted based on in vitro screens, and the only way to ascertain this is to attempt to see how the mAb behaves once in a living organism.

The final question that will be discussed here will be the surprising observation of neutrophil infiltration following CASPR2 mAb injection. Although this was seen only in two mice, with a single image provided per mouse, and was not appropriately quantitated, it is interesting to consider from a neuroimmunological perspective. Neutrophils traffic to the CNS parenchyma following blood-brain barrier breakage during various neuroinflammatory states, including in multiple sclerosis, Alzheimer's disease, experimental autoimmune encephalomyelitis, and ischemic stroke, recruited by factors like TNF-α released by activated mast cells, P-selectin, very late antigen-4 (VLA-4), and LFA-1.[450-455] It is unclear how any of these factors could figure into our disease model, but it is possible that autoantibody deposition in the hippocampus activates tissue-resident macrophages or mast cells via Fcγ-receptor signalling, which release pro-inflammatory cytokines and activated complement components that recruit blood neutrophils from blood.

Neutrophils are thought to contribute to the pathogenesis of autoimmune CNS diseases like multiple sclerosis, their numbers and activity biomarkers prominently increasing with relapses and worsening of disability.[456, 457] Albeit not experimentally confirmed, the mechanisms that have been postulated to aggravate pathogenesis include neutrophil secretion of a diverse array of cytokines (IL-6, TNF-α, IL-1β, IFNγ) that reinforce an inflammatory state, and neutrophil-mediated BBB breakdown via the secretion of matrix metalloproteinases (MMP-9).[450] However, none of these alleyways nor the contribution of neutrophils have been investigated within the context of autoantibody-mediated neurological diseases. In human studies, there has been a single report on the presence of polymorphonuclear cells in post-mortem brains of two patients with CASPR2-antibodies who had medial temporal lobe epilepsy with hippocampal sclerosis, along with infiltration of T-cells and macrophages.[305] Although this limited report in

a small number of patients does not add much weight to the argument, it is worth appreciating that neutrophils have a very short half-life of about 7 hours and as highlighted above, may only contribute to disease at specific stages such as prior to onset or relapses.[450, 458] Therefore, even if they do play a yet-undiscovered role in pathology, this may only coincide with relapses or wane in patients with active disease by the time they are deceased and their tissue can be studied post mortem. Further studies in animals might be needed to substantiate a role of PMN cells in this family of diseases, as well as to elucidate why targeting CASPR2, but not a related brain antigen like LGI1 is associated with neutrophil infiltration.

In summary, throughout this chapter I have presented and discussed a comprehensive, if not always successful effort to understand whether and how CASPR2-specific antibodies could lead to CNS pathology. This represents a continuation of the work presented in Chapter 3, as an attempt to demonstrate a successful passive transfer animal model for Caspr2-autoantibody pathogenicity and untangle some of the underlying molecular mechanisms. The roles of AMPA receptors and innate immune cells have been implicated, although one might argue how convincingly, and the relevance of domain specificity, epitope, affinity, binding across multiple screening assays and, to some extent, subclass, has been addressed at various lengths. Thinking about the implications of this work, it is important to highlight that many of the screening assays mentioned in this chapter are also often used as diagnostic tests for antibodies. The fact that what the results from these tests would imply are 'best candidates' for pathogenicity does not always necessarily align with what's observed upon injection of the antibodies into animals serves as an important reminder that antibody attributes alone do not predict disease severity, as implied from the findings in Chapters 2 and 3. Nonetheless, my hope is that this chapter constitutes reasonably strong evidence for why investigating patient-derived monoclonal antibodies is a worthwhile effort, as it allows us to ask questions and investigate pathogenic mechanisms in a manner that we couldn't with polyclonal serum alone. Indeed, many of the questions and hypotheses we have examined in this chapter would not have been amenable to work with the polyclonal sera. In chapter 5, I will turn my focus away from the antibody itself and toward the main 'culprit' for autoantibody production, the autoreactive B-cell.

CHAPTER 5

STUDYING CASPR2-REACTIVE B-CELLS IN PATIENTS WITH CASPR2-ANTIBODY ENCEPHALITIS

Preface

The use of single B-cell technologies is rapidly changing the face of autoimmune neurology by allowing an unprecedently in-depth look at the very rare but potentially fatal autoreactive B-cells that give rise to disease.[435] At the time of writing of this thesis, no prior published work exists characterising the autoreactive B-cells in Caspr2-antibody encephalitis. It thus remains unclear how B-cells producing CASPR2-autoantibodies escape innate tolerance checkpoints that would otherwise serve to eliminate them. More research has been done on the underlying immunobiology leading to the production of autoantibodies against other neuronal targets. This will be discussed in the Chapter 5 introduction to demonstrate the value in studying these rare antigen-specific B-cells.

INTRODUCTION

Isolation of single B-cells and their respective expansion, activation, and maturation *in vitro* can provide insight into how autoreactive B-cells get sensitised to their target antigens and the unique differences in genotype, phenotype, and secreted antibodies that separate B-cells in patients from those in healthy donors. Furthermore, *in vitro* single B-cell culture experiments could be useful in characterising BCRs and development of clonal B-cell functional assays. Multiple attempts at this have been made across several disease modalities. Luo engineered a culture system of B-cell maturation and differentiation using MS40L cells expressing stable low levels of human CD40L, whose interaction with CD40 on B-cells plays a major role in the control of humoral and cellular immune responses and enhances B-cell proliferation *in vitro* and *in vivo*.[459, 460] Cytokine stimulation of this co-culture system results in maturation and differentiation of B-cells into terminally differentiated plasma cells.[461, 462] This system has had wide-ranging applications, from vaccine development via generation of HIV-neutralizing antibodies, to isolation of influenza-specific memory B-cells to characterise the BCR repertoire, to isolating and cloning individual HLA-specific B-cells from patients sensitised by pregnancy and transplantation to better understand antibody-mediated allograft rejection.[459-464]

In autoantibody-mediated neurological diseases, it is believed that antigen-specific precursor B-cells give rise to autoantibody-secreting plasma cells in the periphery, largely based on the observation that median concentration of target-specific autoantibodies in patients sera is 4-fold to 640-fold higher than that in CSF. While the exact sites of autoimmunisation of the precursor B-cells remain undefined, this notion has been further substantiated by detection of circulating autoantigen-specific B-cells in patients with neuromyelitis optica spectrum disorder (NMOSD), LGI1- and NMDAR-antibody encephalitis, MOG antibody-associated disorders (MOGAD), and autoimmune myasthenia gravis, implicating peripheral sensitisation to antigen accompanied by a failure of B-cell tolerance.[222]

In the case of NMOSD, patient-derived naïve and memory B-cells, but not B-cells from healthy donors, were found to secrete AQP4 antibodies ex vivo, indicating existence of AQP4-reactive B-cells both before and after germinal centre maturation.[395] A different study found that when plasma cell-derived AQP4-mAbs were back-mutated to their unmutated common ancestors (UCAs), the UCAs lost their ability to bind AQP4, but did exhibit some non-AQP4 autoreactivity.[438] Taken together, these studies at the single B-cell level suggested a failure of both central and peripheral tolerance in NMOSD. The medical relevance of this finding is that the persistent generation of autoreactive naïve B-cells and their emigration into the periphery may give way to their recurrent activation and disease relapse. The significance of this is further substantiated by the effectiveness of B cell-modulating therapies such as rituximab in this family of diseases, even as high serological autoantibody levels persist.[224, 241, 243, 285, 465, 466]

In MOG-antibodies associated disease, screening of adult and paediatric seropositive patients for MOG protein reactivity using cell-based assays has helped identify several target epitopes. However, experiments where B-cells were differentiated into antibody-secreting cells (ASCs) suggested more epitopes than those routinely detected by screening sera existed in patients.[397, 467, 468] In patients with autoantibody-mediated MuSK myasthenia gravis who relapsed post-treatment with rituximab, single-cell transcriptional and BCR profiling on longitudinal B-cell samples identified clones present before therapy that persisted during relapse.[253, 469] These persistent B-cell clones included antibody-secreting cells and memory B-cells, a significant subset of which were specific for the MuSK autoantigen and were characterised by gene expression signatures associated with promotion of B-cell survival. Here, the single cell studies

helped demonstrate that rituximab was not fully effective in eliminating autoantibody-producing B-cells long-term, thus providing a mechanistic understanding of post-rituximab relapse in MuSK myasthenia gravis.

In NMDAR-antibody encephalitis, single-cell cloning of full-length immunoglobulin heavy and light chain genes has helped generate a panel of recombinant monoclonal antibodies to the NR1 subunit from CSF memory B-cells and ASCs in patients with the disease.[400] This revealed that while some NR1-reactive cells carried somatically mutated immunoglobulin genes and had undergone class-switching to immunoglobulin G and clonal expansion, a fraction of NR1 antibodies were unmutated, thus resembling naturally occurring (non-pathogenic) antibodies. This indicated that, while present, somatic hypermutation was not essential to the generation of functional NR1 autoantibodies and instead there was a degree of impaired central tolerance against NMDAR, implicating multiple B-cell subsets and tolerance checkpoints in disease pathogenesis. In LGI1-antibody encephalitis, single B-cell studies on patient CSF samples revealed that 84% of ASCs and 21% of memory B-cells in CSF expressed LGI1-reactive antibodies which had undergone affinity maturation, whereas reactivities to other brain epitopes were rare.[399] This suggested intrathecal proliferation of B-cells and maturation of the immune response to LGI1, despite patients usually having normal CSF cell counts and protein levels and no oligoclonal IgG bands. The clinical significance of this is that aggressive immunotherapy may not be eliminating the small but significant numbers of LGI1-reactive B-cells intrathecally, which would not be detected via routine diagnostic testing or targeted via conventional therapies.

Evidently, there is merit in studying antibody-mediated neurological disorders using single cell technologies: it can allow us to examine defects in B-cell tolerance checkpoint fidelity, narrow down the site of disease initiation by examining antigen-reactivity in specific B-cell populations at various stages of the developmental pathway, and finally, it can lead us to interrogating the binding specificity and affinity of resultant antibodies via the generation of recombinant mAbs (Chapter 4). Pinpointing the B-cell subsets responsible for disease-specific antibody production is not only scientifically but also clinically relevant, as it can allow for more specific targeting of the pathogenic B-cell populations with directed immunotherapies, as opposed to the broad immunosuppression currently in use. Several B-cell populations have been implicated as culprits in the pathogenesis of antibody-mediated neurological disorders, among them new emigrant (transitional), naïve, traditional- and double negative memory B-cells, as well as plasmablasts and plasma cells.[470]

Earlier studies focused on characterising antigen-specific monoclonal antibodies from primary infections, vaccination, and autoimmunity largely relied on techniques such as antibody phage display (APD), hybridoma technologies, and amplification of paired heavy and light chain genes from sorted B-cells.[222, 435] The latter has been highly favoured in the case of autoimmune diseases with rare autoantigen-specific B-cells as it allows for an unbiased approach with reasonably high throughput representative of *in vivo* conditions. However, an inherent challenge is the generation of a massive library of antibody sequences requiring individual expression as mAbs in a mammalian cell culture system to screen for target reactivity and identify the few cells specific for the target antigen. A strategy used to circumvent this involves using a soluble recombinant target antigen to stain surface BCRs and thus 'bait' out antigen-specific B-cells during the single cell sort, allowing for their enrichment in culture. This has been insofar attempted for the MuSK and AchR antigens.[471, 472]

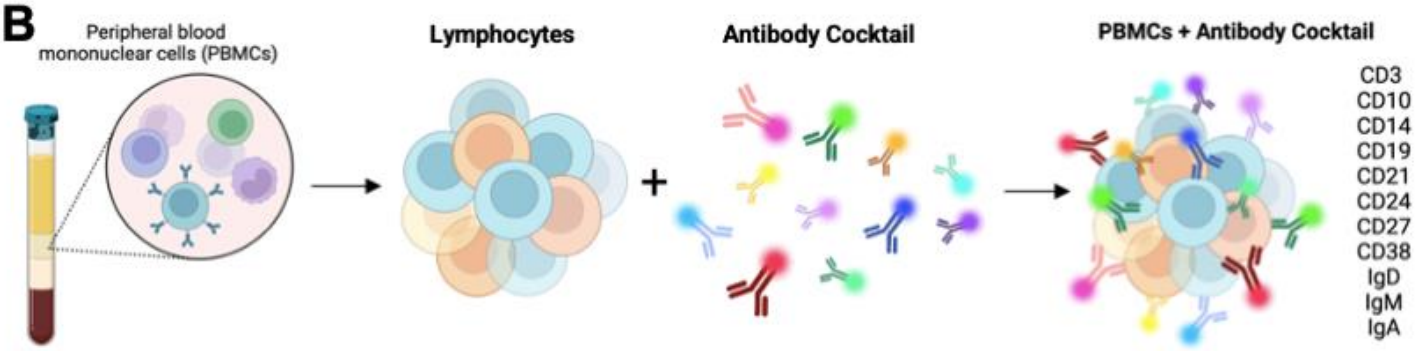
Whilst effective when dealing with rare cells reactive to neuroglial surface antigens, the baiting method is not without its limitations. Firstly, many of the neuroglial membrane proteins targeted in CNS autoimmune disease, CASPR2 included, are complex multi-domain integral cell adhesion molecules or ion channels, leading to concerns about their structural fidelity and stability when expressed as a soluble recombinant protein. Different types of antigen labelling reagents can be used when constructing these 'baits' including fluorescent tags and biotin-streptavidin complexes,[473, 474] which can themselves be the target of promiscuous polyreactive BCRs, thus reducing the specificity of the bait. Finally, considering high affinity binding is a necessity for 'fishing out' the autoreactive B-cell during the single-cell sort, it is possible that this method misses out naïve and anergic B-cells with low affinity BCRs, which is an important population in the study of B cell-mediated autoimmunity. One way to overcome these challenges is to use a 'double bait' system with different fluorescent tags, thus increasing the likelihood that the B cells isolated genuinely bind the antigen of interest. This can be paired with *in vitro* culturing and differentiation of single B-cells into antibody secreting cells, thus allowing to screen for BCR reactivities and affinities prior to downstream antibody cloning. This has been the approach adopted in this thesis.

In this chapter, I will cover an effort to investigate the frequencies and characteristics of pre- and post-germinal B-cell subsets, as well as the properties of the antibodies they secrete. Interrogation of this kind is crucial to pinning down the earliest point of autoantibody genesis of which none is known in the context of CASPR2 autoantibody-associated disease. Studying the CASPR2 reactivity of IgD+CD27- naïve B-cells and IgD-CD27+ class-switched memory B-cells may help to indirectly examine the integrity of pre- and post-germinal centre immune tolerance checkpoints.[475] To this end, existing single-cell B-cell culture protocols were adapted to sort single naïve and memory B-cells and co-culture them with the 3T3-msCD40L feeder cell line in the presence of select cytokines (IL-2, IL-21, IL-4, BAFF), inducing proliferation and differentiation into antibody-secreting cells (ASCs). Four CASPR2 patients and five healthy controls were selected. Importantly, two of the CASPR2 patients had been untreated at the time of sample collection, allowing for a perspective on BCR reactivity and features in the absence of immunotherapy in CASPR2-antibody encephalitis.

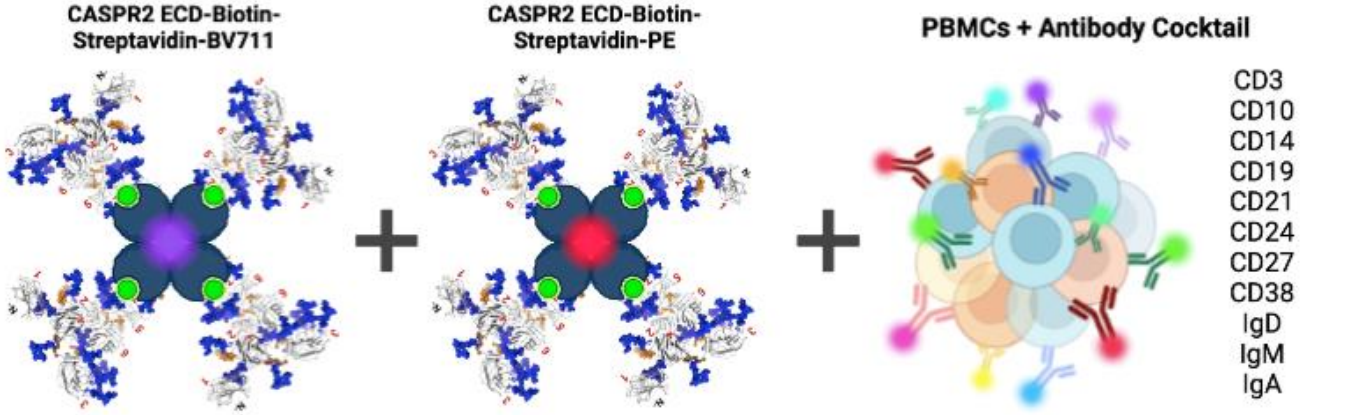
A

	CASPR2 AUTOANTIBODY PATIENTS				HEALTHY CONTROLS				
ID Number	**JR794**	**JR1284**	**JR1300**	**JR1307**	**JR846**	**JR1006**	**JR1179**	**JR1402**	**JR1408**
Age	77	73	69	67	75	63	74	90	63
Sex	Male	Male	Male	Male	Male	Female	Male	Male	Female
DRB1*11:01	No	Heterozygous	No	Homozygous	No	No	No	Heterozygous	No
Clinical Phenotype	Limbic Encephalitis	Limbic Encephalitis	Limbic Encephalitis	Limbic Encephalitis	Healthy Control	Healthy Control	Healthy Control	Healthy Control	Healthy Control
Symptoms	Seizures, Ataxia, Pain, Memory Disturbance	Seizures, Ataxia, Memory Disturbance	Seizures, Memory Disturbance	Seizures, Ataxia, Memory Disturbance	N/A	N/A	N/A	N/A	N/A
Disease Duration	2 years	3 years	1.5 years	1 year	N/A	N/A	N/A	N/A	N/A
Treatment	Untreated	Untreated	Steroids	CP + RTX	N/A	N/A	N/A	N/A	N/A

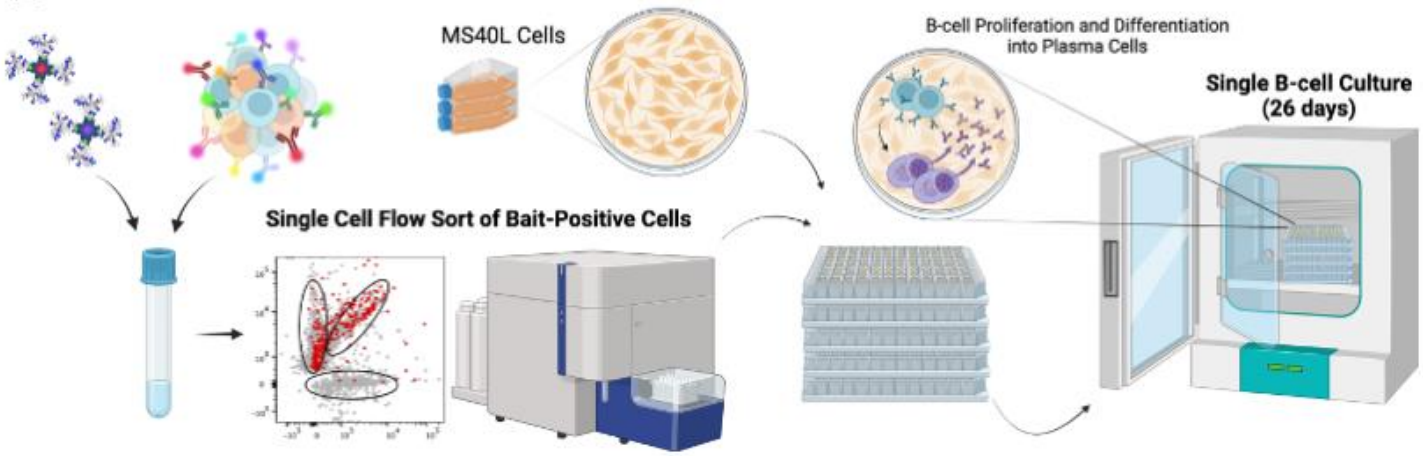
B



C



D



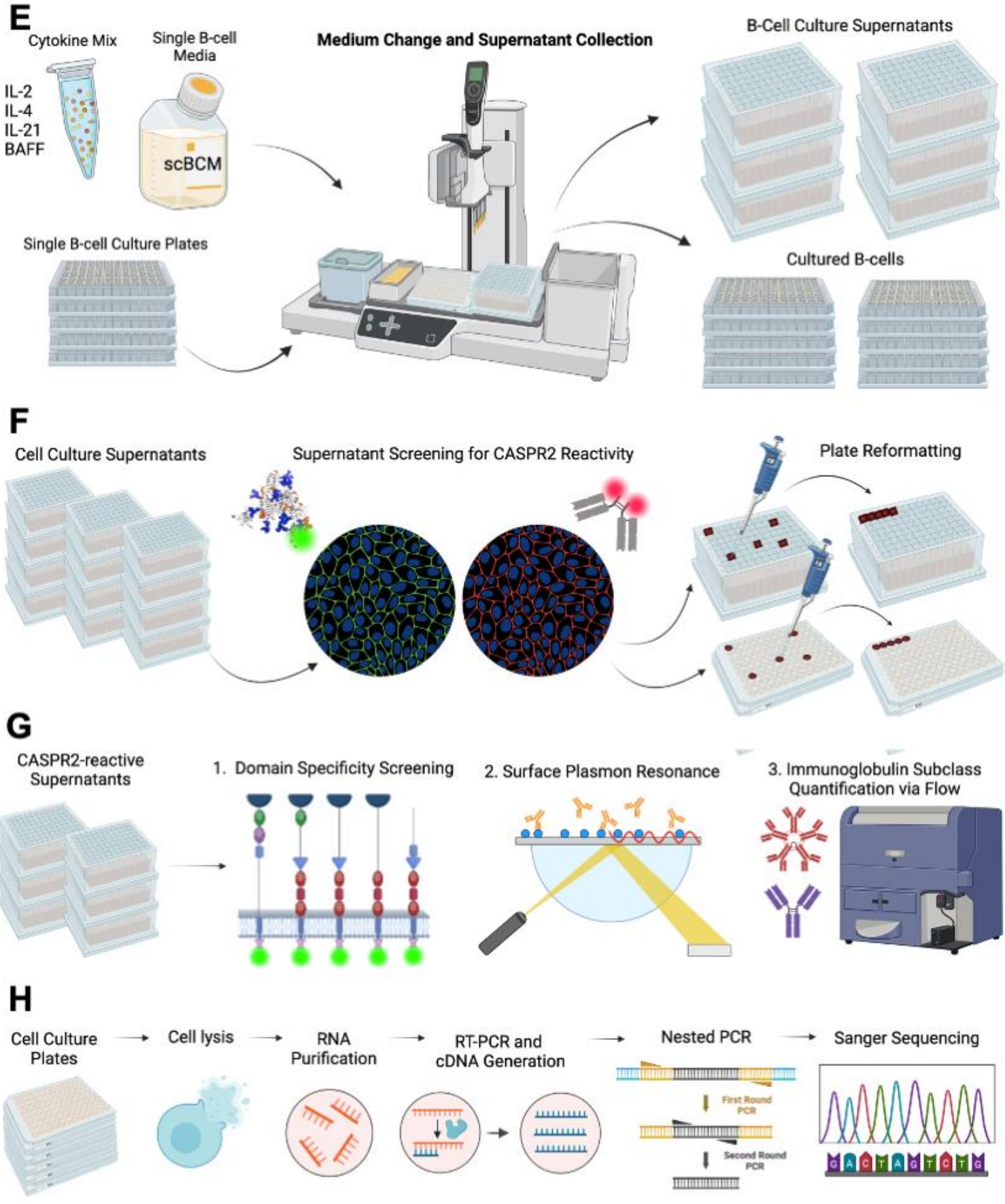


Figure 5.1 Illustrated protocol of the CASPR2 Bait experiment (A) Summary of CASPR2 patients and healthy controls included in the study. (CP – cyclophosphamide; RTX – rituximab) (B) PBMC labelling for key immune cell markers (C) Design of CASPR2-bait and antibody cocktail (D) Fluorescence-activated cell sorting and single B-cell culture (E) Media change on single B-cells and supernatant collection (F) Supernatant screening for CASPR2 reactivity and reformatting of supernatants (and later culture cell lysates) to yield plates of only CASPR2-reactive supernatants (G) Screening supernatants for domain specificity using CASPR2 deletion constructs, measuring affinity using surface plasmon resonance and determining immunoglobulin composition using flow cytometry (H) B-cell culture plates were lysed, followed by RNA purification, RT-PCR, nested PCR and Sanger sequencing of variable heavy and light chain regions of the BCR.

METHODS

Patient Samples

Blood samples from four patients referred to the Oxford Autoimmune Neurology clinic were collected at the John Radcliffe Hospital, Department of Neurology after informed consent was obtained under research ethics approval committee (REC reference 16/YH/0013, IRAS Project ID: 195152). De-identified blood samples from the patients and five age- and sex-matched healthy controls were collected into 9ml sodium-heparin tubes (Greiner Bio-One) and processed for peripheral blood mononuclear cells (PBMCs) isolation, as described in Chapter 4. Principal features of the patients, including sample number, age, sex, treatment status, HLA-DRB1*11:01 status, and clinical phenotype are summarised in Figure 5.1A.

Culturing ms40L Cells

B-cells were co-cultured in 96 well-plates pre-coated with murine stromal cells engineered to express stable low levels of human CD40L (MS40L cells), developed in Dr Baltimore's lab and kindly donated by Dr Garnett Kelsoe for our experiments (Figure 5.1D). The MS40L cells were prepared as described previously.[459] Briefly, MS40L cells stored at -150°C in freeze media (10% DMSO in FBS) were thawed in a water bath and resuspended in 2ml of Iscove's Modified Dulbecco's Medium (IMDM; 21056-023 Invitrogen) supplemented with 10% FCS, 1% Penicillin-Streptomycin, and 55μM β-mercaptoethanol. Cells were then plated at a density of 1 x 10^6 cells in a T25 cell culture flask (ThermoFisher), cultured at 37°C and 5% CO_2 for 3 weeks and passaged twice a week until they reached sufficient numbers for seeding. Two days before the single B-cell sort, MS40L cells were harvested by removing culture media, washing with 5ml PBS, and then treating with 1ml Trypsin (10% in PBS) for 5mins at room temperature to detach the cells. Cells were washed with supplemented IMDM and once pelleted down, resuspended in complete B-cell media, and counted. Finally, to be used as a feeder co-culture, the MS40L cells were plated in flat-bottom 96-well culture plates using an Assist Plus® Pipetting Robot (Integra) at a density of 1,000 cells/ml in 100 μl/well of complete B-cell media and grown at 37°C and 5% CO_2 for 48h prior to the single-cell sort.

Cell Labelling and Fluorescence-Activated Cell Sorting

A total of three single B-cell sorts were performed. First, PBMC suspensions from patients and healthy controls ranging in volume from 500-700μl and containing circa 1.0 x 10^7 cells per sample that had been previously stored in liquid nitrogen were thawed by gently flicking in lukewarm water, washed in RPMI buffer, and resuspended in 10ml FACS buffer (PBS with 3% FCS and 2mM EDTA). To expedite sorting, CD3-expressing T-cells were depleted from the PBMCs samples using Dynabeads™ CD3 (Invitrogen). The T-cell-depleted PBMCs were washed in FACS buffer, and the cell suspension was incubated with the CASPR2 Bait. This consisted of a biotin-tagged soluble extracellular domain of the human Caspr2 protein complexed with streptavidin (SA) conjugated to either a phycoerythrin (PE) or a Brilliant Violet 711 (BV711, Thermo Fisher) fluorophore, as illustrated in Figure 5.1C. The biotin-streptavidin complexes (illustrated as tetramers, but possibly also forming other types of molecular arrangements) were formed by incubating CASPR2-Biotin separately with each streptavidin-fluorophore construct for 20mins on ice prior to combining both baits with the PBMCs. An alternative protocol featured combining the CASPR2 soluble protein with SA-PE and SA-BV711 prior to adding them to the PBMCs suspension already containing biotin and incubating for 10mins prior to staining; the latter version was found to be more effective. PBMCs were incubated with 20-28μl of the C2-Bait preparation, followed by washing with FACS buffer supplemented with normal mouse serum (NMS) and resuspension in 100-150μl of the antibody mix (Figure 5.1B), whose components are summarised below.

Table 5.1 Cell Markers and Antibodies Used in Single Cell Sort

Cell Marker	Antibody Clone	Fluorophore	Supplier	Dilution
CD3	UCHT1	Pacific Blue	BioLegend	1:100
CD14	HCD14	Pacific Blue	BioLegend	1:100
IgA	IS11-8E10	VioBlue	Miltenyi Biotec	1:100
CD21	B-ly4	BV510	BD Biosciences	1:50
CD27	O323	BV605	BioLegend	1:20
CD10	HI10a	PE-Dazzle	BioLegend	1:50
CD24	ML5	PE-Cy7	BD Biosciences	1:50
IgD	IA6-2	FITC	BioLegend	1:20
IgM	MHM-88	AF647	BioLegend	1:10
CD38	HIT2	APR-R700	BD Biosciences	1:100
CD19	SJ25C1	APC-Cy2	BD Biosciences	1:20

The cell markers are summarised in Table 5.2.[475] Following staining, cell suspensions were further diluted in FACS buffer with DAPI, washed and resuspended in PBS with 3% FCS + EDTA and filtered to remove clumps prior to sorting. Cells were sorted on a FACS Aria III where dead cells and unwanted populations were removed by first gating on CD3/CD14/IgA/DAPI prior to cell sorting, after which B-cells within gates corresponding to naïve ($CD19^{+}IgD^{+}IgM^{+}CD27^{-}$), class-switched memory ($CD19^{+}IgD^{-}IgM^{-}CD27^{+}$) and immature B-cells ($CD10^{+}CD21^{+}CD24^{hi}CD38^{hi}$) were positively sorted into flat-bottom 96-well plates lined with MS40L cells and cultured in 150µl/well of enriched single cell B-cell media (scBCM) supplemented with cytokines IL-2, IL-4, IL-21 and BAFF. (Figure 5.1D)

Table 5.2 Cell Markers Used in CASPR2 Bait Experiment

Marker	Expression	B-cell Developmental Stages
CD3	T-cells	n/a
CD10	B- and T-cell precursors, neutrophils	Pre-Pro, Pro-, Pre-, Immature
CD14	Monocytes, macrophages, granulocytes	n/a
CD19	B-cells, Follicular dendritic cells	All except plasma cells
CD21	B-cells, Follicular dendritic cells, T-cells	Immature, Transitional, Follicular
CD24	B-cells, granulocytes, thymocytes	Pro-, Pre-, Immature, Transitional
CD27	B-cells, T-cells, thymocytes, NK cells	Activated, Germinal, Plasma cell, Memory
CD38	Plasma cells, B-cells, T^{ACT} cells	All except memory and follicular B-cells
IgD	Mature naive B-cells	Follicular, activated, germinal centre
IgM	Mature B-cells	All stages past immature B-cell
IgA	Mature B-cells	Post-activation

Table 5.2 Summary of markers used in CASPR2 Bait experiment, their expression across immune cell subsets and B-cell developmental stages.

Single Cell Culture and Supernatant Collection

Cells were cultured at 37°C and 5% CO_2 for a total of 26 days. On day 4 of culture, wells were topped up with another 50µl/well of scBCM, consisting of RPMI 1640 Medium without glutamine and phenol red supplemented with FBS, sodium pyruvate, HEPES, GlutaMAX™, Penicillin-Streptomycin, MEM Non-Essential Amino Acids Solution, and β-mercaptoethanol (all reagents from ThermoFisher). (Figure 5.1E) Prior to being added to the B-cell cultures, the following recombinant human cytokines (all supplied by Peprotech) were added to the scBCM: IL-21 (#200-21), IL-2 (#200-02), IL-4 (#200-04) and BAFF (#310-13), for a final concentration of 50 ng/ml (IL-2) or 10 ng/ml (all others). Starting from day 8 of culture and continuing every 4 days for the culture duration, ~100µl/well of cell supernatant was removed and replaced with an equal volume of freshly prepared complete scBCM and cytokines. The supernatants were stored in 96-well DeepWell™ plates (FisherScientific), maintaining well order and pooling the supernatant for each well collected across the culture period. On day 26 of culture the remaining 190µl/well of culture supernatant was harvested prior to cell lysis.

Supernatant Screening

Supernatants were screened for human CASPR2 reactivity at a 1:5 dilution using a HEK293T live CBA as described in previous chapters. However, the secondary antibody used here was an AlexaFluor® 594-conjugated AffiniPure $F(ab')_2$ specific for human IgA, IgG and IgM (109-586-064; Jackson ImmunoResearch), diluted 1:750 in DMEM/HEPES/ BSA. (Figure 5.1F) Results were read by three independent observers and the positive wells were selected for further B-cell receptor sequencing. In addition to this, supernatants were screened for reactivity to LGI1 and untransfected HEK cells. To determine domain specificity, supernatants were screened on CASPR2 domain deletion constructs donated by the Bennett Lab and generated by Dr Teodora Trendafilova (University of Oxford) (Figure 5.6A-B). Surface plasmon resonance and flow cytometry were used by collaborators to determine antibody affinity and subclass respectively (Figure 5.1G). The results of the latter will not be featured in this thesis.

Cell Lysis

All remaining steps of the protocol are summarised in Figure 5.1H. On culture day 26, single-cell culture plates were spun down (5mins, 300g), supernatants were harvested, and cell pellets were resuspended in 50µl/well of buffer RLT, a cell lysis buffer for RNA isolation (#79216, QIAGEN) with β-mercaptoethanol added to inactivate RNAses in the lysate. Plates were vigorously shaken back and forth in all directions while being kept flat for 20s, sealed with VWR® Aluminium Foil Seals (#391-1282) and kept at -80°C until after supernatants had been screened for Caspr2 reactivity. Once positive supernatants had been identified, the corresponding lysate wells were selectively thawed, and their contents transferred to new 'Reformatted Plates' containing only the CASPR2 CBA-positive wells which were used for downstream RNA extraction and BCR sequencing.

RNA Isolation

To isolate RNA from the lysed B-cells, a RNeasy 96 Kit (#74181, QIAGEN) was used in conjunction with the QIAvac 96 vacuum manifold for processing 96-well plates (#19504, QIAGEN), as per the manufacturer's instructions. Prior to starting the extraction, cell lysates were further diluted in 100 µl/well of buffer RLT without β-mercaptoethanol and 150 µl/well of 70% ethanol. Samples were then loaded into the RNeasy 96 plate, and a 900mbar vacuum pressure was applied. Following washes with the buffers supplied in the kit, RNA was eluted after a 5mins incubation with 45 µl/well RNAse-free water and 5min spin at 6000rpm, a step that was repeated twice to maximise yield. Maximum care was taken throughout to prevent

RNAses contamination, and the plates with eluted RNA were sealed with Aluminium Foil Seals and stored at -80°C until further use for downstream RT-PCR.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)
Prior to starting RT-PCR, RNA plates were thawed on ice, and the random primer mix was prepared consisting of 2.5 µl/well Random Primers (#S1330S, NEB; final concentration 6 µM), 0.25 µl/well CH5 (#200) universal immunoglobulin gene reverse primer (Eurofins, 20 nM working concentration), and 1.75 µl/well of RNAse-free H_2O (#B1500S, NEB). Then, 4.5 µl/well of the random primer mix was combined with 30 µl/well of isolated RNA, plates were sealed, centrifuged for 1min at 400g and 4°C in a benchtop centrifuge, incubated for 5min at 65°C in a preheated thermocycler and finally placed on ice for 10mins. The SuperScript™ RT Mastermix was freshly prepared in a PCR hood on ice, containing the following: 10 µl/well 5X SuperScript™ IV RT Reaction Buffer (#18090050B, Invitrogen), 2.5 µl/well 10 mM dNTP Mix (#U1511, Promega), 2 µl/well IPEGAL® CA-630 (#18896, Merck; 25%), 0.5 µl/well RNAsin (#N2518, Promega), 1 µl/well 0.1 M DTT (Invitrogen), and 1 µl/well of SuperScript™ IV RT Enzyme (#18090010, Invitrogen), which was added last and kept on ice before use. The plates containing RNA and Random Primer Mix were then combined with 15.5 µl/well of the SuperScript™ Mastermix for a final volume of 50 µl/well. Plates were sealed with Adhesive PCR Plate Seals (#AB0558, Thermo Scientific), centrifuged again for 1min at 400g and 4°C and placed in a thermocycler where the following program was run: 25°C for 10mins, 50°C for 60mins, 85°C for 10mins, 10°C on infinite hold. The cDNA generated was diluted 1:100 to be used as a working stock for downstream nested PCR.

Nested PCR, Purification, and Sequencing
To amplify variable immunoglobulin gene heavy chains (IGH) and both possible light chains (IGK and IGL), a two-step nested PCR strategy was performed for each chain, where cDNA generated in the RT-PCRs was used as template for the first PCR step (PCR1) and the PCR1 reaction products were used as the starting reagent for the second PCR step (PCR2). Thus, a total of six PCR reactions was performed per reformatted plate: PCR1 and PCR2 for each of IGH, IGK, and IGL. Prior to starting the PCRs, 200 µl primer aliquots specific to each of the six reactions were prepared where primers were diluted to a working concentration of 5 µM in nuclease-free H_2O (#129114, QIAGEN). Each PCR step was performed using 10 µl/well of GoTaq® G2 Green MasterMix (#M7823, Promega), 2 µl/well of specific primer mix, 6 µl/well of nuclease-free H_2O and 2 µl/well of template DNA. Following nested PCR completion for each of IGH, IGK, and IGL, 2 µl/well of PCR2 reaction product was used to visualise DNA using E-Gel® 96 pre-cast 2% agarose gels with SYBR® Safe DNA gel stain (#G7208-02, Invitrogen) in conjunction with the E-Base™ Electrophoresis Device (#EB-M03, Invitrogen), as per the manufacturer's instructions. DNA electrophoresis bands were visualised under UV light on a ChemiDoc Imaging System (Bio-Rad), and sample gel images are shown in Figure 5.16. The remaining PCR2 product (~18 µl) was then purified using a GenElute™ 96 Well PCR Clean-Up Kit (#PCR9604, Sigma-Aldrich), which removes primers, primer-dimers, nucleotides, salts, and polymerase. Purified PCR products were sent for Sanger Sequencing (Source BioScience). Where sequencing results did not come back of sufficient quality, this was assumed to be due to the presence of multiple gel bands, and the respective cDNA wells were thus subjected to another round of nested PCR, this time followed by gel excision of the bands visualised at 300-500bp and purification using the Monarch® DNA Gel Extraction Kit (#T1020L, NEB). For the heavy chain PCRs specifically, where no DNA band was visible, the respective cDNA wells were sequenced using IGHM gene-specific reverse primers.

Data Analysis

Sequencing data analysis was performed by Dr Adam Handel (University of Oxford). Sanger sequencing calls were first run through IgBLAST (Basic Local Alignment Search Tool), an online tool for immunoglobulin variable domain sequence analysis (NCBI). Sequences were then integrated with FACS data to generate Seurat object plots containing multiple BCR sequence properties such as V/D/J gene usage and mutation rate in the meta-data. Based on several FACS parameters (FSC-A, FSC-H, SSC-A, SSC-H, CD19-APC-Cy7, and CD3-CD14-IgA), Caspr2-positive wells were matched to control Caspr2-negative wells for both patients and healthy controls, which also had their BCRs sequenced as a form of in-patient and in-healthy donor control. Dimensionality reduction was also performed on chosen FACS markers using principal component analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP).

RESULTS

Screening of B-cell supernatants justifies the use of two bait constructs

Following day 26 of culture, collected B-cell supernatants were screened for reactivity to CASPR2 and LGI1. A total of 535 CASPR2-positive supernatants were identified out of the 3664 cells sorted (14.6% of sorted cells), with somewhat fewer positive cells from healthy controls (239/535 or 44.7%) than from patients (296/535 or 55.3%). To understand how these CASPR2-reactive cells related to the C2-bait constructs used, the identity of the positive wells was traced back to the parent B-cells from the flow sort. This revealed that in both patients and in healthy controls, most bona fide CASPR2-positive cells were reactive to both the C2-BV711 and C2-PE bait constructs (Figure 5.2A-B). In patients, some CASPR2-positive cells came from the C2-PE channel only, whereas in both patients and healthy controls there were a few cells that came from the C2-BV711 channel only. By comparison, LGI1-positive cells were seen very rarely in healthy controls and somewhat more prominently in CASPR2 patients, albeit limited to the C2-PE bait channel (Figure 5.2C-D). In fact, all but one of the LGI1-positive wells (defined as CBA score > 0.5) came from a single patient, JR1284. Importantly, upon CASPR2 deletion construct screening, most LGI1-reactive supernatants were found to indiscriminately react to all deletion constructs, indicating likely non-specific binding to HEK cells rather than the antigen of interest. Overall, identifying most of our CASPR2-CBA positive supernatants as hailing from cells in the 'double positive' bait channel justified our use of two C2-bait constructs, which likely increased our chances of finding cells with specific reactivity to the CASPR2 antigen rather than the other molecular moieties present in our bait construct like streptavidin, biotin, and the fluorophores BV711 and PE.

CASPR2-reactive B-cells come from distinct subsets in patients versus healthy controls

Next, I examined the identity and phenotype of the B-cells that produced our CASPR2- and LGI1-positive supernatants. The CASPR2- and LGI1-positive B-cells were identified on FACS plots depicting the IgD vs CD27 signal, traditionally used to distinguish naïve from memory B-cell populations (Figures 5.3 and 5.4). This led to a very neat observation that in all healthy controls the CASPR2-reactive B-cells hailed from the naïve (CD27-IgD+) B-cell compartment, with very rare minor presence in the switched memory (CD27+IgD+) B-cell compartment, as seen in HC 1006 and HC 1408 (Figure 5.3). By contrast, in patients, CASPR2-reactive B-cells were found in both the naïve and memory B-cell compartment (Figure 5.4). Furthermore, the LGI1-reactive (likely non-specific) B-cells identified for patient JR1284 also hailed from the memory B-cell compartment. Pooling together these results for all healthy controls versus all patients revealed that in healthy controls nearly all CASPR2 positive wells came from the naïve B-cell compartment, with very minor presence in the switched memory (CD27+IgD-), IgM memory (CD27+IgD+) and late memory (CD27- IgD-) subsets (Figure 5.5A). By contrast, among patients there was prominent presence of CASPR2-positive cells within all four subsets (Figure 5.5B). Superimposing the identity of these cells on tSNE plots based on multi-dimensional FACS data also reflected this observation, where the positive wells in healthy controls were seen to cluster together more than they did in patients, where they had a more widespread distribution (Figure 5.5C-D)

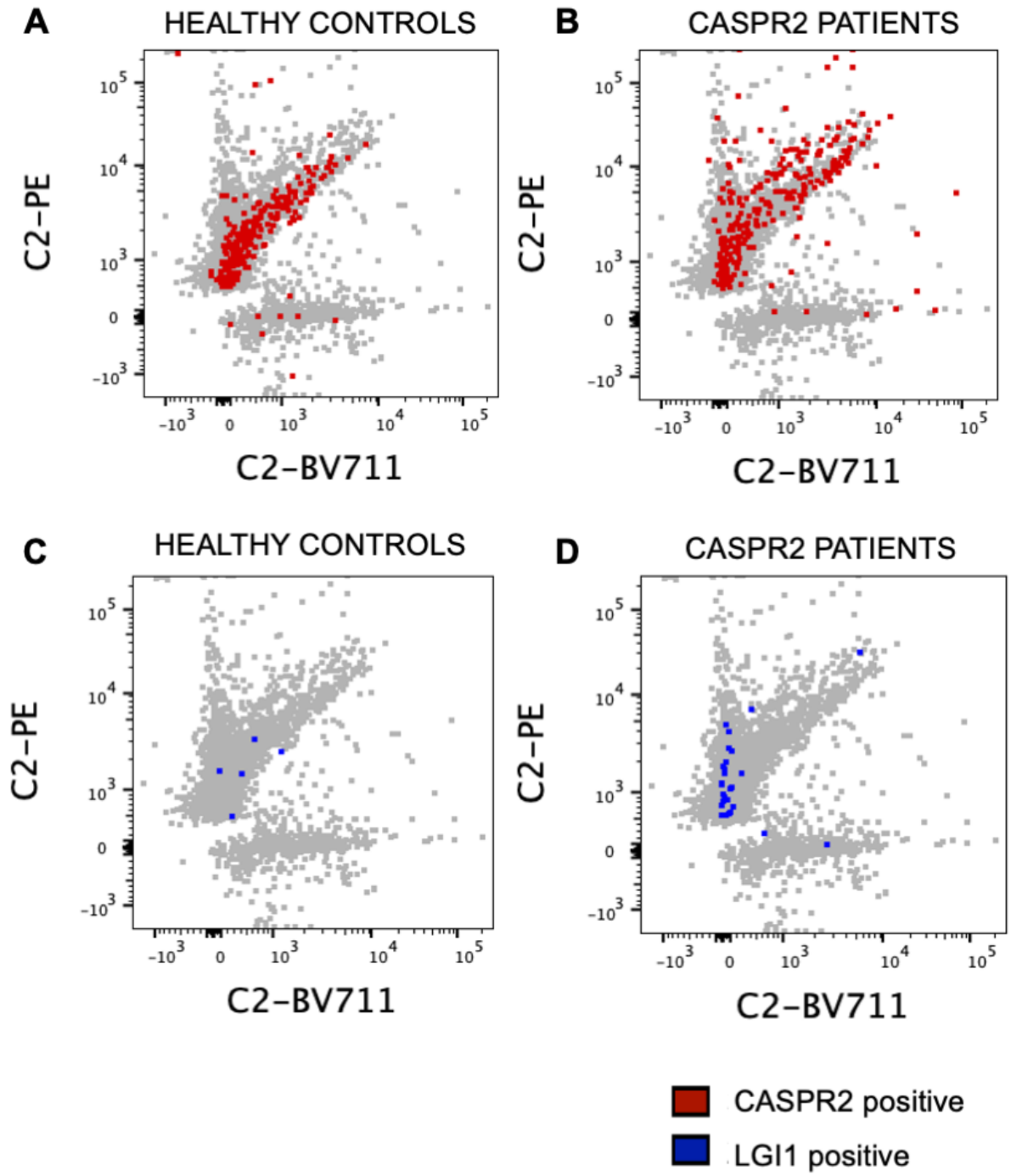


Figure 5.2 Following supernatant screening, information about CASPR2-positive and LGI1-positive wells was related to the FACS data and the positive cells were identified on the CASPR2-Bait channels. Within both patients (B) and healthy controls (A) the majority of CASPR2-positive cells were 'double positive' for reactivity on both bait channels (C2-711 and C2-PE). LGI1-positive cells were rare in healthy controls (C) and somewhat found in patients (D), predominantly in only the C2-PE channel.

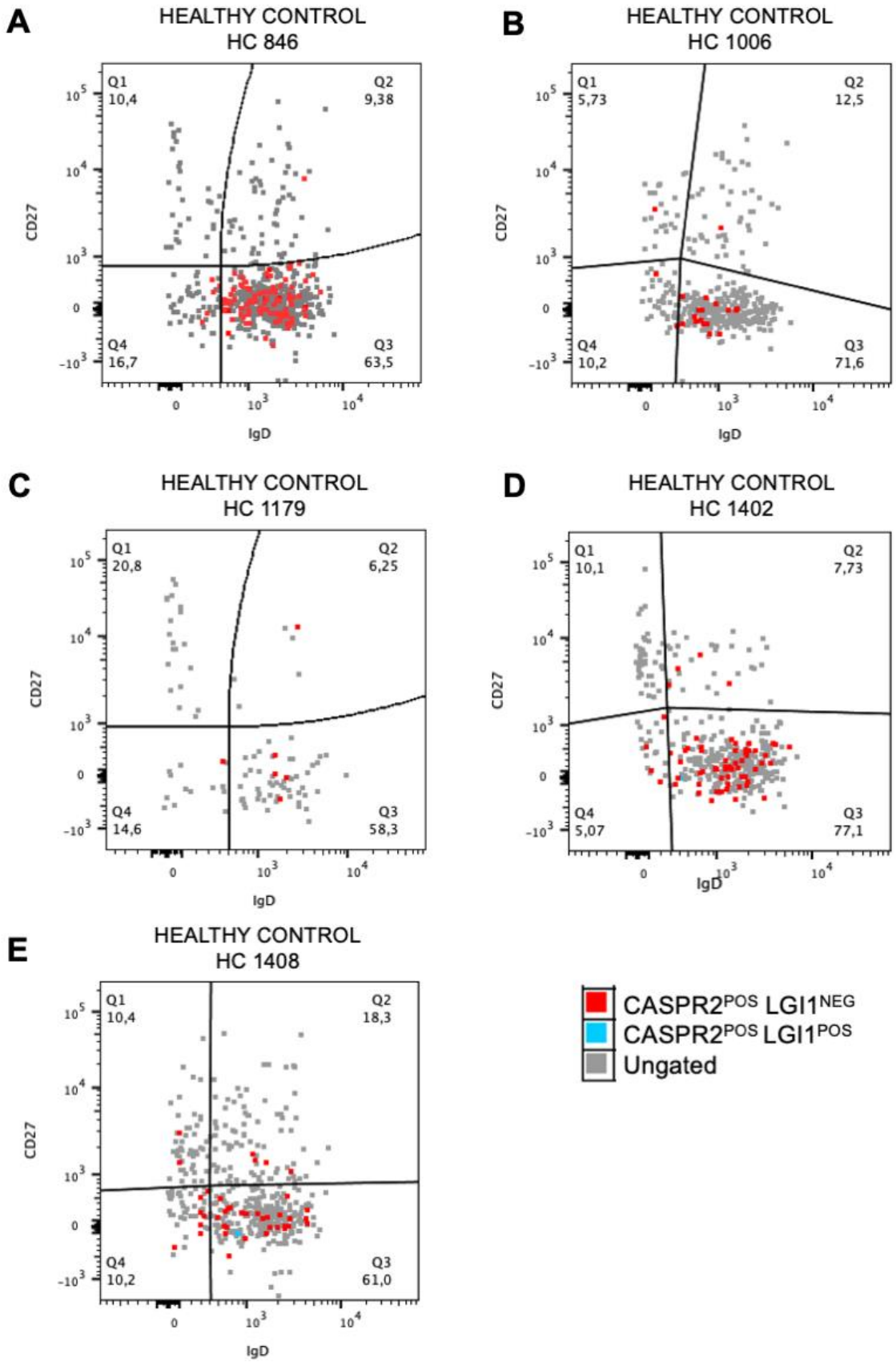


Figure 5.3 Distribution of CASPR2-reactive and LGI1-reactive cells within the naïve (CD27-IgD+) and memory (CD27+IgD-) B-cell compartments for healthy controls (A) HC 846; (B) HC 1006; (C) HC 1179; (D) HC 1402; (E) HC 1408

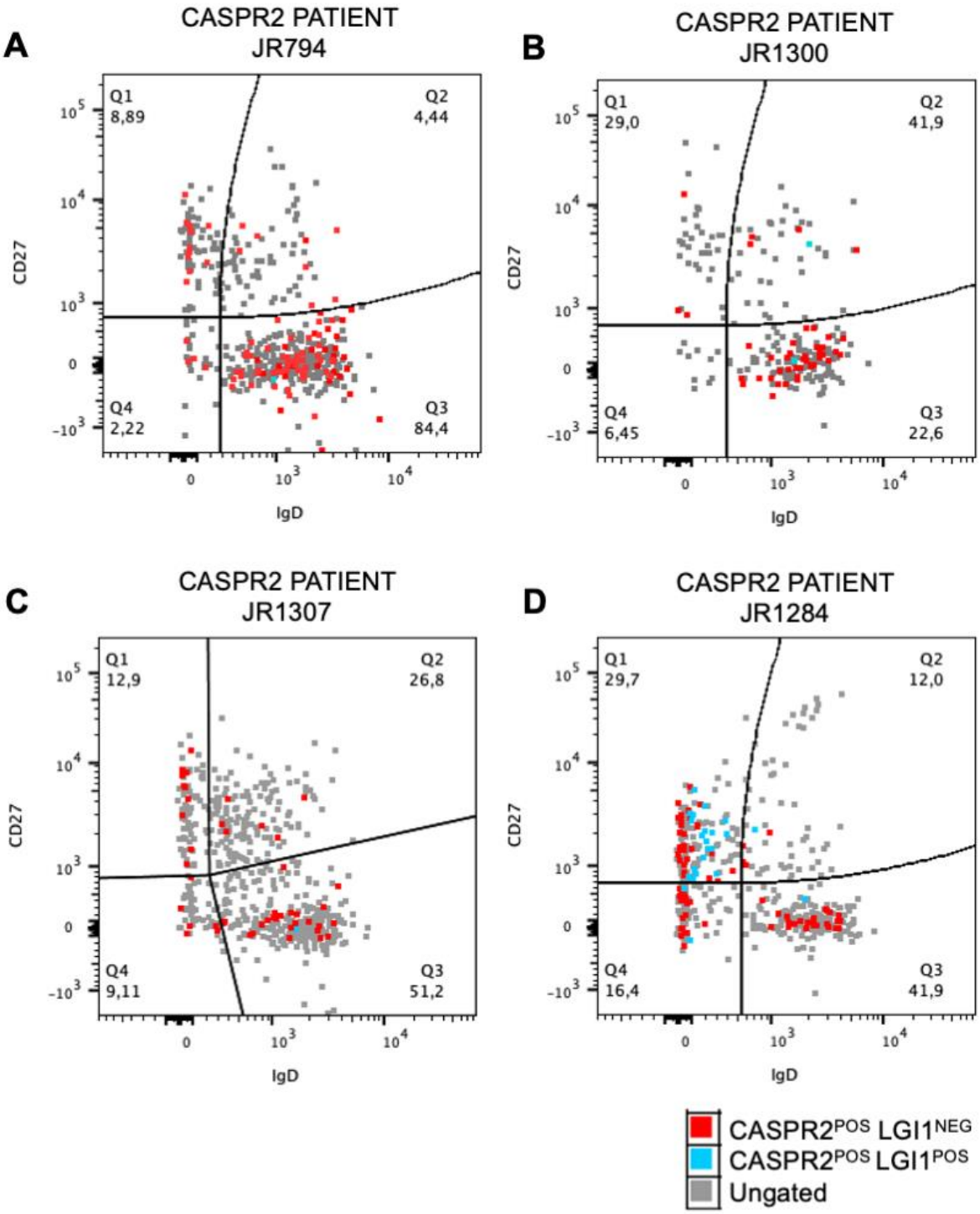


Figure 5.4 Distribution of CASPR2-reactive and LGI1-reactive cells within the naïve (CD27-IgD+) and memory (CD27+IgD-) B-cell compartments for CASPR2 patients (A) JR 794; (B) JR1300; (C) JR1307 and (D) JR1284

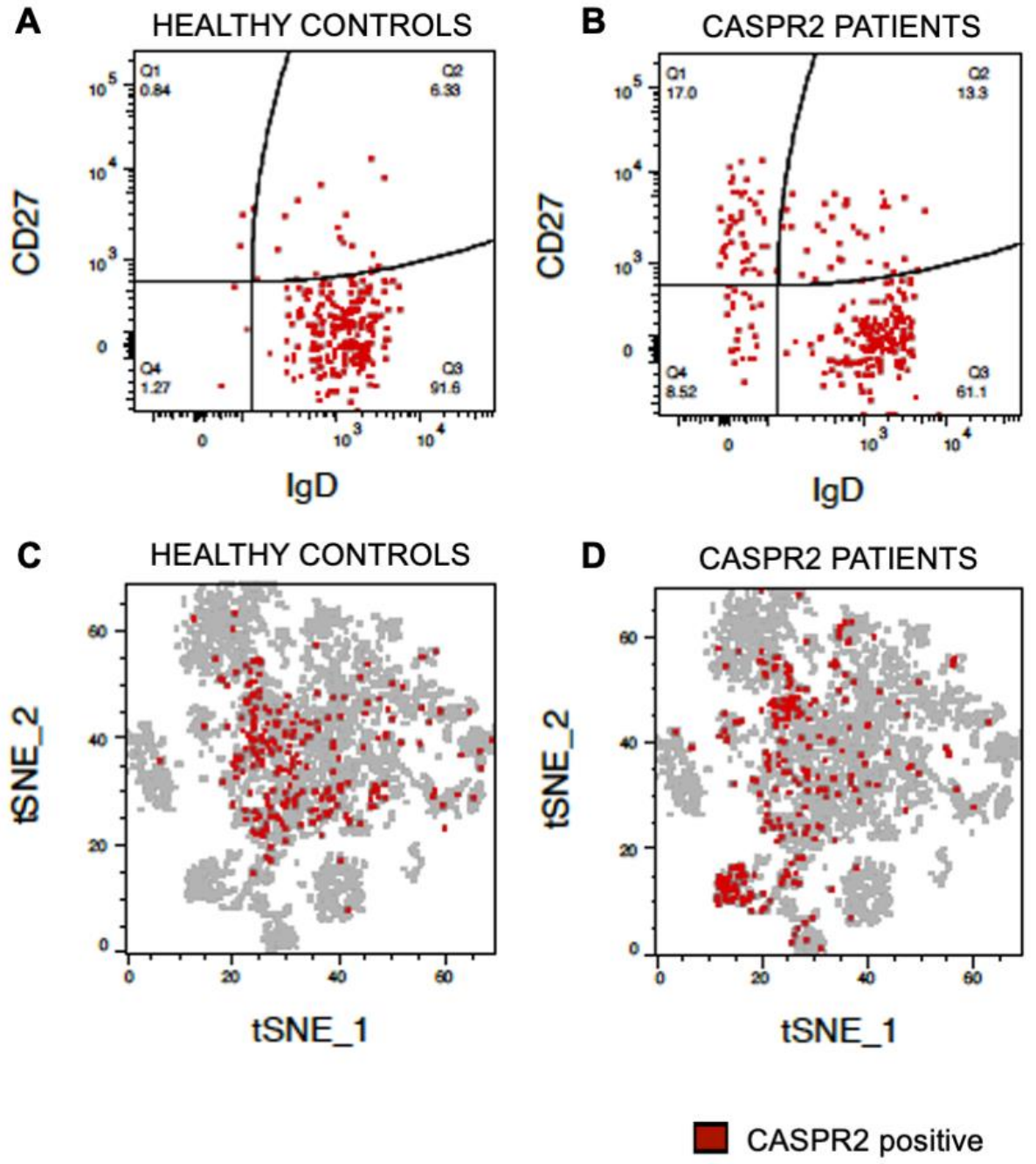


Figure 5.5 Summary of the distribution of CASPR2-positive cells within the naïve and memory B-cell compartments for all healthy controls (A) and patients (B) showed that CASPR2-positive cells could be found only in the naïve B-cell compartment in healthy controls, but in both the naïve and the memory B-cell compartment in patients. (C and D) tSNE plots showing the distribution of CASPR2-positive cells in patients and healthy controls.

Varying domain specificities between patients and healthy controls

The use of CASPR2 deletion constructs allowed me to examine the domain specificity of CASPR2-positive supernatants and relate this to parent B-cell phenotype as well as patient genotype. (Figure 5.6) An unexpected finding from the screening was that certain domain specificities were differently represented in patients versus in healthy controls. (Figure 5.6D) Collectively, patients had twice as many discoidin-reactive B-cells as healthy controls, whereas conversely EGF1/FibC reactivity was twice as prevalent in healthy controls as it was in patients. Non-specific reactivities, defined as binding indiscriminately to all deletion constructs, was also more prevalent in patients, as was L2 reactivity. L1 and L3/EGF2/L4 reactivity were nearly equally represented in both groups. I next wondered if distinct domain reactivities were specific to individual patients. Plotting domain reactivities for the individual patients did not reveal any notable trends, apart from L1 and EGF1/FibC reactivity being much more prominent in patient JR794, and discoidin reactivity and non-specific binding being more prominent in patient JR1284 than the remaining patients. (Figure 5.6E) Of note, patients JR794 and JR1284 were much further into their illness than patients JR1300 and JR1307 at the time of sample collection, were both untreated, and patient JR794 was the only patient exhibiting neuropathic pain. Curiously, these two untreated patients also had higher overall numbers of CASPR2-reactive cells than patients JR1300 and JR1307, who were earlier into their illness and had been treated at the time of sample collection. No clear patterns were identified in healthy controls, apart from the obvious predominance of EGF1/FibC reactivity. (Figure 5.6F) After the EGF1/FibC domain, the L1 domain was the second most targeted in healthy controls. Curiously the L3/EGF2/L4 domain reactivity was very sparsely represented in both patients and healthy controls, with only one member of each group (JR1307 and HC 1402, respectively) showing any notable reactivity to this domain. Interpreting these results in light of the structure of the CASPR2 protein, as predicted by Google AlphaFold, (Figure 5.6C) revealed that the two domains most targeted in patients, discoidin and L1, are closely exposed and form a significant portion of the N-terminal (membrane-distal) portion of the protein, whereas the domains most prominently targeted in healthy controls, EGF1/FibC were closer to the C-terminal (membrane-proximal) portion, suggesting the CASPR2-reactive B-cells in patients and healthy controls may have distinct domain reactivities of potentially varying pathophysiological significance.

Relating B-cell phenotypes of distinct domain specificities

Next, I wanted to examine how domain specificity relates to B-cell phenotype in patients and healthy controls, so I plotted domain specificities against IgD:CD27 status for both groups (Figures 5.7 and 5.8). Doing this for individual patients and healthy controls did not reveal any notable patterns, so I looked more globally at the domain specificity as a function of IgD:CD27 status for all patients versus all healthy controls combined. (Figure 5.9) Among healthy controls all domain specificities were largely contained within the naïve B-cell subset (Figure 5.9), with occasional 'escape' into other subsets, like late memory (CD27- IgD-) for discoidin- and EGF1/FibC-reactive cells (Figure 5.9B,H), and IgM memory (CD27+IgD+) for EGF1/FibC-reactive cells (Figure 5.9H). Escape into the switched memory compartment was obvious only for EGF1/FibC-reactivities and to a very minor extent for discoidin and L2 (Figure 5.9F) in healthy controls. In CASPR2 patients, the L1-, L2-, EGF1/FibC- and L3/EGF2/L4-reactive B-cells permeated all four compartments, being most represented in the naïve and least in the IgM memory compartment (Figure 5.9, left). Discoidin-reactive B-cells followed a different trend, barely featuring in the naïve B-cell subset, and prominently featuring in the switched memory and late memory compartment (Figure 5.9A). This suggested that in patients B-cells with different CASPR2-reactivities might follow distinct developmental trajectories from each other and from their 'counterparts' in healthy controls.

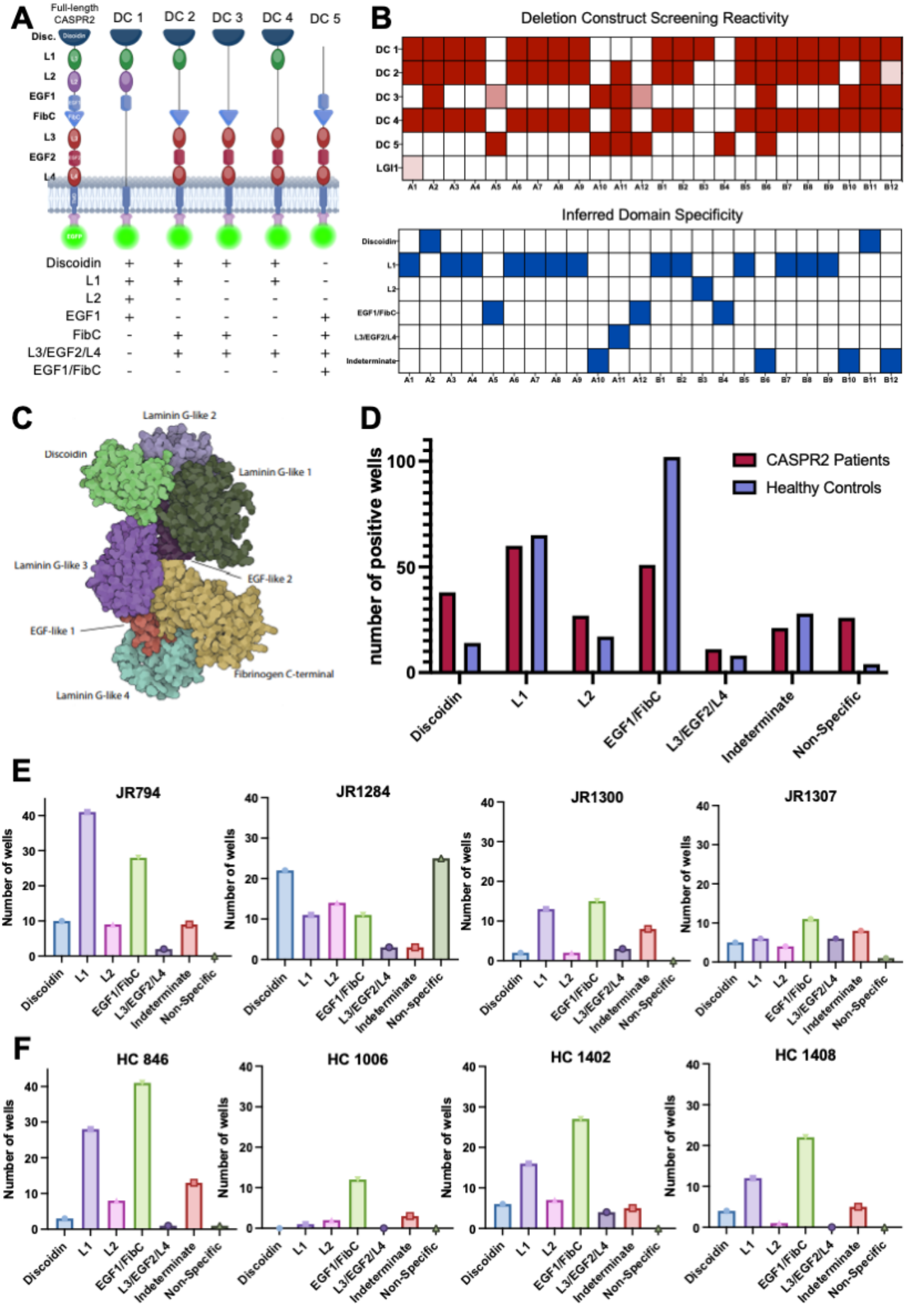

A
Full-length CASPR2
DC 1
DC 2
DC 3
DC 4
DC 5
Disc.
L1
L2
EGF1
FibC
L3
EGF2
L4
EGFP
Discoidin + + + + -
L1 + + - + -
L2 + - - - -
EGF1 + - - - +
FibC - + + - +
L3/EGF2/L4 - + + + +
EGF1/FibC - - - - +
B
Deletion Construct Screening Reactivity
DC 1
DC 2
DC 3
DC 4
DC 5
LGI1
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12
Inferred Domain Specificity
Discoidin
L1
L2
EGF1/FibC
L3/EGF2/L4
Indeterminate
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12
C
Laminin G-like 2
Discoidin
Laminin G-like 1
Laminin G-like 3
EGF-like 2
EGF-like 1
Fibrinogen C-terminal
Laminin G-like 4
D
number of positive wells
100
50
0
CASPR2 Patients
Healthy Controls
Discoidin
L1
L2
EGF1/FibC
L3/EGF2/L4
Indeterminate
Non-Specific
E
JR794
JR1284
JR1300
JR1307
Number of wells
40
30
20
10
0
Discoidin
L1
L2
EGF1/FibC
L3/EGF2/L4
Indeterminate
Non-Specific
F
HC 846
HC 1006
HC 1402
HC 1408
Number of wells
40
30
20
10
0
Discoidin
L1
L2
EGF1/FibC
L3/EGF2/L4
Indeterminate
Non-Specific

Figure 5.6 ***(previous page)*** Domain specificity of CASPR2-positive B-cell supernatants. (A) Design of CASPR2 deletion constructs used for determining domain specificity of supernatants. (B) Example of how domain specificities were inferred based on the reactivity pattern of a supernatant against the different deletion constructs. In the upper panel, each column is a different supernatant and each row a CASPR2 deletion construct (or LGI1, used as a control). Red indicates binding and white no binding, with pink indicating putative binding (CBA result inconclusive). The lower panel shows the domain specificities inferred based on the upper panel in blue. (C) Structure of CASPR2 as predicted by Google AlphaFold with the individual domains labelled, shown here for comparison to the schematic structure in (A). (D) Plotting distribution of domain specificities among patients and healthy controls showed discoidin reactivity and non-specific binding (defined as binding to all deletion constructs) to be more prevalent in patients, whereas EGF1/FibC reactivity was twice more common in healthy controls than in patients. (E and F) Domain reactivity shown for individual patients (E) and healthy controls (F). Results from HC 1179 were excluded due to very low overall and CASPR2-reactive cell count, as seen in Figure 5.3(C).

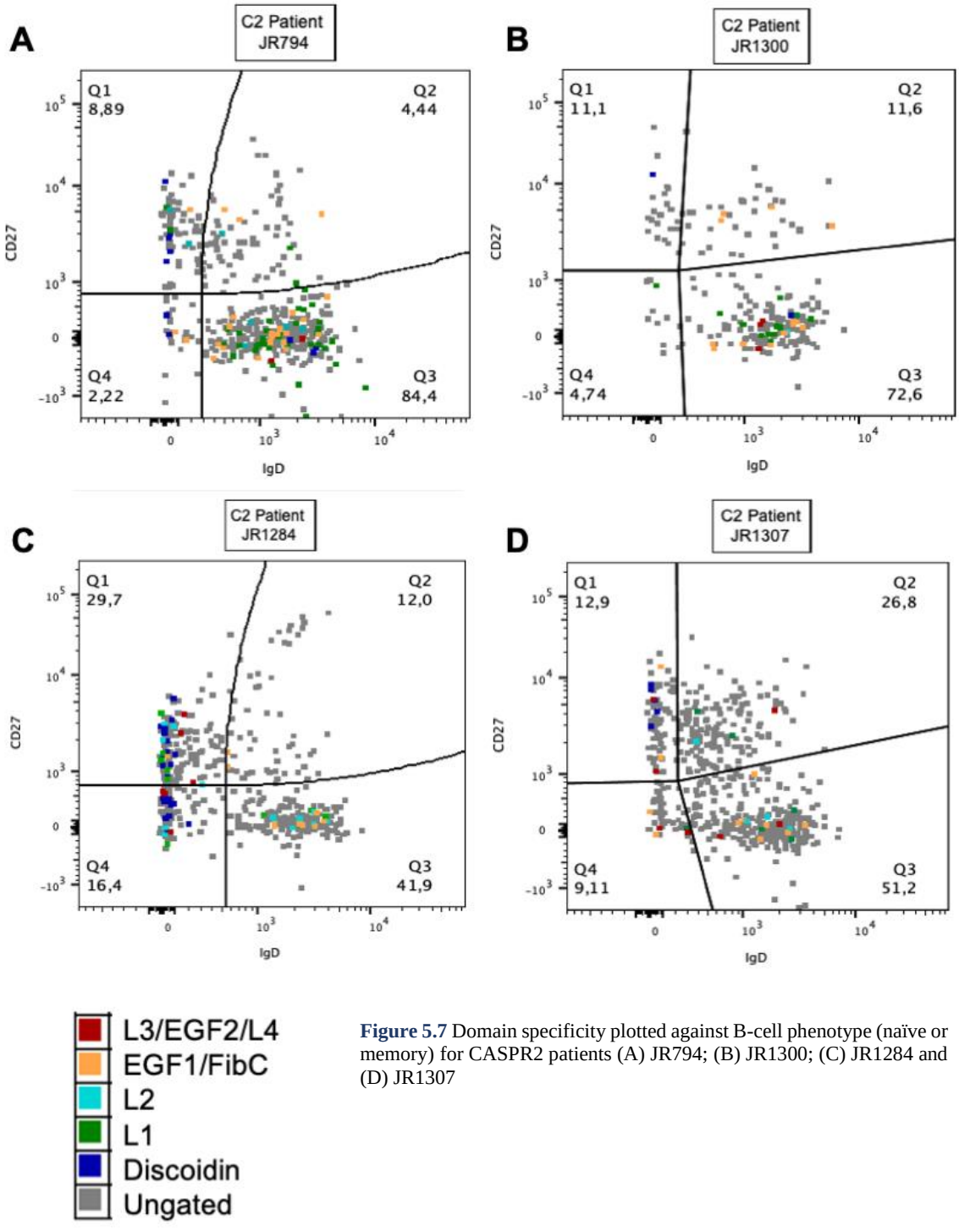


Figure 5.7 Domain specificity plotted against B-cell phenotype (naïve or memory) for CASPR2 patients (A) JR794; (B) JR1300; (C) JR1284 and (D) JR1307

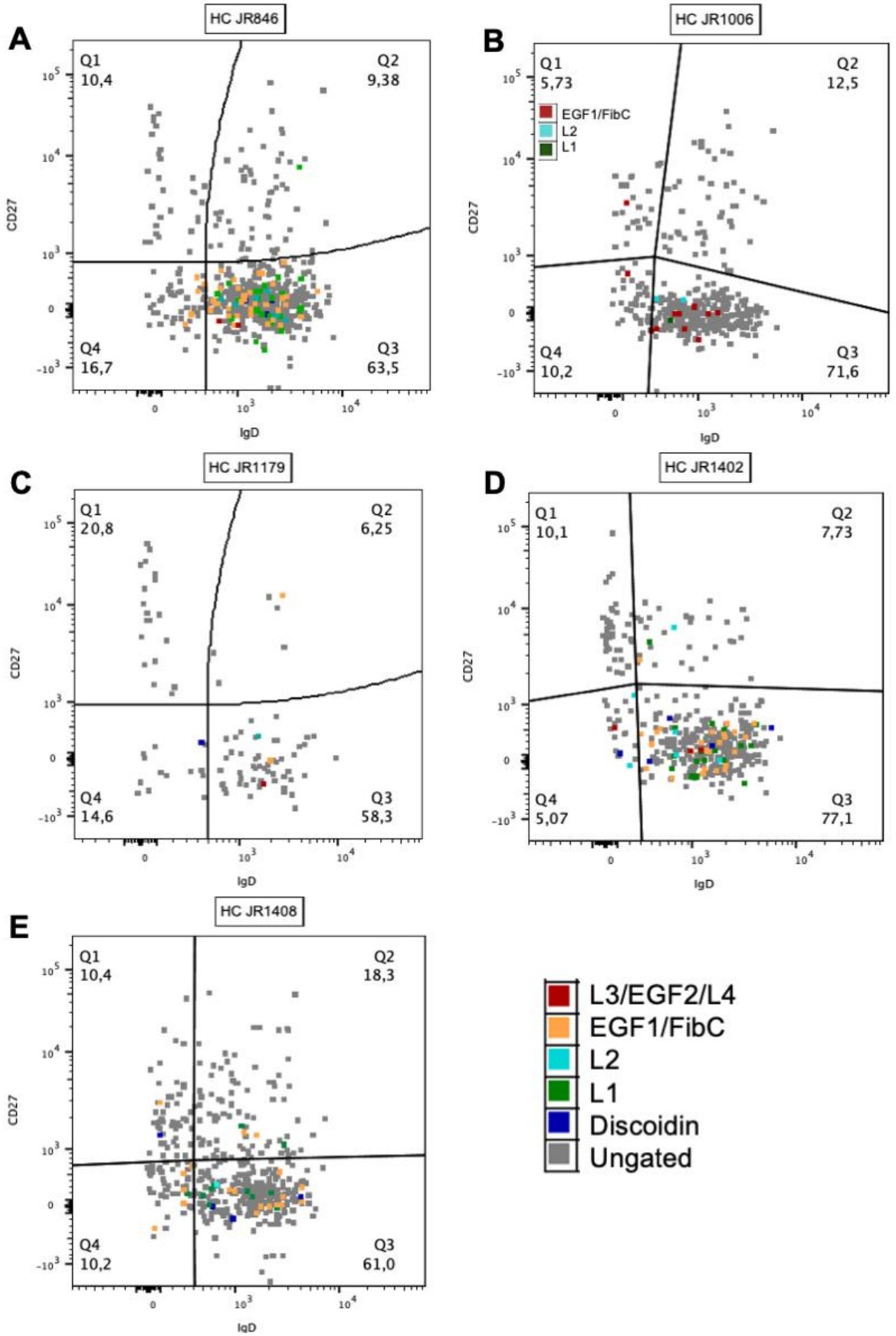
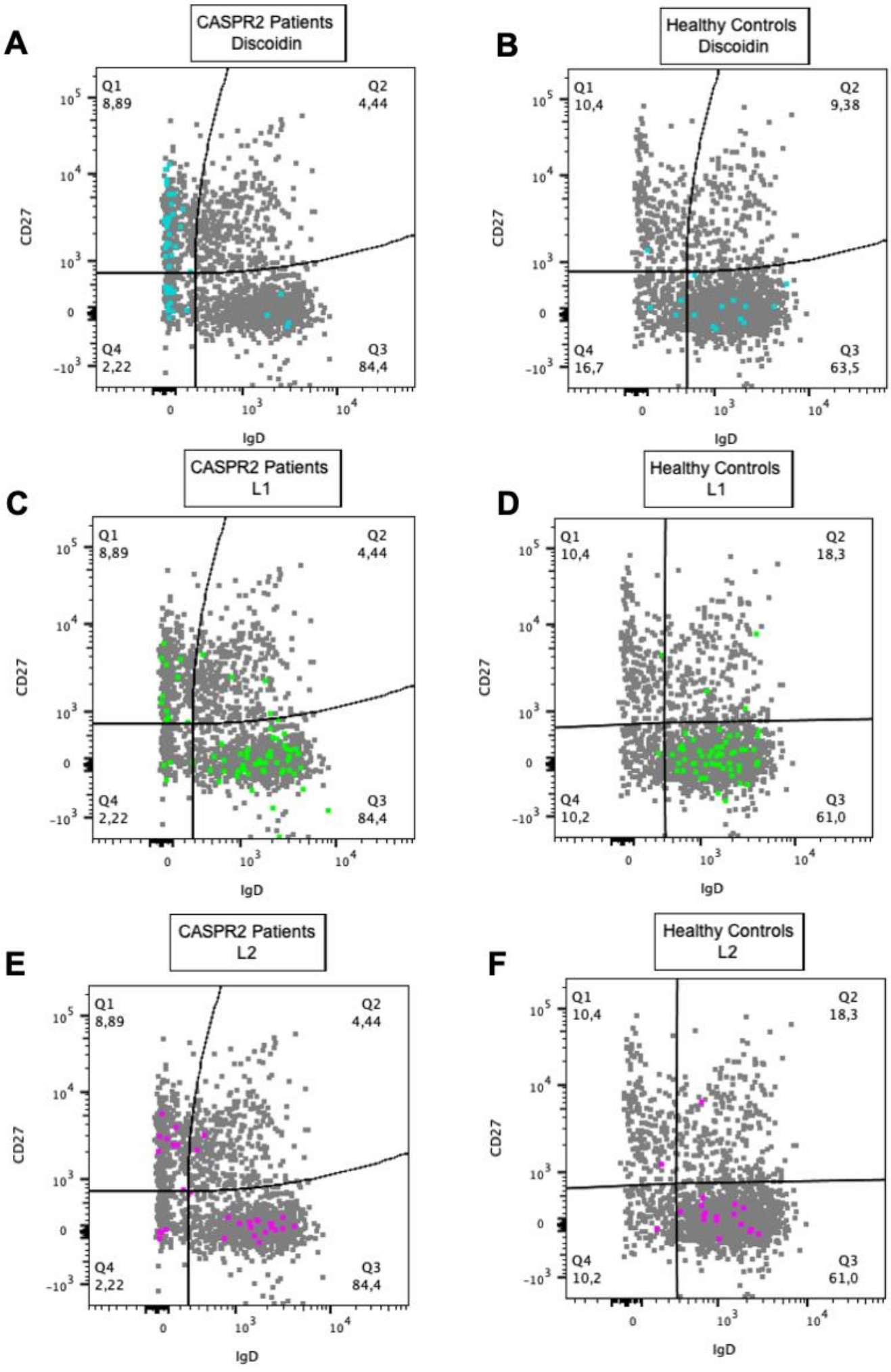


Figure 5.8 Domain specificity plotted against B-cell phenotype (naïve or memory) for healthy controls (A) HC 846; (B) HC 1006; (C) HC 1179; (D) HC 1402 and (E) HC 1408


A
CASPR2 Patients
Discoidin
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
B
Healthy Controls
Discoidin
Q1
10,4
Q2
9,38
Q4
16,7
Q3
63,5
CD27
IgD
C
CASPR2 Patients
L1
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
D
Healthy Controls
L1
Q1
10,4
Q2
18,3
Q4
10,2
Q3
61,0
CD27
IgD
E
CASPR2 Patients
L2
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
F
Healthy Controls
L2
Q1
10,4
Q2
18,3
Q4
10,2
Q3
61,0
CD27
IgD

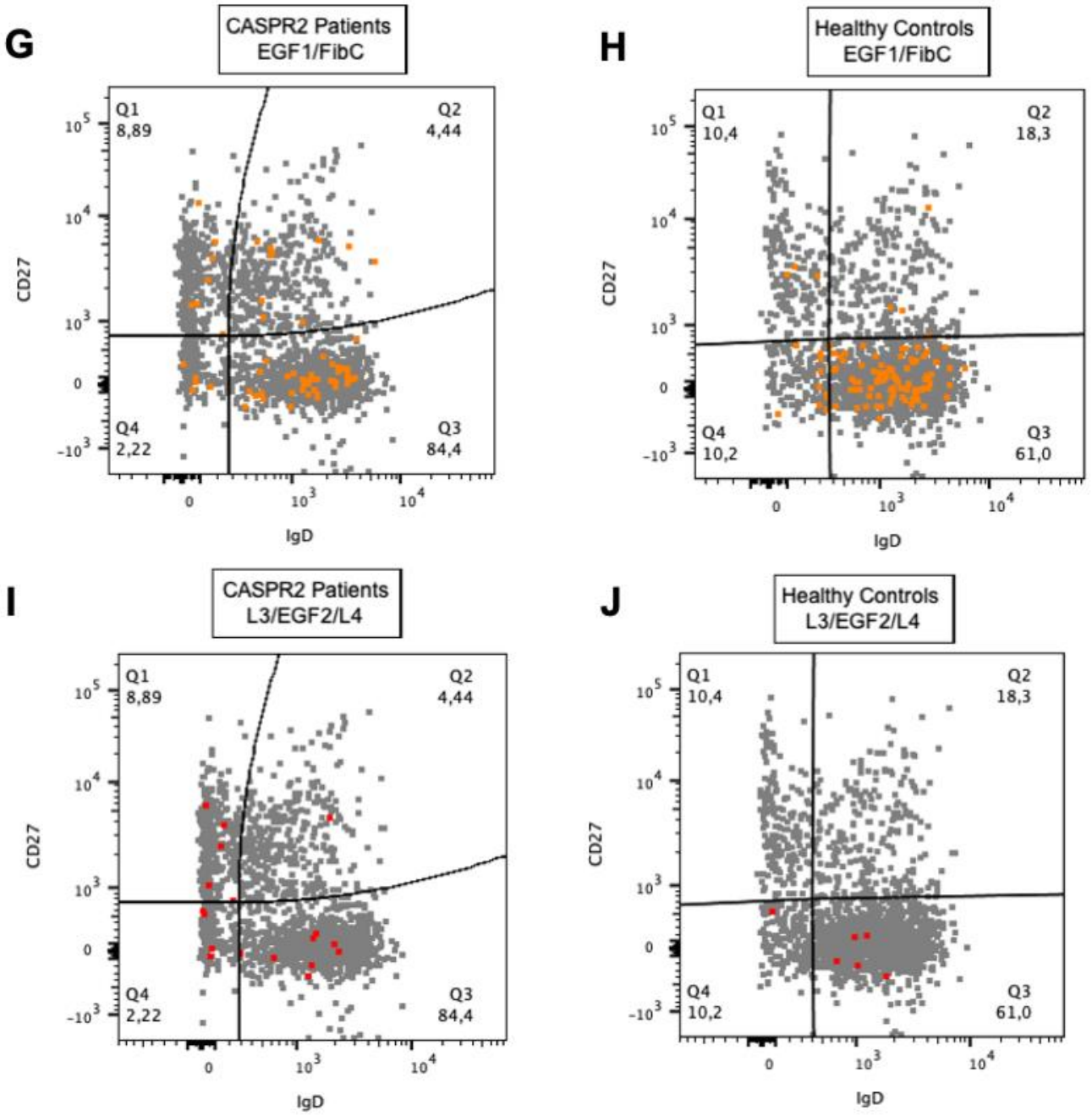
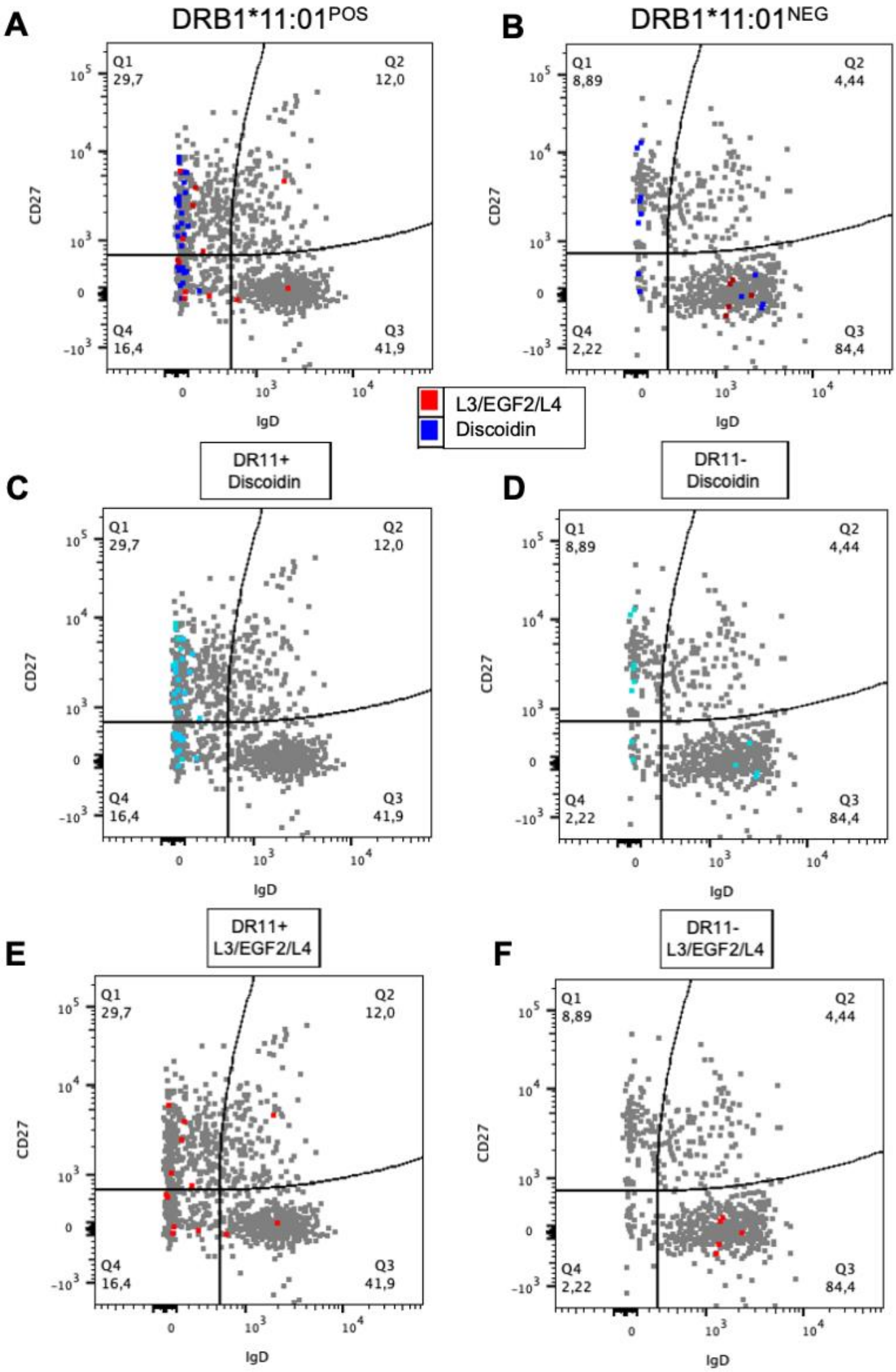
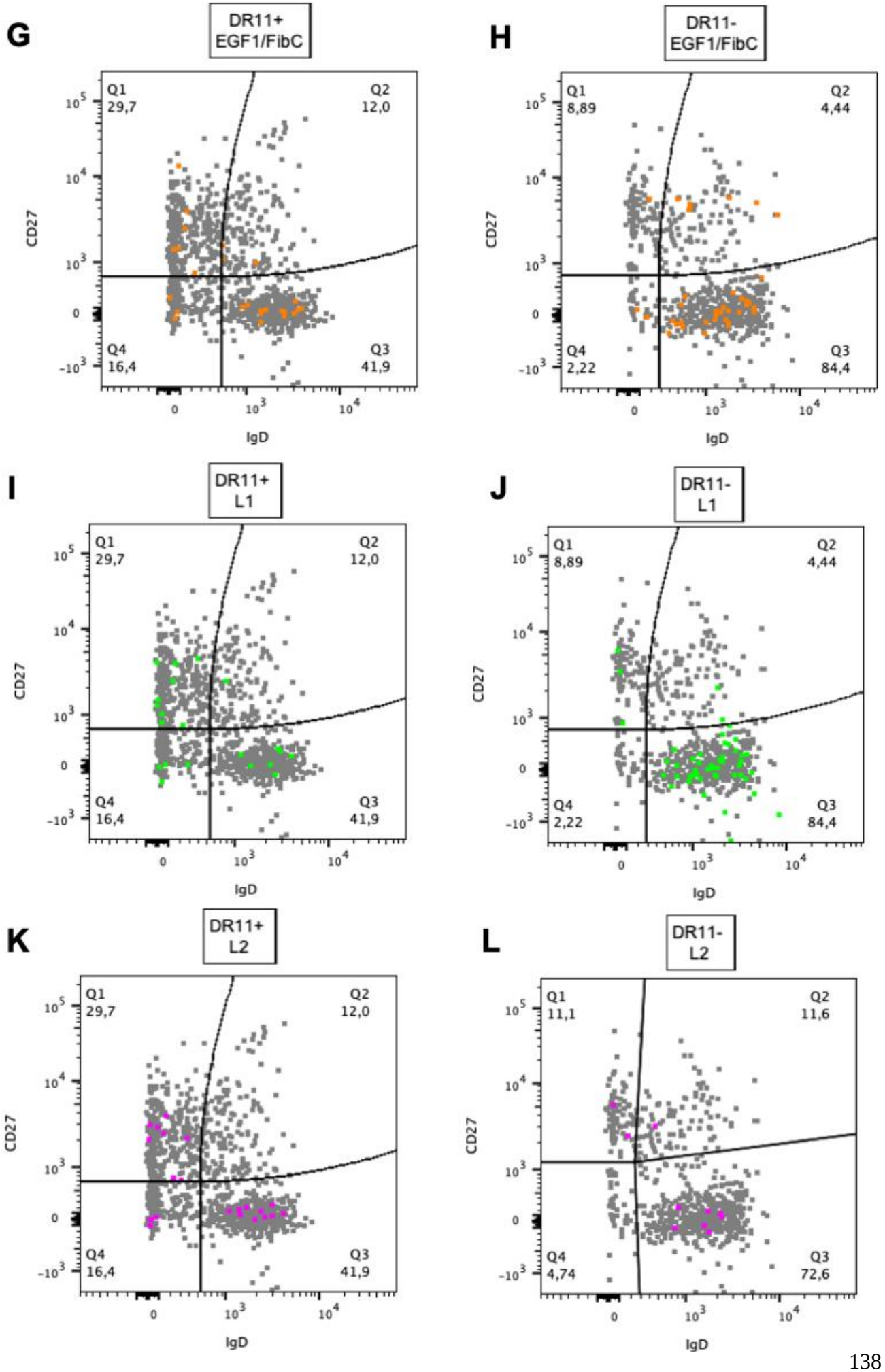


Figure 5.9 Domain specificities are plotted superimposed upon B-cell phenotypes (naïve vs memory) for CASPR2 patients (left-hand panels) and healthy controls (right-hand panels) for (A-B) Discoidin reactive; (C-D) L1 reactive; (E-F) L2 reactive; (G-H) EGF1/FibC reactive; and (I-J) L3/EGF2/L4 reactive cells

Figure 5.10 ***(next two pages)*** Domain specificities are plotted superimposed upon B-cell phenotypes (naïve vs memory) for CASPR2 patients thar are either positive (left-hand side) or negative (right-hand side) for the HLA-DRB1*11:01 haplotype. This is first done for Discoidin- and L3/EGF2/L4-specific cells, plotted first together (A-B) and then individually (C-D and E-F, respectively), then EGF1/FibC- (G-H), L1- (I-J), and L2-specific cells (K-L). For graphs C-L, the haplotypes HLA-DRB1*11:01^{POS} and HLA-DRB1*11:01^{NEG} are abbreviated to DR11+ and DR11-, respectively, followed by the domain specificity represented in the graph.


A
DRB1*11:01POS
B
DRB1*11:01NEG
Q1
29,7
Q2
12,0
Q4
16,4
Q3
41,9
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
L3/EGF2/L4
Discoidin
C
DR11+
Discoidin
D
DR11-
Discoidin
E
DR11+
L3/EGF2/L4
F
DR11-
L3/EGF2/L4


G
DR11+
EGF1/FibC
Q1
29,7
Q2
12,0
Q4
16,4
Q3
41,9
CD27
IgD
H
DR11-
EGF1/FibC
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
I
DR11+
L1
Q1
29,7
Q2
12,0
Q4
16,4
Q3
41,9
CD27
IgD
J
DR11-
L1
Q1
8,89
Q2
4,44
Q4
2,22
Q3
84,4
CD27
IgD
K
DR11+
L2
Q1
29,7
Q2
12,0
Q4
16,4
Q3
41,9
CD27
IgD
L
DR11-
L2
Q1
11,1
Q2
11,6
Q4
4,74
Q3
72,6
CD27
IgD

*Relationship between domain specificity and DRB1*11:01 status*

I next wanted to see what relationship reactivities bore to the patient HLA haplotype (Figure 5.10). The HLA-DRB1*11:01 haplotype has been found to be associated with the presence of CASPR2 autoantibodies.[296, 306] In our study group, two patients were carriers of this allele, one of whom was heterozygous (JR1284) and one homozygous (JR1307), while the other two were negative for this allele (JR794 and JR1300). Among healthy controls, only one (JR1402) was a heterozygous carrier for the allele (Figure 5.1A) To examine the relationship between HLA-DRB1*11:01 status and domain reactivity, I separated patients into DRB1*11:01^{POS} (JR1284 and JR1307) and DRB1*11:01^{NEG} (JR794 and JR1300) and examined the relationship between domain reactivity and IgD:CD27 status for both groups. (Figure 5.10) First, I looked at how genotype and IgD:CD27 status related to discoidin and L3/EGF2/L4 reactivity, (Figure 5.10A-B) as previous work in the group had suggested a relationship between haplotype and reactivity to these domains (Varley and Irani, unpublished). The first trend that became apparent from this classification was that discoidin- and L3/EGF2/L4-reactive cells were much more prevalent in the DRB1*11:01^{POS} group, where they were mostly distributed in the switched memory and late memory compartment, as opposed to the DRB1*11:01^{NEG} group where they were fewer in number and more common in the naïve B-cell compartment. Indeed, plotting only the discoidin reactivities for the two groups revealed that the only discoidin-reactive B-cells belonging to the naïve B-cell subset hailed from the DRB1*11:01^{NEG} group, whereas these cells were limited to the switched and late memory B-cell subsets in the DRB1*11:01^{POS} group. (Figure 5.10C-D) For the other domain reactivities, cells positive for each domain would be largely limited to the naïve B-cell compartment for the DRB1*11:01^{NEG} patients but seemed to infiltrate all four compartments for the DRB1*11:01^{POS} group. (Figure 5.10E-L) Altogether, these findings suggest the DRB1*11:01 allele predisposes to permissiveness of the central and peripheral B-cell tolerance checkpoints and to greater reactivity to the discoidin domain. In patients that lacked the DRB1*11:01 allele, it seemed that CASPR2-reactive B-cells were more restricted to the naïve subset, reminiscent of healthy controls, whereas in DRB1*11:01^{POS} patients, the reactivities were spread out across all four subsets. The exception were discoidin-reactive B-cells, which could be seen *only* in the switched and late memory subsets for DRB1*11:01^{POS} patients, and in subsets other than the naïve in DRB1*11:01^{NEG} ones.

UMAP Analysis allows for identification and characterisation of distinct Seurat clusters

We next wanted to take a more detailed look at the FACS data and relate this to what we had learned about the B-cell reactivities, as well as BCR sequence characteristics. UMAP analysis on the FACS data integrating information across all channels apart from the CASPR2 bait (C2-BV711 and C2-PE) led to the identification of nine distinct clusters, numbered 0-8. (Figure 5.11A and 5.13A) Superimposing results from the CBA screening of the supernatants showed CASPR2-reactive B-cells were uniformly distributed across multiple clusters reflecting a diversity of phenotypes. (Figure 5.12A) The exception was cluster 8, which seemed to contain virtually no CASPR2-positive B-cells. However, when looking at the K_D affinity values for the CASPR2-positive B-cell supernatants, it became apparent that the B-cells whose cognate antibodies had the highest affinity for CASPR2 as determined using SPR nearly all came from cluster number 3. (Figure 5.12B) This cluster predominantly consisted of cells reactive to the discoidin and L1 domains (the two domains most represented in patients), as well as cells that were positive for all deletion constructs, and contained nearly all the LGI1-positive cells within our dataset (Figure 5.12C-D). When looking at just the discoidin-reactive cells for patients versus healthy controls, it became apparent that discoidin-reactive B-cells from CASPR2 patients were concentrated in cluster 3, whereas in healthy controls they were nearly absent from cluster 3 and more uniformly distributed across the entire UMAP plot. (Figure 5.12E) Furthermore, when looking at these clusters within the context of the CASPR2-bait channels,

cluster 3 was the one with the highest enrichment for both bait constructs. (Figure 5.13B-C) By contrast, cluster 8 was most enriched for only the C2-BV711 channel, likely suggesting the lack of bona fide CASPR2-reactive B-cells was due to cells in this cluster reacting to BV711 rather than the antigen. Indeed, examining bait status against FACS Seurat cluster identity confirmed most cells with high expression for both antigens ($PE^{HIGH}BV^{HIGH}$) were localised to cluster 3, but also that this cluster was represented in patients but not healthy donors. (Figure 5.14A-C) The majority of IGHG4 BCRs were also localised to this cluster. (Figure 5.14D) Conversely, cells with low reactivity to both constructs ($PE^{LOW}BV^{LOW}$) were more prominently featured in healthy donors than in patients, forming cluster 4. (Figure 5.14B) Finally, plotting IgD:CD27 status against the UMAP plots revealed most switched memory ($IgD^{NEG}CD27^{POS}$) and late memory ($IgD^{NEG}CD27^{NEG}$) B-cells to be localised to cluster 3, with IgM memory B-cells ($IgD^{POS}CD27^{POS}$) occupying most of clusters 2 and 8, and naïve B-cells ($IgD^{POS}CD27^{NEG}$) being uniformly distributed across the remaining clusters (Figure 5.15A). When relating IgD:CD27 status to bait reactivity, switched and late memory B-cells were most enriched for both CASPR2-PE and CASPR2-BV711. (Figure 5.15B-D) Relating domain specificity to IgD:CD27 status for patients and healthy controls revealed patients to have a more diverse representation of all four different B-cell populations across the various domain specificities. (Figure 5.15E-F) Specifically for discoidin-reactive B-cells, patient data saw an expansion of the CD27+IgD- (switched memory) phenotype compared to controls, as expected.

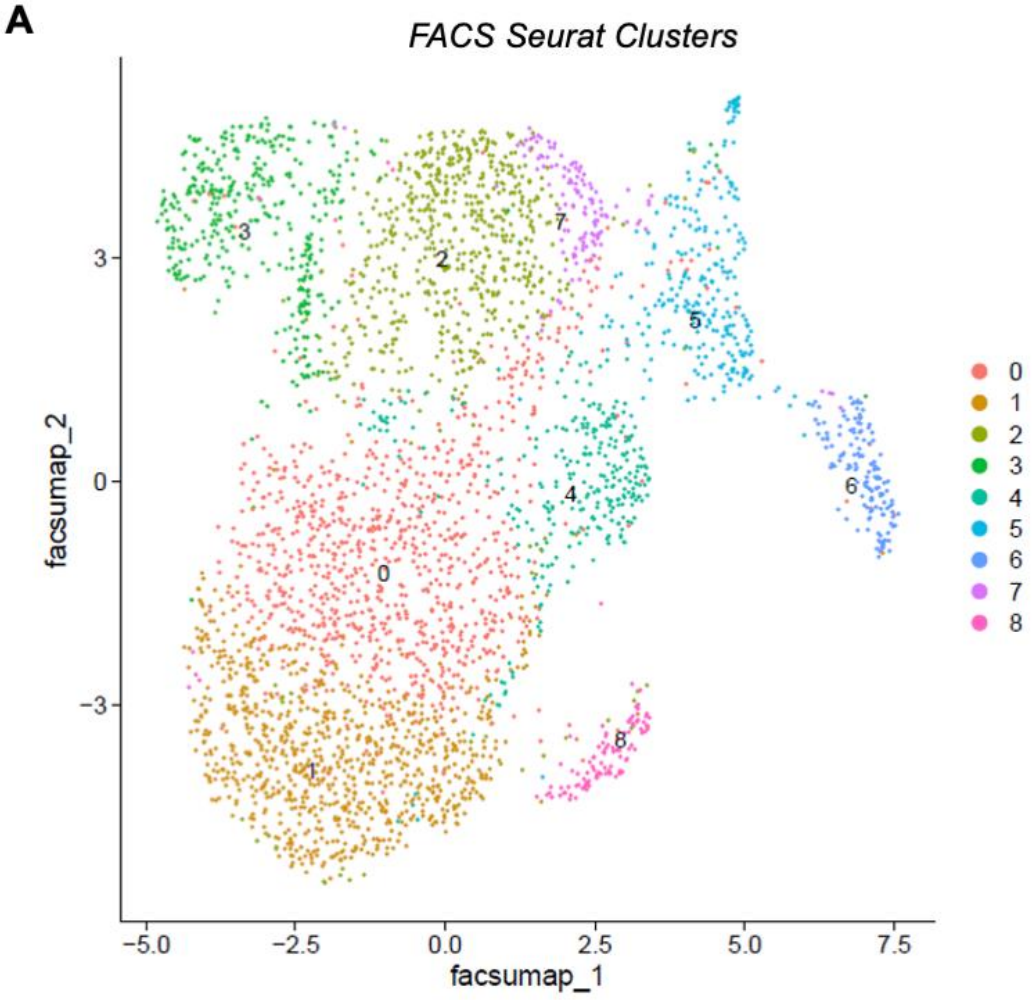


Figure 5.11 (A) Seurat clusters following UMAP analysis of FACS data

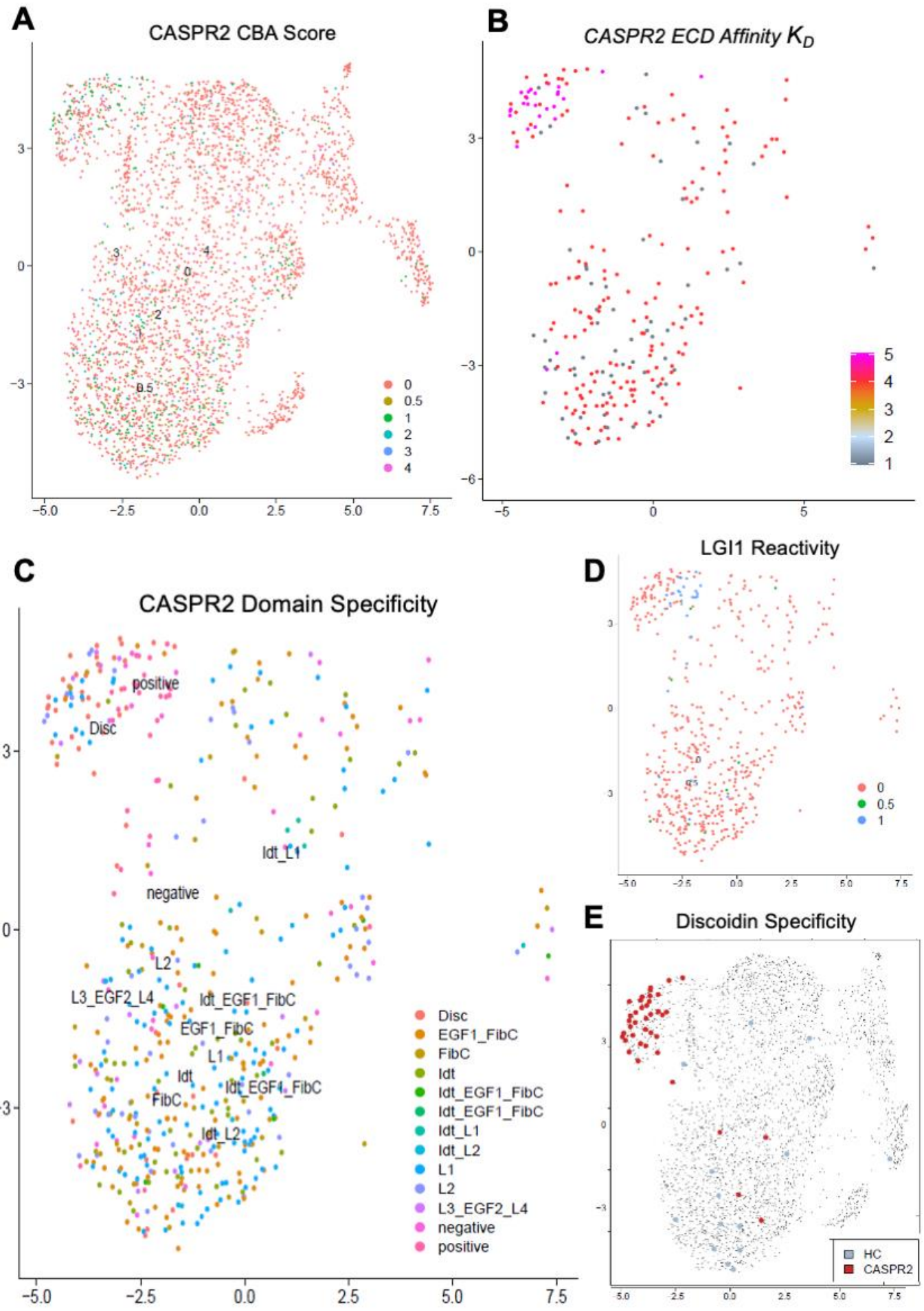


Figure 5.12 (A) CASPR2-CBA scores, (B) Affinity K_D measurements, (C) Domain specificities, (D) LGI1 reactivity and (E) Discoidin reactivity for CASPR2 patients (red dots) and healthy controls (grey dots) superimposed upon the FACS Seurat Clusters from Figure 5.11(A)

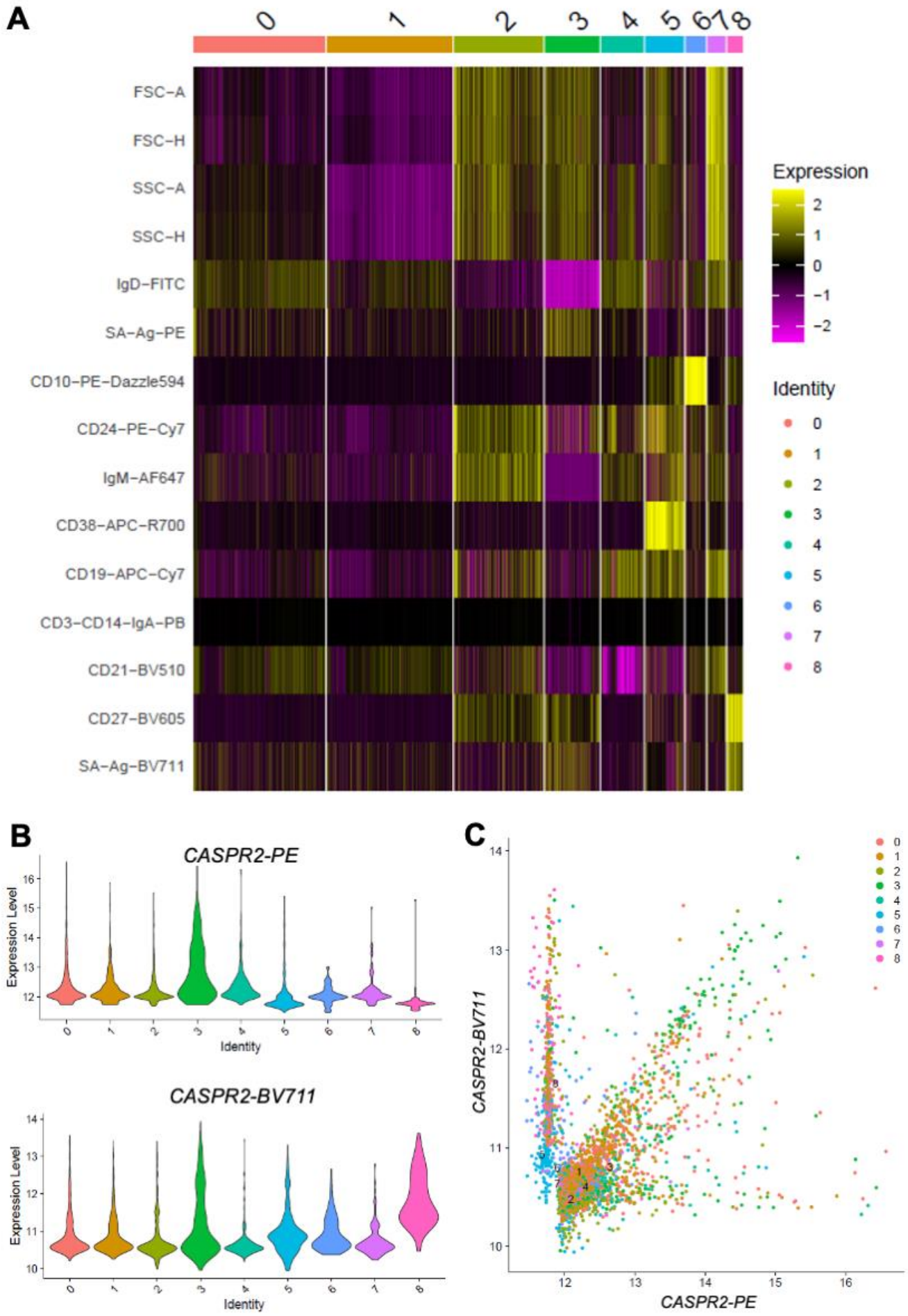


Figure 5.13 (A) Cluster identity based on Forward- and Side-scatter as well as the expression levels of the various cell markers used in the experiment. The CD3-CD14-IgA channel does not feature any expression data as cells positive for these markers were excluded from the sort. The bait channels (SA-Ag-BV711 and SA-Ag-PE) are also not featured as they were not used in defining cluster identity. (B) Bait signal by Seurat cluster for both bait constructs. (C) Bait signal by FACS Seurat Cluster, with CASPR2-PE plotted on the x-axis and CASPR2-BV711 on the y-axis.

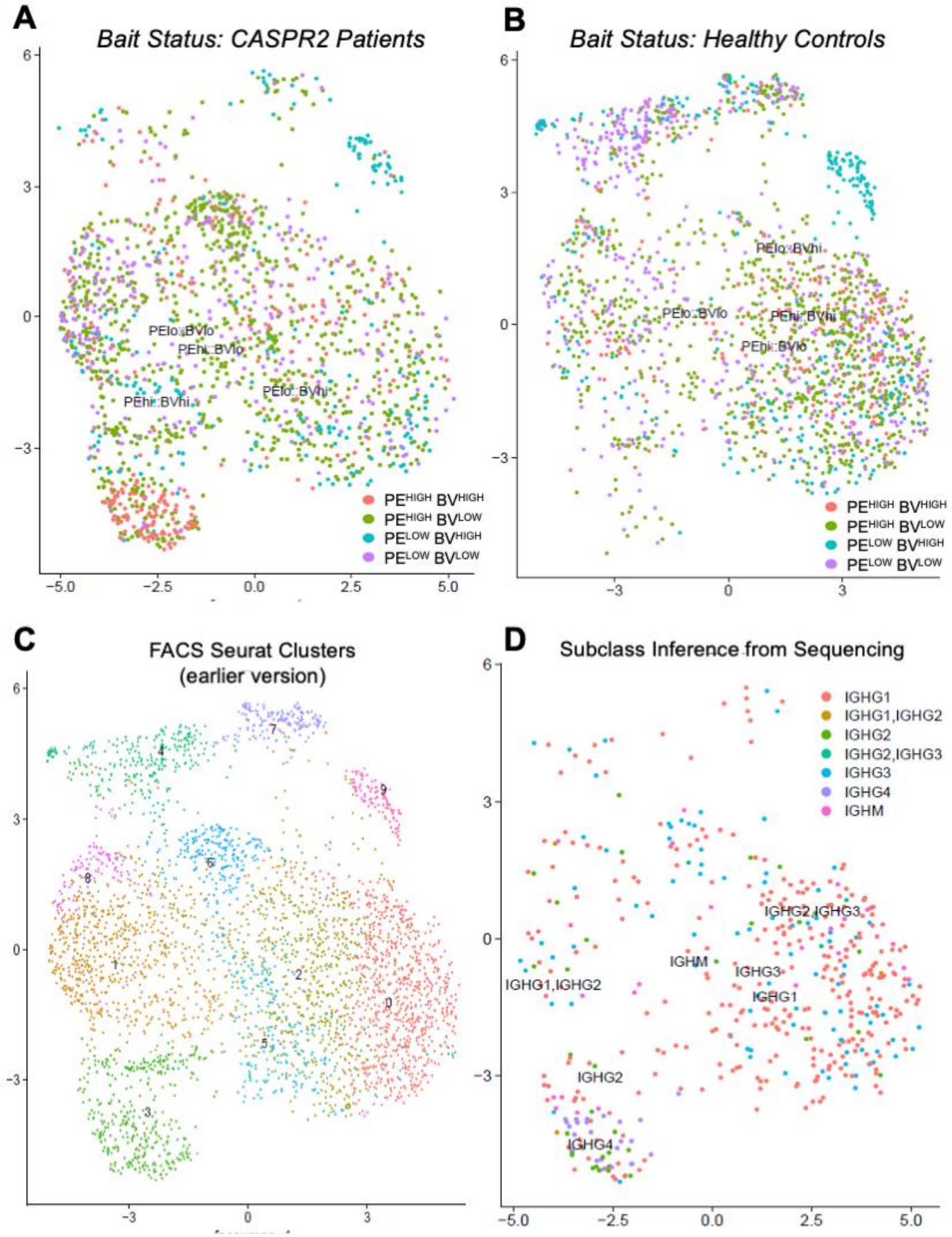


Figure 5.14 (A-B) Plotting the distribution of cells with variable reactivity to the two bait constructs in CASPR2 patients (A) and healthy controls (B) using an earlier UMAP analysis of the same dataset (C) showed clustering of cells with high reactivity to both channels ($PE^{HIGH}BV^{HIGH}$) in CASPR2 patients (A) within cluster 3, but not in healthy controls (B). In healthy controls, cells with low reactivity to both channels ($PE^{LOW}BV^{LOW}$) clustered together in cluster 4. (D) Superimposing IgG subclass inferred from sequencing data on this UMAP plot showed the majority of CASPR2-reactive cells in cluster 3 to be expressing IGHG4.

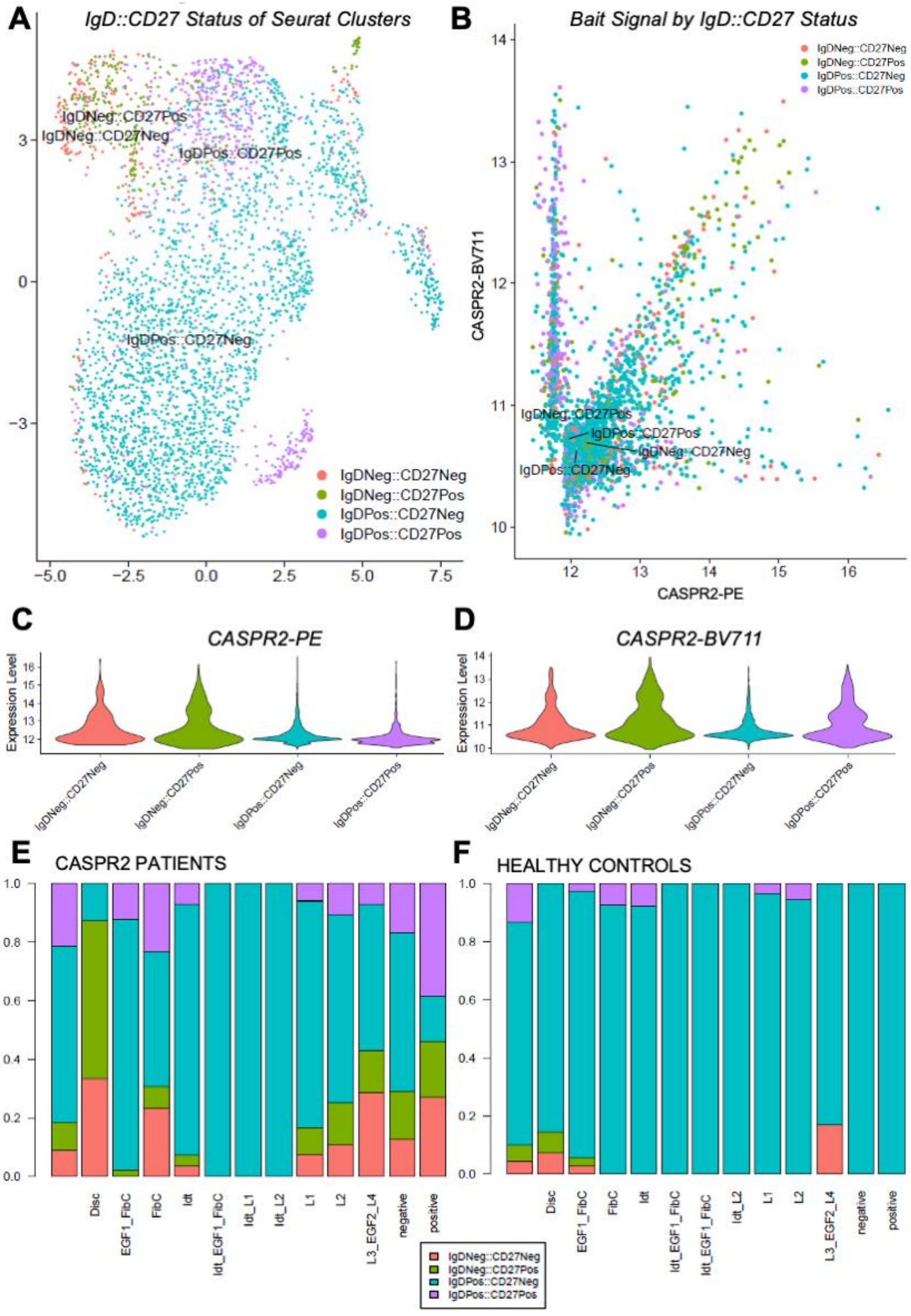


Figure 5.15 (A) Expression of IgD and CD27, defined as IgG:CD27 status for the Seurat clusters identified in 5.11. (B) Bait signal by IgD:CD27 status, with CASPR2-PE on the x-axis and CASPR2-BV711 on the y-axis. (C-D) Bait signal by IgD:CD27 status for both bait constructs showed highest reactivity in the $IgD^{NEG}CD27^{POS}$ population. (E-F) Domain reactivity by IgD:CD27 status for CASPR2 patients (E) and healthy controls (F)

Distinct BCR sequence characteristics between patients and healthy controls

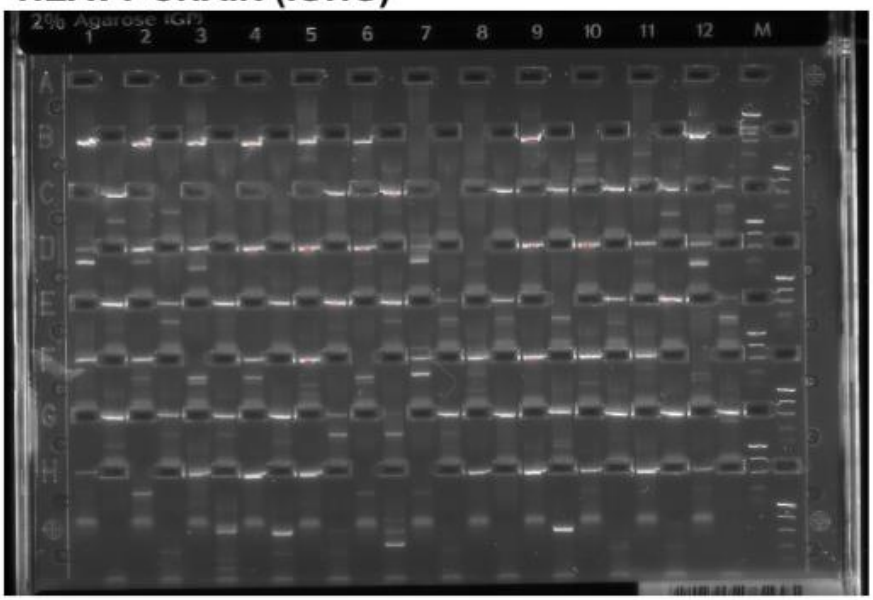
I next turned to examining the BCR variable heavy and variable light chain sequences for the CASPR2-positive cells. (Figure 5.16) Initial Sanger sequencing results revealed an interesting observation whereby a notable number of samples (40.37%) yielded two light chain sequences, (Figure 5.17B) which could also be observed individually for CASPR2 patients only (41.02%) (Figure 5.17D) and healthy controls only (39.78%) (Figure 5.17F). Heavy chain distribution revealed most sequences to contain IgG constant regions (88.04%), with some having the IgM constant region (4.11%), and some having both IgG and IgM (5.42%), likely indicating some class-switching occurring in vitro. (Figure 5.17A) This too was consistent across patients and healthy controls. (Figure 5.17C,E) The observation of a prominent presence of cells expressing both a kappa and a lambda light chain was an interesting one, but it was still possible that one of the two light chains was not actually expressed at the protein level due to the sequence being unproductive. The trend persisted once sequences were run through IgBLAST, with ~30% of cells having two productive light chains (Figure 5.17G). The overall sequencing returns were over 63% productive paired heavy and light chain sequences, comparable to previous work done in the group. Looking at the light chain distribution between patients and healthy controls for samples where a single productive light chain sequence was detected (roughly 57% of all productive sequences), healthy controls had a roughly equal representation of both kappa and lambda light chains, but in patients the kappa light chain was twice as prevalent as the lambda among CASPR2-reactive BCRs. (Figure 5.17H)

BCR Clonal Analysis

Examining BCR clonotypes revealed no BCR clonotype expansion in healthy controls (not shown) but prominent clonotype expansion in patients JR1284 and JR794, and somewhat less so in patients JR1300 and JR1307. (Figure 5.18) Interestingly, patients JR1284 and JR794 were also untreated at the time of sample collection, while JR1300 and JR1307 had received disease-modifying treatments (steroids or cyclophosphamide + rituximab). We wondered whether BCR clonotype expansion was a general feature of autoimmune disease, or this was an observation specific to CASPR2-positive cells. To address this, we selected CASPR2-negative cells with similar FACS phenotypes to the CASPR2-positive B-cells from patients and healthy controls. (Figure 5.19F) These '$CASPR2^{NEG}$' B-cells had their BCRs sequenced in the same way as $CASPR2^{POS}$ B-cells. Sequence analysis revealed BCR clonotype expansion to be restricted to $CASPR2^{POS}$ cells in patients. In healthy controls, there was little to no clonal expansion, with no difference between $CASPR2^{POS}$ and $CASPR2^{NEG}$ cells. This revealed BCR clonotype expansion could be a feature of CASPR2-reactive B-cells uniquely seen in the disease state (Figure 5.19A-D). We were also interested in how BCR mutation rates differ between patients and healthy controls. Mutation rates were examined relative to CASPR2 CBA score, domain specificity, and Seurat cluster (Figure 5.20A-C). Nearly without exception, mutation rates were higher among patients than healthy controls across all these categories. Plotting mutation rates by IgD:CD27 status showed lowest mutation rates in the CD27-IgD+ (naïve) population and highest in the CD27+IgD- (switched memory) population (Figure 5.20D), as expected.

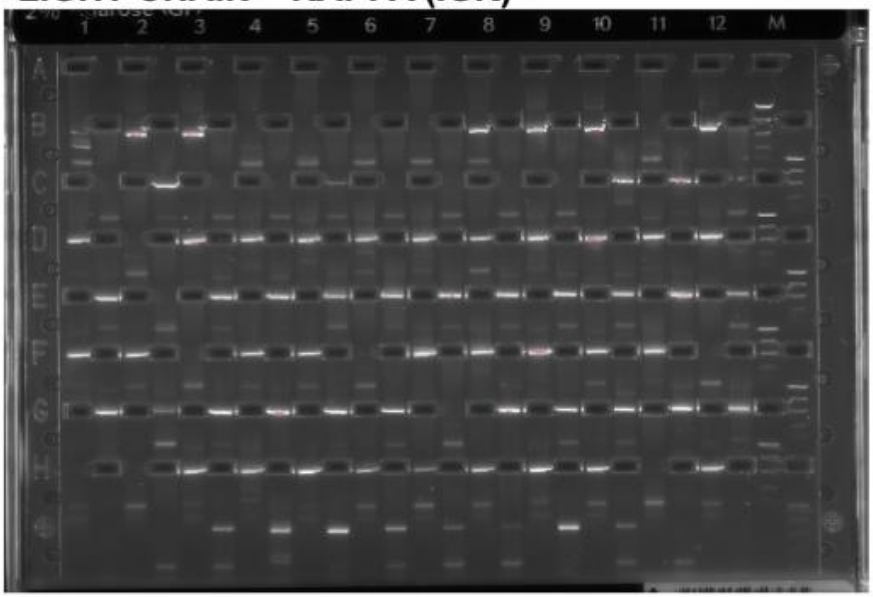
A

HEAVY CHAIN (IGHG)



B

LIGHT CHAIN – KAPPA (IGK)



C

LIGHT CHAIN – LAMBDA (IGL)

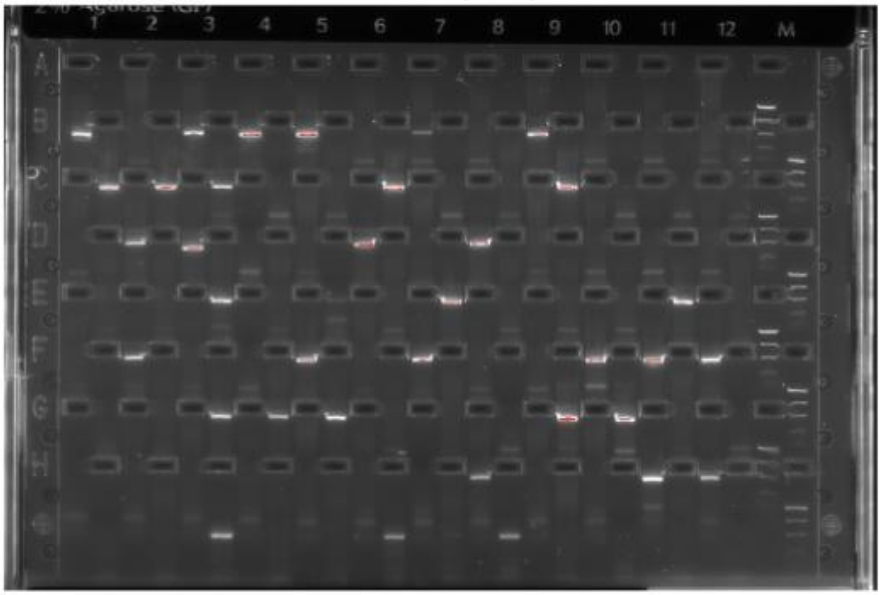


Figure 5.16

Sample scans of DNA electrophoresis gels using PCR product following second stage of nested PCR for IgG heavy chain IGHG (A), kappa light chain IGK (B) and lambda light chain IGL (C) for a single 96-well plate

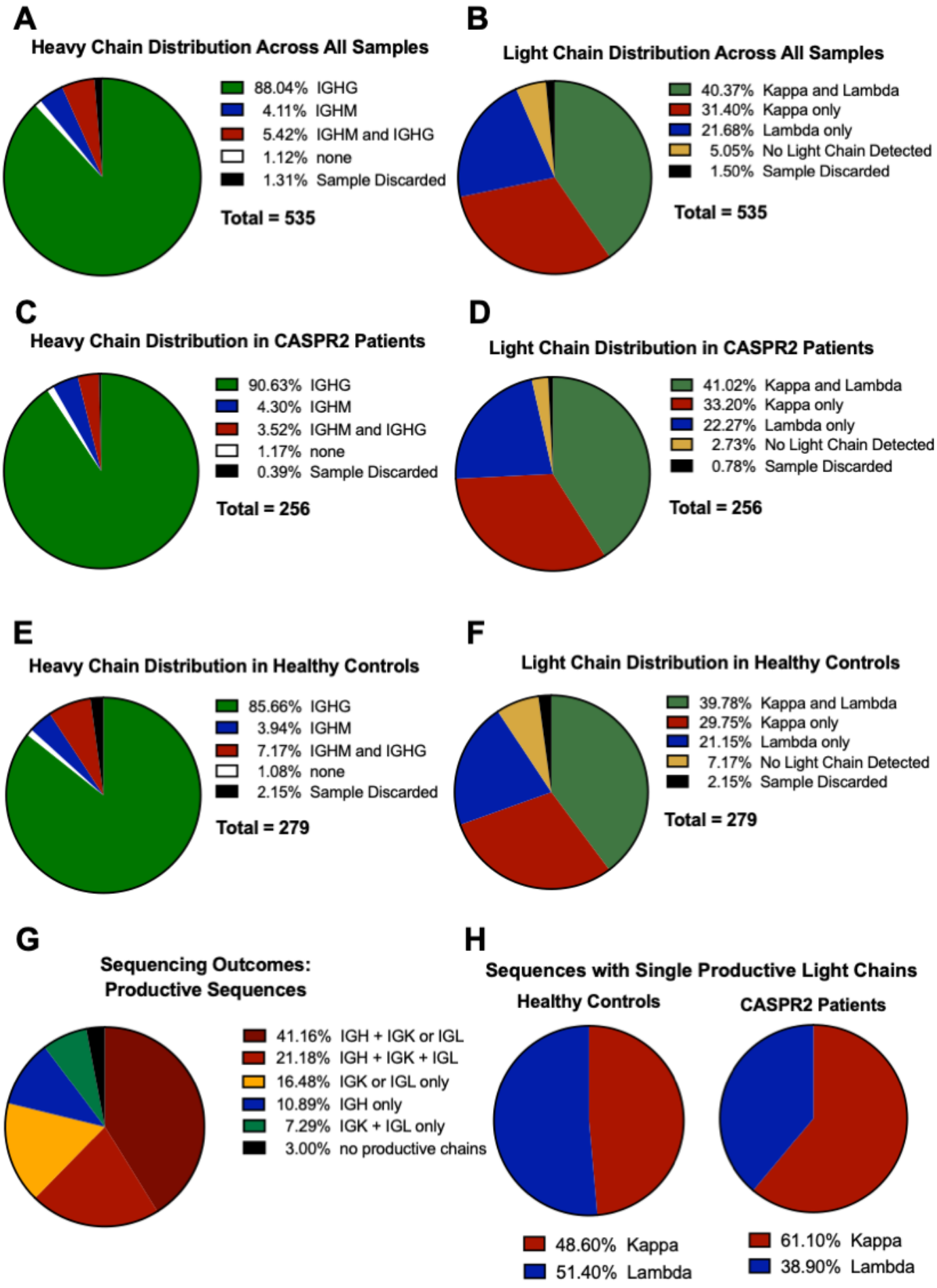


Figure 5.17 Distribution of IGHG and IGHM heavy chains and IGK and IGL light chains across entire dataset (A-B), in CASPR2 patients only (C-D) and in healthy controls only (E-F) as determined based on Sanger sequencing outcomes. (G) Final productive sequences outcomes following IgBLAST analysis. (H) Number of kappa vs lambda productive light chains in healthy controls vs CASPR2 patients

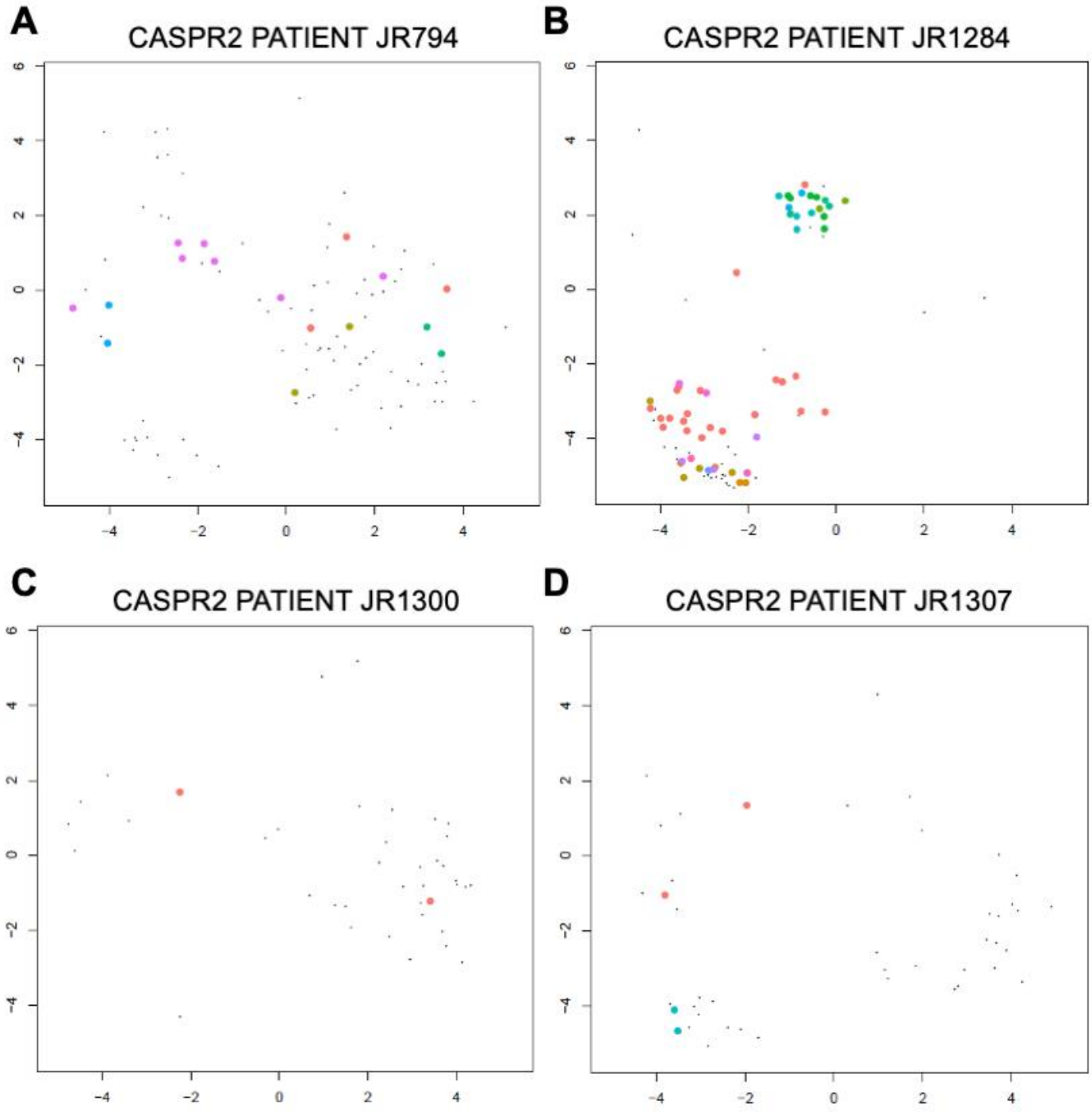


Figure 5.18 BCR clonotype expansion in CASPR2 patients (A) JR794, (B) JR1284, (C) JR1300, and (D) JR1307. Prominent BCR clonotype expansion was more evident in the untreated patients. No expanded CASPR2-positive BCR clonotypes were found in healthy controls (data not shown) BCR clonotype data is presented superimposed upon Seurat Cluster plot (bottom right corner)

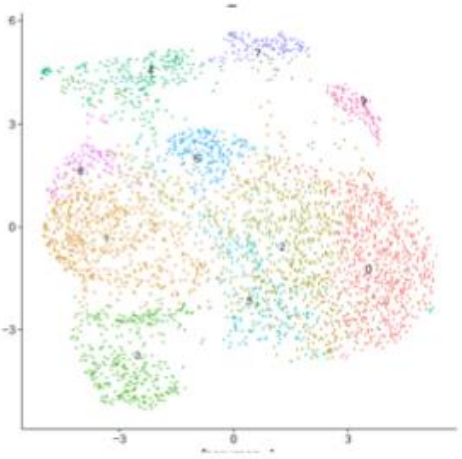
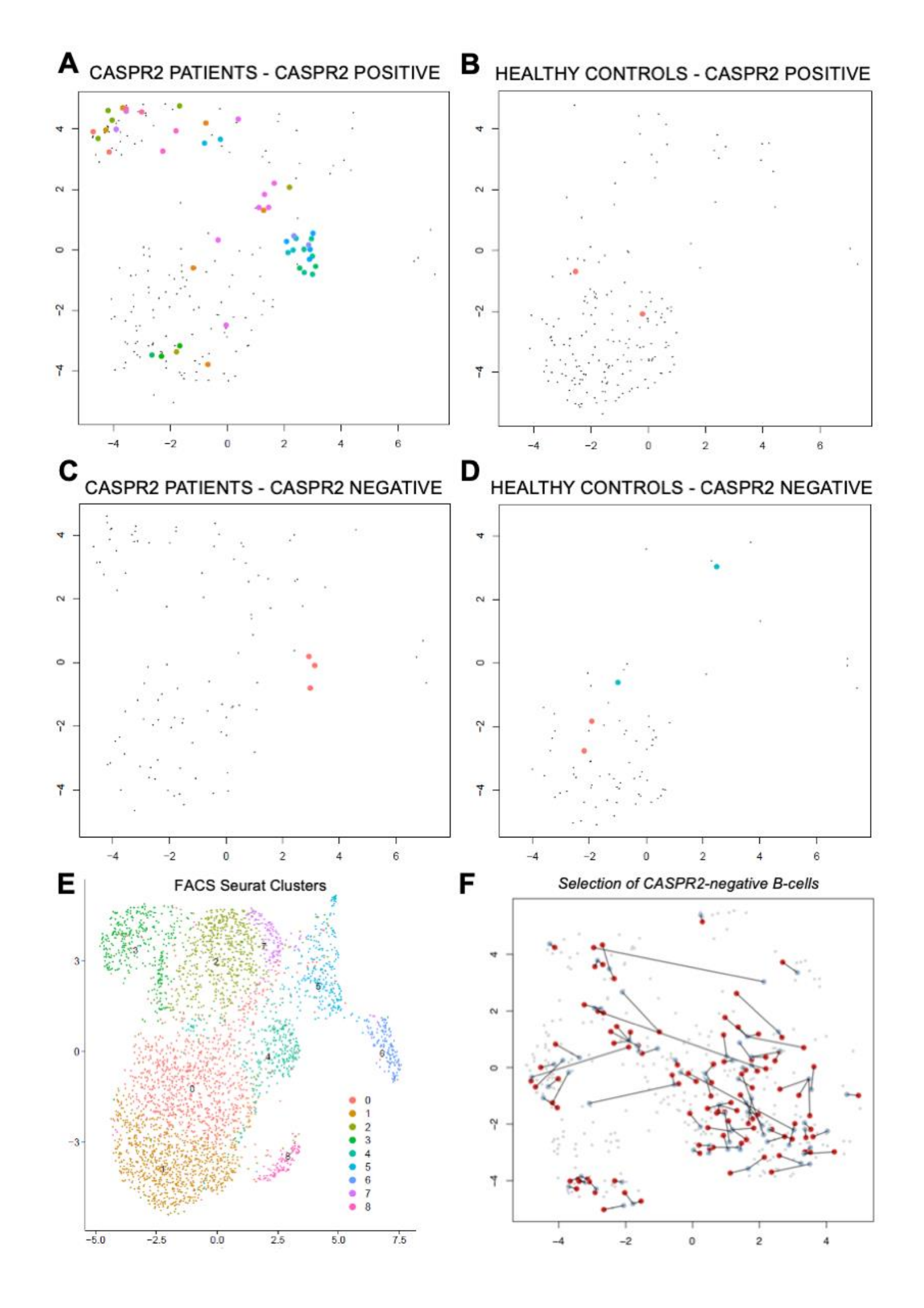


Figure 5.19 ***(next page)*** (A-D) Clonal networks as defined by identical paired heavy and light VJ genes for CASPR2 positive (A-B) and CASPR2-negative cells (C-D) in CASPR2 patients (A,C) and healthy controls (B,D). Clonal network information was superimposed on FACS Seurat cluster UMAP plots (E). (F) Strategy for selection of CASPR2-negative control B-cells based on similarity of FACS phenotypic data, visualised here as proximity on the UMAP plot.


A
CASPR2 PATIENTS - CASPR2 POSITIVE
B
HEALTHY CONTROLS - CASPR2 POSITIVE
C
CASPR2 PATIENTS - CASPR2 NEGATIVE
D
HEALTHY CONTROLS - CASPR2 NEGATIVE
E
FACS Seurat Clusters
0
1
2
3
4
5
6
7
8
F
Selection of CASPR2-negative B-cells

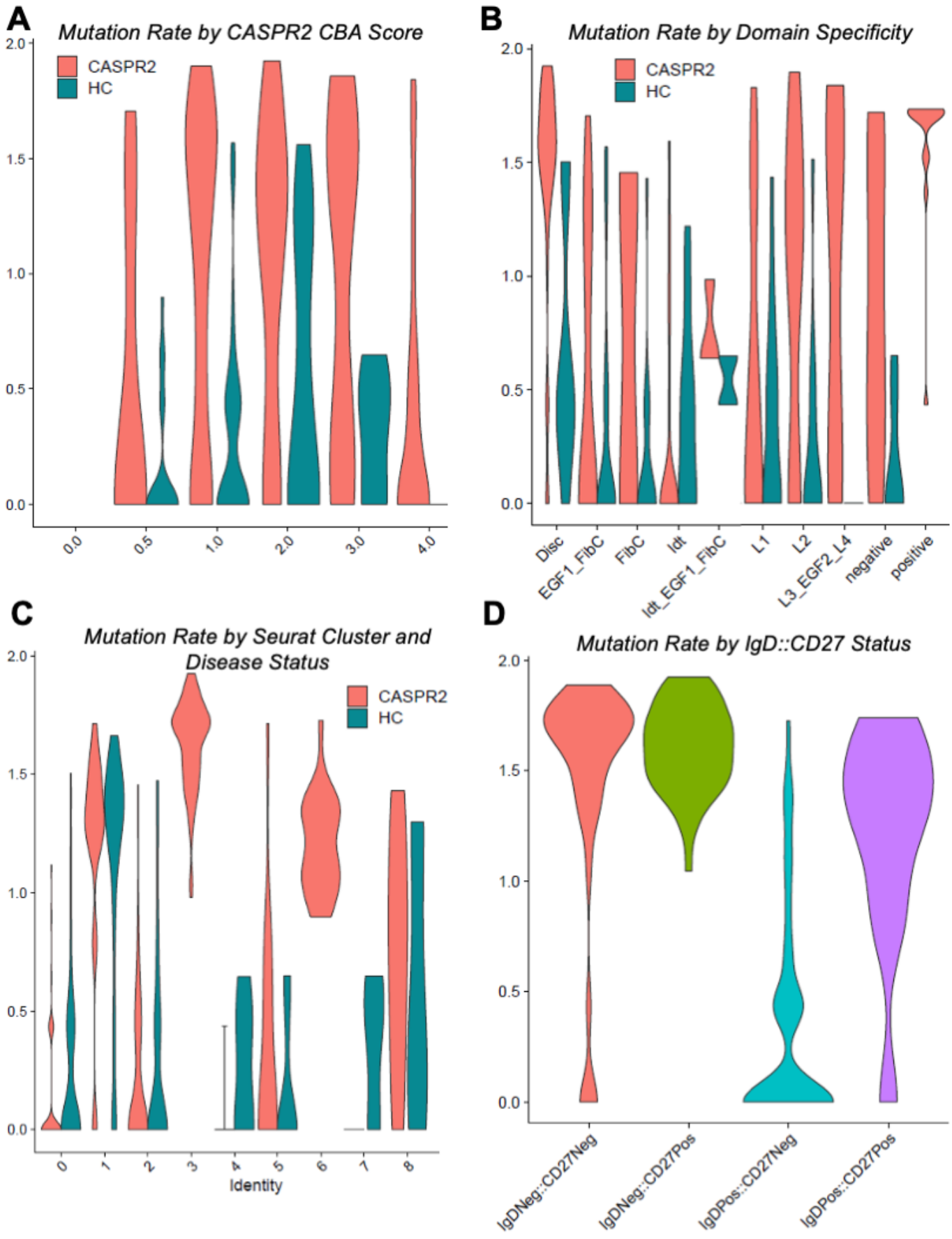


Figure 5.20 (A) BCR mutation rate expressed as log10 of mutations per kilobase pairs in the IGH, IGK and IGL variable regions plotted by CASPR2-CBA score for patients (orange) versus healthy controls (green). Mutation rates were then plotted by B) domain specificity, (C) Seurat cluster identity and (D) IgD:CD27 status for patients (orange) and healthy controls (green)

DISCUSSION

In this chapter, I have used a single B-cell culture experiment to investigate the frequencies, phenotypes, and antibody and sequence characteristics of CASPR2-specific B-cells in both patients with CASPR2-antibody encephalitis and healthy controls. I have demonstrated the existence of permissive early B-cells tolerance checkpoints that allow for the formation of a CASPR2-reactive repertoire among naïve B-cells in both groups. However, only in patients was CASPR2-reactivity also prominently observed among switched and late memory B-cells, suggesting a disease specific failure in post-germinal centre B-cell tolerance mechanisms. Clinically, this finding suggests the natural presence of circulating autoantigen-reactive B-cells which, upon patient-exclusive permissive class-switch events that allow for their activation, may become a source for persistent de novo generation of autoantibody-secreting cells.

The study is the first of its kind in the Caspr2 field and demonstrates a generally unprecedented capacity to isolate and characterise autoantigen-specific B-cells in autoimmune neurology. One of its strengths has been the selection of patients: both treated and untreated, both more and less genetically susceptible, and with a range of clinical features and disease durations. Inclusion of age- and sex-matched healthy controls, as well as CASPR2-negative B-cells matched on the FACS data in both the patients and the healthy donors added another layer of elegance to the study design where we could interrogate which B-cell features were disease state-specific, and which ones reflected CASPR2 reactivity. This was especially important considering CASPR2-reactive B-cells were also identified in healthy donors. The use of a 'double-bait' system allowed for pre-enrichment and culture of over 500 CASPR2-specific B-cells split roughly equally between patients and healthy controls. A streamlined protocol where the single cell sort was followed by antibody screening and BCR sequencing generated a wealth of information about the character of the autoreactive B-cells and their cognate antibodies that could then be related back to disease status.

The use of a bait system allowed isolation of an incredibly large number of rare antigen-specific cells. However, it also resulted in one of the major caveats and potential disadvantages of our study, which is that all B-cells sorted and cultured had been pre-enriched for reactivity to the CASPR2-bait constructs. Not only did this mean that many cultured cells did not bind Caspr2, it also means we cannot use our data to draw broader conclusions about the frequency and abundance of Caspr2-reactive B-cells within the B-cell repertoire, as our approach was biased. As the chapter introduction discussed, there are two main approaches to isolating autoantigen-specific B-cells: initial pre-selection of antigen-specific B-cells using the target antigen as bait or unbiased culturing and later cloning of all sorted B-cells (Chapter 4). As exemplified in this thesis, the latter allows global and impartial sampling of the B-cell and antibody repertoire but may also mean that very few or indeed no target-specific antibodies are identified. The former on the other hand can serve to statistically increase the chance of obtaining disease-specific antibodies while reducing the workload but may impose a selection bias on the B-cells isolated, or indeed miss certain reactivities due to the antigenic bait not retaining the conformation of the native protein. Earlier work in the lab using an unbiased approach identified only 0.2-0.4% of CASPR2-reactive B-cells from the sort, whereas in our study the frequency of CASPR2-reactive B-cells was higher than 14%.

The discovery of CASPR2-reactive naïve B-cells in both the patients and healthy controls was a surprising one, but it is worth mentioning that a degree of autoreactivity has been observed in ~20% of the naïve B-cell subset in healthy donors, probably a natural consequence of the semi-stochastic nature of VDJ-recombination.[476] Indeed, the level of CASPR2 reactivity seen

among healthy donors here was roughly on the same scale. However, two features of the study mitigate the risk of conflating the aforementioned naïve B-cell autoreactivity phenomenon with genuine specificity for CASPR2: (1) the use of a double-bait system, which increases the likelihood of specific binding and (2) screening for reactivity to LGI1 and five CASPR2 deletion constructs, which allows for identification of BCRs with indiscriminate binding.

Another potential disadvantage of using the bait constructs as opposed to an unbiased sorting approach is the use of a soluble version of the extracellular domain of an otherwise membrane-spanning protein. The construct does not include the transmembrane anchoring portion of the protein, which may convey small but biochemically relevant modifications to protein structure. This in turns might miss some CASPR2 antibodies, as has been shown for the MOG antigen where an extracellular domain of the protein used for affinity purification of anti-MOG antibodies was unable to bind all serum anti-MOG antibodies that were detectable by CBA.[477] This could explain why we didn't get many antibodies against the L3/EGF2/L4 domains, which are closest to the transmembrane region. The experiment which yielded L3 antibodies (Chapter 4) relied on unbiased sorting and identification by CBA, although it identified fewer antibodies overall. To circumvent these disadvantages of the bait method, a more recent technique adopted in the field has been membrane antigen capture activated cell sorting (MACACS), which makes use of the ability of B-cells to bind and internalise antigen. The fluorescently tagged antigen is expressed on the surface of a target cell line and exposed to patient B-cells, which upon antigen internalisation can be identified via flow cytometry. This approach has already been used for transmembrane antigens that would otherwise be difficult to bait due to a complex tertiary and quaternary structure, such as the acetylcholine receptor.[472]

The discovery of differential domain prevalence among patients and healthy controls was an interesting and surprising one. In concordance with their immunodominance among serum samples, the discoidin and L1 domains were the most frequently targeted by CASPR2-reactive B-cells from patients. The L3/EGF2/L4 domains, despite occupying a non-negligible portion of the protein, were the least frequently targeted by autoreactive B-cells in patients. This finding comes as a surprise considering an L3 domain-specific patient-derived mAb was the centrepiece of Chapter 4, demonstrating high pathogenic potential across multiple assays. It is possible that L3/EGF2/L4-reactive cells are rare but have high affinity for the protein, but this was not substantiated by SPR experiments, which identified the discoidin-reactive antibodies to have the highest affinity. A more likely explanation was the difference between the way CASPR2-reactive B-cells were identified: in the previous chapter cells were sorted using an unbiased approach and then screened on CASPR2 while here cells were pre-selected using the CASPR2-bait constructs. Considering the CASPR2 extracellular domain was linked to the rest of the bait construct via the C-terminus end, it could be that the L3/EGF2/L4 domains, which are nearest to the C-terminus, are partially obscured by the remaining bait components due to steric hindrance, thus making it less likely for cells reacting to these domains to be picked up during the sort.

The discoidin domain has been the 'standout' of this chapter's work, but it is important to consider that *in vivo* this domain might be involved in interactions with Caspr2's expression partner, contactin-2, or alternatively obscured via the extracellular domain of Caspr2 folding on itself, therefore not actually being available to Caspr2-antibodies.[316, 334] Based on the data in Chapter 5, I propose a mechanism whereby early immune tolerance checkpoints eliminate discoidin-reactive B-cells either completely or nearly completely in patients with autoimmune encephalitis and in healthy controls, hence their notable rarity in the naïve B-cell compartment in both groups. However, in patients with Caspr2-antibody encephalitis, and even more so in

those that are carriers of the DRB1*11:01 allele, later immunisation events occur, likely involving CD4+ T-cell-mediated MHC class II-dependent help, that lead to the development of discoidin-reactive B-cells in the late memory and switched memory subset. Persistence of this subpopulation of cells in the circulation might explain the immuno-dominance of the discoidin domain in patient sera, as well as the highest affinities and mutation rates among discoidin-reactive BCRs, demonstrated in this chapter. However, the question remains of whether the discoidin-autoantibodies can access their target and elicit pathogenic effects in vivo. As shown in the previous chapter, while both a discoidin and an L3 mAb could disrupt Caspr2 function in a neuronal culture model, it was only the L3 antibody that could reproduce features of the disease in an animal model.

While this chapter poses more questions than it answers, the data presented here provides a hitherto unseen resolution and detail in the study of Caspr2-antibody encephalitis and makes some important clinical discoveries. This includes the observation of BCR clonal expansion in Caspr2 patients but not healthy controls, which among patients was seen to be more prominent in those who had been untreated at the time of sample collection. The clinical significance of several other findings, such as the predominance of the kappa over the lambda light chain among Caspr2-reactive BCRs in patients, remains to be elucidated in the future. For the time being, I hope that Chapter 5 has provided both a rationale as to why and a template as to how rare autoreactive B-cells in autoimmune neurology could be studied on the single-cell level.

CHAPTER 6

DISCUSSION

Preface

Each data chapter within this thesis was followed by its own discussion section where the results were further elaborated upon and interpreted, and had their value and significance discussed. Here, I will briefly summarise the key findings once more, and then discuss their significance, especially considering what is already known in the Caspr2-antibody encephalitis field. Then, I will move on to the main shortcomings of this thesis, many of them already mentioned in the respective data chapter discussions. Finally, I will make proposals for how this work could be developed further, highlighting potential future experiments.

6.1 Summary of Findings

In Chapter 2, I have described an experiment that investigated the subclass prevalence among Caspr2-specific autoantibodies and attempted to relate this to features like CASPR2-IgG titre, clinical phenotype, and exposure to immunotherapy. Caspr2-specific autoantibodies were found to be overwhelmingly of the IgG4 subclass in our cohort, with most samples having only CASPR2-IgG4, and the IgG4 subclass being more prevalent among those patients who had both the IgG1 and IgG4 subclass. The remaining two IgG subclasses, IgG2 and IgG3, were not investigated. In contrast to existing studies on this topic which predominantly relied on cell-based assays employing subclass-specific secondary antibodies, thus reporting only the mere presence or absence of each subclass, our study relied on a flow cytometric method which allowed us to not only detect, but also quantify the relative levels of each subclass. This allowed me to plot the levels of CASPR2-IgG1 and -IgG4, as well as CASPR2 -IgG over time, though no relationships between the three variables and disease course was revealed. However, grouping patients into those who only had CASPR2-IgG4, and those who at any point throughout the course of their illness has CASPR2-IgG1 revealed that while the former group was more frequently associated with neuromyotonia, the latter was more frequently associated with ataxia. Such a clinical association had previously not been found in the Caspr2-field, and its mechanistic basis remains to be elucidated.

In Chapter 3, I used the sera of two patients from the cohort presented in Chapter 2, purified total immunoglobulin G from these samples and attempted to use it to develop a passive-transfer animal model in experimental mice. To this end, I injected purified IgG from the patient samples or from healthy controls bilaterally into the CA3 region of the hippocampus under stereotactic guidance and followed this up with a plethora of behavioural tests at various time points post-injection. Reproducing any aspects of the disease in the mouse model proved to be no trivial pursuit, and it became clear very early on that CASPR2 IgG failed to produce cognitive deficits in the mouse, as evidenced via performance on the spatial, working, and object-based memory tasks. By contrast, the injection of purified IgG *did* alter performance on assays for anxious behaviours, such as the open field, light-dark box, and elevated zero maze. Ironically, these tasks were initially included in the testing battery as 'controls' for the cognitive-based tasks, as my initial hypothesis was that CASPR2-IgG would produce memory deficits but have no effects on anxiety levels. The 6-hours' time-point post-injection seemed to be crucial for the manifestation of the phenotype and the two patient samples used to develop the model interestingly produced different behavioural effects. I have summarised my passive transfer model as an acute behavioural change following a surgical procedure which delivers a small but physiological level of Caspr2-autoantibodies, in contrast to previous publications on this subject which tended to employ chronic systemic administration of supraphysiological levels of antibody. While demonstrating that patient Caspr2-autoantibodies do have pathogenic potential, this chapter did not answer the question of *how* this is achieved, crucially because polyclonal serum is not an ideal tool for such investigations. This matter was addressed in the next data chapter with the use of patient-derived monoclonal antibodies.

In Chapter 4, myself and others attempted to dissect the pathophysiological basis of Caspr2-antibody encephalitis with the use of patient-derived mAbs, some of them even cloned from the peripheral B-cells of the patient whose immunoglobulin was used to develop the passive-transfer animal model in Chapter 3. Many challenges popped up in this pursuit, to be expected considering no prior attempts had been made in the field to generate and characterise Caspr2-specific mAbs. The mAbs were generated using a single-cell culture following an unbiased B-cell sort from two patients, and initial experiments aimed to characterise their domain specificity, binding strength, and reactivity to rodent CASPR2, tissue sections, and neurons in

culture. Collaborators examined the effects of the mAbs on cortical neurons in culture, showing that two mAbs (H01 and E08) altered the levels of Caspr2 and AMPA receptors and influenced synaptic firing. My work aimed to examine mAb pathogenicity in vivo, and indeed mAb E08 was found to produce effects comparable to that of polyclonal IgG in a mouse model. A different mAb, E07, was found to recruit neutrophils to the hippocampus at the same time as the behavioural effects observed. Although this was seen in a very limited sample size, I have speculated an immunological basis for the altered behaviour. The demonstration that the mAbs affect AMPAR levels in cortical neurons could also suggest that disrupted glutamatergic neurotransmission, mediated by reduced AMPAR currents and/or AMPAR expression levels, could underlie the behavioural changes observed both with mAbs and with polyclonal immunoglobulin G. Of course, substantiating this claim would require several additional experiments, discussed later.

Finally, in chapter 5, I turned my attention from the antibodies to the B-cells responsible for their production and conducted a single B-cell culture experiment which allowed me and others to characterise native B-cell reactivities and BCR genetic sequences and relate these to B-cell phenotype. The cells were selected for using a soluble form of the extracellular domain of Caspr2 as antigenic 'bait'. This sort of experiment has hitherto not been done in the Caspr2 field, and as such the research was led primarily by a 'hypothesis blind' approach wherein we aimed to gather as much information as possible on the Caspr2-reactive B-cells in both patients and healthy donors. The most surprising and interesting finding of this chapter was that Caspr2-reactive B-cells were present in the naïve B-cell repertoire even among healthy donors but, in contrast to what is observed in patients, these cells never develop into antibody-secreting plasma cells or memory B-cells. Several curious 'minor' findings accompanied this 'major' finding of Chapter 5. Perhaps the most intriguing one was that discoidin-reactive B-cells seem to follow different trends from B-cells with reactivities to the remaining Caspr2 domains, likely following a distinct developmental pathway that underpins the immunodominance of the discoidin domain in patient sera, also demonstrated here for the patient B-cell supernatants. The significance of this from a pathophysiological standpoint is covered in the Discussion section of Chapter 5.

A major question that emerges is how the findings in Chapter 5 fit in with those from the remaining chapters. While the in vitro experiments described in Chapter 4 did find both a discoidin and an L3 mAb to alter cortical neuron function, only for the L3 mAb (E08) did this carry forward in the animal model. How do we reconcile these seemingly conflicting findings? My argument would be that 'uniqueness' does not necessarily signify 'pathogenicity'. While demonstrating the highest affinities and mutation rates in our dataset and being the domain reactivity that most clearly separates patients from healthy donors, it is possible that discoidin-reactive antibodies are not actually pathognomonic and, while present at high levels, may not actually access their target in vivo due to its obscurement by the Caspr2 interaction partner contactin-2. This could explain why neither their presence, nor the titres of Caspr2 autoantibodies in general, seems to correlate with disease severity in patients. Conversely, discoidin-specific B-cells and discoidin antibodies may be the ones most likely to be picked up both by our Caspr2-bait setup and by Caspr2 cell-based assays respectively, both of which employ artificial conditions that may not necessarily reflect the native conformation and interactions of the protein. By contrast, antibodies targeting the L3 domain may be less prevalent (or, crucially, less detectable by standard diagnostic or experimental means) but carry much more weight in the disruption of Caspr2 function. Of course, current clinical approaches rarely investigate the domain immunoprevalence in patient samples, and perhaps one of the key takeaways from this thesis is that this could provide valuable insight into the disease.

Another fascinating observation in data chapter 5 that warrants further investigation is the contrast in clonal expansion among Caspr2-specific B-cells between patients and healthy donors, as well as between treated and untreated patients – with healthy donors and treated patients showing virtually no expansion of the BCR clonotypes. Not only could this explain why healthy donors do not exhibit pathology despite presence of Caspr2-reactive B-cells, it could also provide resolution to the perennial conundrum of how treatment seems to eliminate symptoms in patients without necessarily reducing Caspr2 immunoglobulin titres.

6.2 What have we learned? Significance for Caspr2- and CNS-(auto)immunity

With the summary and commentary in the previous section in mind, how has the work presented in this thesis advanced our understanding of Caspr2-antibody encephalitis and contributed to the field as a whole? As emphasised multiple times across this text, virtually none of the experiments listed here have been done in the field before, so nearly all results presented are novel by default, but this of course does not guarantee they would be significant. The key contribution of this thesis to the field of auto-antibody-mediated neurological diseases is exploring directly the question of to what extent Caspr2 autoantibodies are pathogenic. As discussed in the thesis introduction, this matter has been called into question recurringly since the first characterisation of Caspr2-antibody encephalitis. In comparison to autoimmune neurological diseases where other neuronal proteins are targeted, Caspr2 has insofar not had its time in the spotlight, especially in regards to the use of monoclonal antibodies to study disease pathology, as has been done for targets like LGI1, NMDAR and even the GABA receptor. Furthermore, the studies done with polyclonal patient sera were either focused solely on pathology of the peripheral nervous system or, when addressing the CNS, were limited in scope or found little to no effects of the antibodies. Consequently, it was evident that interest in the field had begun to decline, perhaps overtaken by attention being increasingly devoted to other targets. Indeed, most Caspr2-related studies being published in the past several years had a clinical perspective, with very limited original basic science research. Hopefully the findings reported in this thesis could contribute towards reversing this trend and further fuelling interest in Caspr2 pathology, by demonstrating that Caspr2-autoantibodies are indeed disease-relevant. As early as Chapter 2, it was shown that Caspr2-autoantibody subclass prevalence might be of clinical relevance and relate to overall Caspr2-IgG levels, while at the same time not being affected by immunotherapies. The method for quantifying levels of Caspr2-autoantibodies shown here could also find applications in diagnostic studies as it allows for much higher-throughput, reproducible and quantitative detection of target-specific antibodies, and further allows characterisation of the autoantibody subclass within the same assay.

Next, Chapters 3 and 4 showed that Caspr2-autoantibodies isolated from patients have some pathogenic potential in vivo, although perhaps not in ways one might have anticipated. Crucially, the two chapters employed vastly different means of accessing the antibodies: in the former antibodies were isolated directly from serum whereas in the latter they were obtained indirectly, by cloning the BCRs of Caspr2-specific circulating B-cells isolated and cultured from patient PBMCs. The fact that congruent results were obtained across two very different experimental modalities lends further support to the claim that Caspr2-specific antibodies are disease-contributors and not just mere bystanders in this subtype of autoimmune encephalitis. This is not to say that the results in these two chapters were in any way foreseen or predictable: perhaps one of the biggest surprises of Chapter 3 was that serum antibodies had no effects on mouse memory performance but did increase anxiety-related behaviours (the opposite direction of effects was hypothesised prior to starting the experiments), and even seemed to produce a hyperactive phenotype. In Chapter 4, an L3-specific antibody and not a discoidin-specific one induced behavioural deficits in the mouse model (once again, the inverse was

postulated prior to starting experiments), but the size of this effect was overshadowed when the mAb-injected mice were compared to naïve controls. While Caspr2-antibodies had been suspected to confer their effects via disruption of AMPA receptor function, it had not been shown before that mAbs targeting different domains of the protein could have opposing effects on AMPARs, and whether these influences may 'cancel each other out' in vivo remains to be seen. Finally, recruitment of innate immune cells by an antibody targeting neuronal proteins has previously not been shown for any other neuronal antigenic target and thus constitutes an entirely novel finding of this thesis that has to nonetheless be confirmed with a larger sample size and more extensive quantification. Altogether, these findings point to a pathophysiological mechanism whereby the acute presence of the Caspr2-antibodies in the CNS parenchyma leads to short-lasting and reversible behavioural changes manifesting in the form of anxiety and hyperactivity, that could possibly be mediated by disrupted glutamatergic neurotransmission and recruitment of innate immune components.

Finally, Chapter 5 has shown a 'first of its kind' study of Caspr2-reactive B-cells, the scale and extent of which is a novelty both in the specific area of Caspr2-antibody encephalitis and more widely for the field of autoimmune neurology. Besides the unique insights offered by the study design, the experiment I've described here could serve as a template on how to study target-specific B-cells in related autoimmune neurological diseases. The findings also lend credibility to the well-researched genetic association of Caspr2-antibody encephalitis, showing clear distinctions between carriers and non-carriers of the HLA-DRB1*11:01 allele. Furthermore, a very clear and neat effect of disease-modifying treatment is observed at the single cell level. The results indicate that an innate susceptibility to development of Caspr2-specific B-cells exists both in patients and in healthy donors, but only in the latter do these autoreactive cells fail to progress to latter developmental stages. This indicates breakdown in peripheral tolerance checkpoints and maturation in germinal centres, possibly with CD4+ T-cell involvement, as the drivers of Caspr2-autoimmunity. A possible further significance of this finding could be the refinement of current immunotherapies to target only memory B-cells and plasma cells, thus sparing the naïve B-cell population. The first three data chapters also bear some clinical or diagnostic significance, suggesting a possible benefit of testing for subclass prevalence and domain specificities among anti-Caspr2 antibodies in patient samples.

While pointing out the contributions of this thesis to the field of Caspr2-autoimmunity has been fairly straightforward, perhaps due to its relative novelty and narrowness, pinning down what these findings mean within the wider context of CNS-(auto)immunity has proven to be slightly more challenging. For one, the animal models developed here were of the passive-transfer kind (Chapters 3 and 4), whereby a ready-made purified antibody is injected into the CNS. This is almost like a 'snapshot' of the full disease process, whereby the steps that actually lead to immunisation against a neuronal target are circumvented and only the final result of a pathological immune response is observed. What's more, the section of this thesis that *does* address how these immunological agents of destruction might have come to be by taking a look at their parent B-cells (Chapter 5), only samples cells from the peripheral blood of affected patients and controls. Whether these autoreactive B-cells ever have or ever will reach the CNS is not known, nor is the manner in which they might have first encountered their target and 'communicated' its detection to other immune cells, CNS-resident or otherwise.

It may be frustrating to not have answers to some of the fundamental questions regarding the origins, development, and persistence of this (and other) autoimmune diseases of the nervous system. At the same time, this permits a certain degree of creativity and lateral thinking, allowing us to employ what we know about CNS immune privilege (or the lack thereof) and

apply it, along with some of the findings in this thesis, in our attempt to understand the chief players in neurological autoimmunity. The thesis Introduction (Chapter 1) covered in a lot of detail the various cellular and molecular players that orchestrate CNS immunity, but how do these pertain to Caspr2-antibody encephalitis? Past literature has informed that dendritic cells and macrophages are rarely seen in the uninflamed brain parenchyma, but readily inhabit the neuroimmune interfaces such as the meninges, choroid plexus, and CSF. Furthermore, the CNS parenchyma does not harbour any resident T- or B-cells, and while it is rich in resident cells such as microglia and long-lived macrophages in the perivascular and subarachnoid spaces, these rarely migrate to CNS-draining lymph nodes the way resident immune cells in peripheral tissues tend to. How then, would immunity against Caspr2 arise? And do any of the findings of this thesis aid in addressing this question?

From Chapter 5, we have learned that Caspr2-autoreactivity can be observed even in the B-cell repertoires of healthy people, but only among naïve 'antigen inexperienced' B-cells. There exists therefore, one may posit based on this finding, a potential to develop reactivity against this neuronal target even within individuals who don't eventually develop disease. On the other hand, studies have demonstrated that it is possible for the extracellular domain of the Caspr2 protein to be 'shed' from neuronal cells in vitro, and detected within the surrounding medium. While the significance of this mechanism and even its validity in vivo are far from established, it is conceivable that rather than the immune cell coming to Caspr2, the protein may make its way to the cell. The pathways of 'brain drain' and antigenic shuttle into the cervical lymph nodes were extensively described in the thesis Introduction and it is possible that during its transit through one of these alleyways, the Caspr2 protein encounters a keen immune cell, be it a dendritic cell, a helper T-cell, or perhaps even one of the rare Caspr2-reactive B-cells that might inhabit the neuroimmune interfaces. Keeping in mind the extreme rarity of this and other autoimmune neurological conditions, there exists a minuscule (but non-negligible) chance that the selection and maturation of the pathogenic B-cells is in fact a random event that is the result of an accidental encounter between a naïve B-cell and its target neural antigen.

The occurrence of this 'encounter' might be completely by chance, yet its likelihood could be increased by events such as an injury, stroke, viral infection, a state of neuroinflammation or indeed old age. What follows after could be the chief factor that separates those who do from those who do not develop disease. While the occurrence of B-cell receptors that recognise the Caspr2 protein might be an innate 'error' of early immunological development and the encounter of such B-cells with their target antigen might be a random event whose incidence is exacerbated by other biological processes, the key is whether the B-cells with the potential to become pathogenic are allowed to further develop and secrete autoantibodies. Here, it is probably the contribution of CD4+ T-cells, regulatory T-cells and lymph node-resident helpers such as follicular dendritic cells that either prevents or encourages somatic hypermutation, affinity maturation, and clonal expansion, thus defining the border between health and disease. In particular, the known HLA-associations of Caspr2-antibody encephalitis provide support for a key role of T-cells and MHC class II molecules in the disease. Sadly, the roles of immune cell populations other than B-cells, both within the brain and outside of it, remain woefully understudied in Caspr2-AE and the related encephalitides. While this thesis has not filled these particular knowledge gaps, the extent to which the relevant B-cells and antibodies in Caspr2 disease have been characterised here hopefully leaves enough space and provides enough questions for future studies to branch into other cell populations and immune molecules. Apart from its focused examination of only a single cell population and immune molecule within a disease that possibly has many other interacting contributors, this thesis has several other shortcomings, discussed in the next section.

6.3 Shortcomings of this thesis

The previous two sections have hopefully highlighted both the importance and the novelty of the work presented here, but this should not detract from its shortcomings and the need for refinement. The most obvious weakness of this thesis has been its scope: casting my net quite broadly in terms of the kinds of experiments performed and the questions investigated meant that certain findings were not followed upon with sufficient detail to provide a coherent story, sometimes risking that the message of the thesis might be diluted or scattered. Hence, chapters 3 and 4 could have on their own been developed into a project looking solely at the pathogenic mechanisms employed by Caspr2 autoantibodies. To an extent this was not achieved due to the initial failure to produce meaningful behavioural changes, especially with the mAbs, and to an extent it was the product of devoting time and resources elsewhere in conducting the study covered in Chapter 5. This experiment could have in turn been further developed by cloning and characterising the native antibodies produced by the cultured and isolated B-cells, and injecting these in experimental animals. While perhaps lacking in focus, this thesis did examine the question of how Caspr2-antibody encephalitis arises and produces the deficits that it does from multiple perspectives and across different experimental modalities, thus perhaps compensating with breadth for the lack of depth in certain areas. In addition to this overall aspect in which the thesis could be improved, each chapter had on its own several disadvantages that have been covered in the respective chapter discussion sections and will be briefly reflected upon here.

The most notable deficit of Chapter 2 was the lack of information on the IgG2 and IgG3 subclasses among Caspr2-specific antibodies – while this could have been easily addressed by a follow-up experiment using an identical strategy, I chose to instead narrow down on the two subclasses which have been widely believed to have the highest contribution to pathology in this family of diseases, henceforth being the most frequently studied ones. Another deficit that became apparent when analysing results was that flow cytometry did not detect as positive several samples characterised as 'low titre' via the cell-based assay. In general, flow cytometric methods are considered to be more sensitive in the detection of target-specific autoantibodies, so it is possible that here this discrepancy occurred because the low-titre samples were not true positives, as the cell-based assay is known to carry forward a higher bias toward false positive results due to the non-quantitative nature of interpreting results. This perceived shortcoming of the flow experiment could thus be an advantage in disguise. It is worth also pointing out that the patients who were negative on the flow assay were not significantly distinguishable in the clinical context from those that were identified as positive, calling into question the previous argument. The study in chapter 2 could also benefit from a larger patient cohort as, despite the reasonably high number of samples used, many come from the same patients in the form of longitudinal data. A more refined definition of the symptoms observed could possibly reveal new clinical associations, as would information on symptom and disease severity.

Chapter 3 focused solely on behavioural data as proof of concept for antibody pathogenicity but did not provide insight on its mechanistic basis. This chapter would have greatly benefited from further histopathological, neurophysiological, and molecular studies, some of which are discussed in the next section. A wide range of behavioural tests was used in this chapter, with several variations in the timings post-injection, but all tasks presented were fairly simple and, it could even be argued, primitive when compared to state-of-the-art techniques employed in the field of behavioural neuroscience. While this does not detract from their value, indeed the open field is one of the most widely used and robust behavioural tests, it is perhaps the use of single-trial limited-time memory tests that did not emphasise the learning behaviours of the animal that could have concealed a possible effect of the antibody on the animals' memory.

An extensive catalogue of tests for learning and memory exists in the field, but these normally necessitate preparation and training across multiple days, which would have not been compatible with my 'acute' disease model. The open field, light-dark box, and elevated zero maze are standard, well-characterised and widely used tests, although perhaps the 'gold standard' in the field of anxiety research has been the elevated plus maze test, which has not been employed here. Furthermore, most of the results I have presented here describe the behaviour of the animals across the entire test, when in fact it is often preferred for behavioural parameters to be plotted longitudinally across the entire duration of testing. Finally, most results were analysed manually, as this was often shown to be better at interpreting animal behaviour and locating the animal in the maze than the software.

Chapter 4 could have significantly benefited from experiments that would further substantiate neutrophil cell involvement following antibody injection, slice electrophysiology studies to bridge the gap between the neuronal culture data and the in vivo behavioural findings, and more diligent characterisation of the antibodies. Notably, while the antibodies were first identified using human Caspr2-expressing HEK293T cells, tissue and neuron binding were determined using rat neurons, and functional studies were performed either in rats when examining effects on AMPA receptor function or in mice when examining behavioural effects. It is possible that the many inconsistencies in this chapter arise precisely due to this experimentation across multiple species. A significant portion of the chapter was also occupied by the initial optimisation of the behavioural protocols, which should have ideally been done prior to and not after the pilot study. The pilot study itself was performed on CD1 mice, which are normally not as well-suited for cognitive tests as the C57Bl/6J strain and it was done using unilateral injections, which rarely manage to produce any behavioural alterations due to the compensatory employment of the non-injected brain hemisphere. A glaring disadvantage of both this and the previous chapter was the use of female experimental mice to model a disease that overwhelmingly affects men. The female mice were chosen from a pragmatic point of view as they are much more amenable to behavioural testing and less likely to exhibit fighting behaviours which necessitate single-animal housing, a measure that can negatively affect animal wellbeing and performance on behavioural tests. It is however entirely possible that immune and endocrine differences between male and female mice underpinned the failure to produce more drastic cognitive behavioural changes, and the higher susceptibility of female mice to anxiety was in turn what produced the behavioural deficits that were observed.

Among other weaknesses of the Chapter 4 study design was the use of a commercial control antibody which, despite allegedly having the same constant region as our patient-derived monoclonal antibodies, was generated in a different species and most likely using very different means. Knowing what we do now from Chapter 5 about the occurrence of Caspr2-reactive antibodies in healthy controls, an ideal control would have been a Caspr2-antibody cloned from a naïve B-cell isolated from a healthy donor, though whether *this* antibody would be pathogenic might constitute an entirely new research question. This kind of a healthy donor Caspr2-specific control would have been especially prescient when investigating the immune cell recruitment to the hippocampus following antibody injection, in itself a finding that was insufficiently expounded upon. While the levels of endotoxin in our antibodies produced 'in house' were negligible and likely insufficient to elicit such a pronounced immune response in the CNS, it remains possible that there were other contaminants in the antibody preparation that are not accounted for by the chromogenic assay used, thus not eliminating entirely the possibility that neutrophil recruitment was the result of LPS. The claims made in Chapter 4 would have been substantially strengthened by accompanying testing for the presence of systemic acute phase proteins in the circulation, as well as local quantification for the

expression of pro-inflammatory genes. Finally, as all controls used were commercially obtained, the most convincing evidence for the 'purity' of in-house generated mAbs would have been producing and injecting an in-house control mAb and demonstrating its failure to elicit an immune response. In the neurophysiological experiments, the effects of the mAbs on AMPAR and Caspr2 levels were studied by collaborators, but all of this work was done on neurons in culture and the extent to which it applied to the observations in vivo is questionable. Experiments employing slice electrophysiology, such as the ones briefly shown but not further developed in Chapter 4 would have been very valuable for the mechanistic understanding of Caspr2 pathology. Showing no deficits in LTP in acute hippocampal slices would have lent further support to my argument that Caspr2-mAbs don't produce memory deficits, while the converse would have suggested that the cognitive tasks used were simply not sensitive enough to detect this change, or perhaps that testing was not done at the correct time-point. Finally, slice electrophysiology could have allowed for the use of AMPAR agonists or antagonists in conjunction with the Caspr2 mAbs to see whether the former counter or the latter exacerbate the effects of the mAbs and thus argue in favour of disrupted glutamatergic neurotransmission as the mechanism underlying Caspr2 autoantibody pathogenicity.

Lastly, Chapter 5, while extensive and well-designed, relies entirely on the results of a single experiment, its first major issue thus being that its reproducibility is yet uncertain. Logically, the next step would be to widen this study to include more patients and healthy donors, though it should be emphasised that this would incur substantial costs and manual labour. The second major disadvantage of the study was its use of a biased cell-selection and isolation approach that relied on a non-native soluble form of the extracellular domain of the Caspr2 protein. This has been extensively discussed in the chapter itself and must be kept in mind when making any conclusions based on the data presented here. Another perhaps less emphasised weakness was that the control cells selected to draw conclusions about differences between Caspr2-reactive and non-Caspr2 reactive cells were not 'true negatives' as they likely found their way into the cell sort due to reactivity to other components of the Caspr2 bait construct such as the fluorophores or the biotin-streptavidin complex. As these too would be 'rare reactivities' in the human B-cell repertoire it is questionable whether these cells truly represent the 'normal' non-autoreactive B-cell population. Perhaps a more refined way of approaching this question would have been to do an unbiased single B-cell sort using the same samples, though an advantage of using the previously mentioned 'negative control' cells were that they were isolated and cultured under the exact same conditions as the Caspr2-reactive B-cells, thus allowing for their selection based on the FACS data, which would have been impossible had a second unbiased sort been performed. A final weak point of the study was the use of Sanger sequencing. While still the gold standard for reading the sequence of small, targeted regions of the genome when focusing on a single gene of interest, this method tends to be expensive and very labour intensive, especially when conducting studies on the same scale as ours. More recent approaches to sequencing such as 10X Genomics could be far more sustainable methods for studies of this kind by substantially reducing demands on time, costs, and resources.

6.4 Suggestions for future experiments

Proposals for follow-up experiments have already been made across the respective chapter discussions, as well as within the previous sections of this chapter. Here, they will be listed in bullet-point format for quick future reference.

The significance of Caspr2-autoantibody subclass could be supported by in vitro and in vivo injections. Samples containing only Caspr2-IgG4, only Caspr2-IgG1, or both subclasses could be applied to neurons in culture, hippocampal slices, or entire animals as shown in Chapter 4.

Addition of blood-derived human complement could allow for investigation of downstream pathogenic mechanisms. In vivo injections could be delivered either directly to the CNS via the ventricles, to specific regions like the hippocampus or cerebellum or systemically to target the peripheral nervous system, to examine whether the different subclasses have distinct effects on different parts of the nervous system.

The fidelity of the anxious behaviours exhibited by mice injected with polyclonal Caspr2-Abs could be tested by administrating anxiolytics or drugs that specifically target AMPA receptor function. The impact of these interventions on defecation levels could also address the question of whether this observation reflects increased anxiety in the mice or the effects of Caspr2 on the enteric nervous system and bowel. The latter could also be examined by dissecting mouse intestine and staining for antibody deposition.

The mechanistic basis of the behavioural deficits observed following antibody administration could be further probed using more detailed histopathological studies of Caspr2 and AMPAR expression, as well as immune cells and pro-inflammatory cytokine expression in the brains of mice that had been treated with antibodies. An obvious improvement would also be increasing the sample size and images acquired per sample in the neutrophil staining experiment, and using a semi-quantitative approach to get an idea of the extent of immune cell infiltration.

The experimental design can be refined by using either intracerebroventricular injections of a larger amount of antibody to achieve wider penetration of the antibodies in the CNS, or via the use of an osmotic pump implanted intraperitoneally. Both approaches are fairly invasive and bear risks for animal welfare which should be carefully considered. However, they could allow for longitudinal studies of the effects of chronic prolonged exposure to Caspr2-antibodies, thus being more reflective of the nature of the disease as seen in patients. To bring the animal model even closer to the human disease demographic, the young adult female mice may be substituted with much older male mice.

Injection of a patient derived Caspr2 mAb wherein the constant region of the mAb is exchanged for a mouse constant region, thus creating a murinized Caspr2 mAb might be more successful in recruiting secondary immune components. Alternatively, an ‘active immunization’ model, whereby a soluble antigen is systemically injected into the animal combined with inflammatory adjuvants to elicit an immune response might produce a far more severe deficit and allow for study of not just antibody effects, but also their origins.

In the single B-cell study discussed in Chapter 5, it would be fascinating to inject either the supernatants or cloned native antibodies of the Caspr2-reactive B-cells from both patients and healthy donors to see whether there are any discrepancies in their pathogenic potential. In the same study, the sorting of a certain number of cells which were negative for the bait construct would have allowed for a bona fide ‘negative control’ cell population and thus circumvented many of the weaknesses of the study highlighted earlier.

BIBLIOGRAPHY

1. Ampie L, McGavern DB. Immunological defense of CNS barriers against infections. Immunity. 2022;55(5):781-799.

2. Swanson II PA, McGavern DB. Viral diseases of the central nervous system. Current opinion in virology. 2015;11:44-54.

3. Swanson PA, Hart GT, Russo MV, et al. CD8+ T cells induce fatal brainstem pathology during cerebral malaria via luminal antigen-specific engagement of brain vasculature. PLoS pathogens. 2016;12(12):e1006022.

4. Kim JV, Kang SS, Dustin ML, et al. Myelomonocytic cell recruitment causes fatal CNS vascular injury during acute viral meningitis. Nature. 2009;457(7226):191-195.

5. Galea I, Bechmann I, Perry VH. What is immune privilege (not)? Trends in Immunology. 2007;28(1):12-18.

6. Carson MJ, Doose JM, Melchior B, et al. CNS immune privilege: hiding in plain sight. Immunol Rev. 2006;213:48-65.

7. Ahn JH, Cho H, Kim J-H, et al. Meningeal lymphatic vessels at the skull base drain cerebrospinal fluid. Nature. 2019;572(7767):62-66.

8. Aspelund A, Antila S, Proulx ST, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. Journal of Experimental Medicine. 2015;212(7):991-999.

9. Fitzpatrick Z, Frazer G, Ferro A, et al. Gut-educated IgA plasma cells defend the meningeal venous sinuses. Nature. 2020;587(7834):472-476.

10. Louveau A, Smirnov I, Keyes TJ, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337-341.

11. Rua R, McGavern DB. Advances in meningeal immunity. Trends in molecular medicine. 2018;24(6):542-559.

12. Rustenhoven J, Drieu A, Mamuladze T, et al. Functional characterization of the dural sinuses as a neuroimmune interface. Cell. 2021;184(4):1000-1016. e1027.

13. Shirai Y. On the transplantation of the rat sarcoma in adult heterogenous animals. Jap Med World. 1921;1:14-15.

14. Murphy JB, Sturm E. Conditions determining the transplantability of tissues in the brain. The Journal of experimental medicine. 1923;38(2):183-197.

15. Medawar P. Immunity to homologous grafted skin. III. The fate of skin homographs transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. British journal of experimental pathology. 1948;29(1):58.

16. Matyszak M, Perry V. Demyelination in the central nervous system following a delayed-type hypersensitivity response to bacillus Calmette-Guerin. Neuroscience. 1995;64(4):967-977.

17. Stevenson P, Hawke S, Sloan D, et al. The immunogenicity of intracerebral virus infection depends on anatomical site. Journal of virology. 1997;71(1):145-151.

18. Byrnes AP, MacLaren RE, Charlton HM. Immunological instability of persistent adenovirus vectors in the brain: peripheral exposure to vector leads to renewed inflammation, reduced gene expression, and demyelination. Journal of Neuroscience. 1996;16(9):3045-3055.

19. Billingham RE, Boswell T. Studies on the problem of corneal homografts. Proceedings of the Royal Society of London Series B-Biological Sciences. 1953;141(904):392-406.

20. Goldmann EE. Vitalfärbung am Zentralnervensystem: Beitrag zur Physio-Pathologie des Plexus chorioideus und der Hirnhäute: Königl. Akademie der Wissenschaften 1913.

21. Mastorakos P, McGavern D. The anatomy and immunology of vasculature in the central nervous system. Science immunology. 2019;4(37):eaav0492.

22. MacAulay N, Keep RF, Zeuthen T. Cerebrospinal fluid production by the choroid plexus: a century of barrier research revisited. Fluids and Barriers of the CNS. 2022;19(1):26.

23. Liddelow SA. Development of the choroid plexus and blood-CSF barrier. Frontiers in neuroscience. 2015;9:32.

24. Da Mesquita S, Louveau A, Vaccari A, et al. Functional aspects of meningeal lymphatics in ageing and Alzheimer's disease. Nature. 2018;560(7717):185-191.

25. Eide PK, Vatnehol SAS, Emblem KE, et al. Magnetic resonance imaging provides evidence of glymphatic drainage from human brain to cervical lymph nodes. Scientific reports. 2018;8(1):7194.

26. Haddad-Tóvolli R, Dragano NR, Ramalho AF, et al. Development and function of the blood-brain barrier in the context of metabolic control. Frontiers in neuroscience. 2017;11:224.

27. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. Nature reviews neuroscience. 2006;7(1):41-53.

28. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. Nature medicine. 2013;19(12):1584-1596.

29. Andreone BJ, Lacoste B, Gu C. Neuronal and vascular interactions. Annual review of neuroscience. 2015;38(1):25-46.

30. Chow BW, Gu C. The molecular constituents of the blood–brain barrier. Trends in neurosciences. 2015;38(10):598-608.

31. Tietz S, Engelhardt B. Brain barriers: crosstalk between complex tight junctions and adherens junctions. Journal of Cell Biology. 2015;209(4):493-506.

32. Barkalow FJ, Goodman MJ, Gerritsen ME, et al. Brain endothelium lack one of two pathways of P-selectin-mediated neutrophil adhesion. 1996.

33. Ransohoff RM, Engelhardt B. The anatomical and cellular basis of immune surveillance in the central nervous system. Nature Reviews Immunology. 2012;12(9):623-635.

34. Bentivoglio M, Kristensson K. Tryps and trips: cell trafficking across the 100-year-old blood-brain barrier. Trends Neurosci. 2014;37(6):325-333.

35. Williams K, Alvarez X, Lackner AA. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. Glia. 2001;36(2):156-164.

36. Louveau A, Harris TH, Kipnis J. Revisiting the Mechanisms of CNS Immune Privilege. Trends Immunol. 2015;36(10):569-577.

37. Ransohoff RM, Kivisäkk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nature Reviews Immunology. 2003;3(7):569-581.

38. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood–brain barriers. Trends in immunology. 2012;33(12):579-589.

39. Takeshita Y, Ransohoff RM. Inflammatory cell trafficking across the blood–brain barrier: chemokine regulation and in vitro models. Immunological reviews. 2012;248(1):228-239.

40. Lindahl P, Johansson BR, Leveen P, et al. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science. 1997;277(5323):242-245.

41. Daneman R, Zhou L, Kebede AA, et al. Pericytes are required for blood–brain barrier integrity during embryogenesis. Nature. 2010;468(7323):562-566.

42. Ben-Zvi A, Lacoste B, Kur E, et al. Mfsd2a is critical for the formation and function of the blood–brain barrier. Nature. 2014;509(7501):507-511.

43. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Developmental cell. 2011;21(2):193-215.

44. Bell RD, Winkler EA, Sagare AP, et al. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. Neuron. 2010;68(3):409-427.

45. Andres K, Von Düring M, Muszynski K, et al. Nerve fibres and their terminals of the dura mater encephali of the rat. Anatomy and embryology. 1987;175:289-301.

46. Brioschi S, Wang WL, Peng V, et al. Heterogeneity of meningeal B cells reveals a lymphopoietic niche at the CNS borders. Science. 2021;373(6553).

47. Cugurra A, Mamuladze T, Rustenhoven J, et al. Skull and vertebral bone marrow are myeloid cell reservoirs for the meninges and CNS parenchyma. Science. 2021;373(6553).

48. Cserr HF, Harling-Berg CJ, Knopf PM. Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. Brain pathology. 1992;2(4):269-276.

49. Kida S, Pantazis A, Weller R. CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. Neuropathology and applied neurobiology. 1993;19(6):480-488.

50. Schwartz M, Raposo C. Protective autoimmunity: a unifying model for the immune network involved in CNS repair. The Neuroscientist. 2014;20(4):343-358.

51. Steinman L. Elaborate interactions between the immune and nervous systems. Nature immunology. 2004;5(6):575-581.

52. Walsh JT, Zheng J, Smirnov I, et al. Regulatory T cells in central nervous system injury: a double-edged sword. The Journal of Immunology. 2014;193(10):5013-5022.

53. Evans FL, Dittmer M, de la Fuente AG, et al. Protective and Regenerative Roles of T Cells in Central Nervous System Disorders. Front Immunol. 2019;10:2171.

54. Mason D, Charlton H, Jones A, et al. The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. Neuroscience. 1986;19(3):685-694.

55. Walsh JT, Watson N, Kipnis J. T cells in the central nervous system: messengers of destruction or purveyors of protection? Immunology. 2014;141(3):340-344.

56. Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. Annu Rev Immunol. 1990;8:579-621.

57. Bradbury M, Cole D. The role of the lymphatic system in drainage of cerebrospinal fluid and aqueous humour. The Journal of Physiology. 1980;299(1):353-365.

58. Andersson P, Perry V, Gordon S. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues. Neuroscience. 1992;48(1):169-186.

59. Matyszak M, Perry V. A comparison of leucocyte responses to heat-killed bacillus Calmette-Guerin in different CNS compartments. Neuropathology and applied neurobiology. 1996;22(1):44-53.

60. Weller RO, Engelhardt B, Phillips MJ. Lymphocyte targeting of the central nervous system: a review of afferent and efferent CNS-immune pathways. Brain Pathol. 1996;6(3):275-288.

61. Carare RO, Hawkes CA, Weller RO. Afferent and efferent immunological pathways of the brain. Anatomy, Function and Failure. Brain, Behavior, and Immunity. 2014;36:9-14.

62. Matyszak MK, Perry VH. Bacillus Calmette–Guerin sequestered in the brain parenchyma escapes immune recognition. Journal of neuroimmunology. 1998;82(1):73-80.

63. Engelhardt B, Carare RO, Bechmann I, et al. Vascular, glial, and lymphatic immune gateways of the central nervous system. Acta Neuropathologica. 2016;132(3):317-338.

64. Bothwell SW, Janigro D, Patabendige A. Cerebrospinal fluid dynamics and intracranial pressure elevation in neurological diseases. Fluids and Barriers of the CNS. 2019;16(1):9.

65. Zhang E, Richards H, Kida S, et al. Directional and compartmentalised drainage of interstitial fluid and cerebrospinal fluid from the rat brain. Acta neuropathologica. 1992;83:233-239.

66. Carare R, Bernardes-Silva M, Newman T, et al. Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathology and applied neurobiology. 2008;34(2):131-144.

67. Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. Nat Immunol. 2017;18(2):123-131.

68. Laman JD, Weller RO. Drainage of Cells and Soluble Antigen from the CNS to Regional Lymph Nodes. Journal of Neuroimmune Pharmacology. 2013;8(4):840-856.

69. Matyszak M, Perry V. The potential role of dendritic cells in immune-mediated inflammatory diseases in the central nervous system. Neuroscience. 1996;74(2):599-608.

70. De Vos AF, Van Meurs M, Brok HP, et al. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. The Journal of Immunology. 2002;169(10):5415-5423.

71. Kivisäkk P, Mahad DJ, Callahan MK, et al. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. Proceedings of the National Academy of Sciences. 2003;100(14):8389-8394.

72. Kivisäkk P, Tucky B, Wei T, et al. Human cerebrospinal fluid contains CD4+ memory T cells expressing gut-or skin-specific trafficking determinants: relevance for immunotherapy. BMC immunology. 2006;7:1-8.

73. Kaminski M, Bechmann I, Pohland M, et al. Migration of monocytes after intracerebral injection at entorhinal cortex lesion site. Journal of leukocyte biology. 2012;92(1):31-39.

74. Goldmann J, Kwidzinski E, Brandt C, et al. T cells traffic from brain to cervical lymph nodes via the cribroid plate and the nasal mucosa. Journal of leukocyte biology. 2006;80(4):797-801.

75. Louveau A, Herz J, Alme MN, et al. CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. Nature neuroscience. 2018;21(10):1380-1391.

76. Laaker C, Baenen C, Kovács KG, et al. Immune cells as messengers from the CNS to the periphery: the role of the meningeal lymphatic system in immune cell migration from the CNS. Frontiers in Immunology. 2023;14.

77. Mohammad MG, Tsai VW, Ruitenberg MJ, et al. Immune cell trafficking from the brain maintains CNS immune tolerance. The Journal of clinical investigation. 2014;124(3):1228-1241.

78. Merlini A, Haberl M, Strauß J, et al. Distinct roles of the meningeal layers in CNS autoimmunity. Nature Neuroscience. 2022;25(7):887-899.

79. Barua NU, Bienemann AS, Hesketh S, et al. Intrastriatal convection-enhanced delivery results in widespread perivascular distribution in a pre-clinical model. Fluids and Barriers of the CNS. 2012;9:1-12.

80. Carare RO, Teeling JL, Hawkes CA, et al. Immune complex formation impairs the elimination of solutes from the brain: implications for immunotherapy in Alzheimer's disease. Acta neuropathologica communications. 2013;1:1-11.

81. Yamada S, DePasquale M, Patlak CS, et al. Albumin outflow into deep cervical lymph from different regions of rabbit brain. American Journal of Physiology-Heart and Circulatory Physiology. 1991;261(4):H1197-H1204.

82. Harling-Berg CJ, Park JT, Knopf PM. Role of the cervical lymphatics in the Th2-type hierarchy of CNS immune regulation. Journal of neuroimmunology. 1999;101(2):111-127.

83. Pashenkov M, Teleshova N, Kouwenhoven M, et al. Recruitment of dendritic cells to the cerebrospinal fluid in bacterial neuroinfections. Journal of neuroimmunology. 2002;122(1-2):106-116.

84. Hatterer E, Davoust N, Didier-Bazes M, et al. How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes. Blood. 2006;107(2):806-812.

85. Harling-Berg CJ, Knopf PM, Cserr HF. Myelin basic protein infused into cerebrospinal fluid suppresses experimental autoimmune encephalomyelitis. Journal of neuroimmunology. 1991;35(1-3):45-51.

86. Wenkel H, Streilein JW, Young MJ. Systemic immune deviation in the brain that does not depend on the integrity of the blood-brain barrier. The Journal of Immunology. 2000;164(10):5125-5131.

87. Szentistvanyi I, Patlak CS, Ellis RA, et al. Drainage of interstitial fluid from different regions of rat brain. American Journal of Physiology-Renal Physiology. 1984;246(6):F835-F844.

88. Arbel-Ornath M, Hudry E, Eikermann-Haerter K, et al. Interstitial fluid drainage is impaired in ischemic stroke and Alzheimer's disease mouse models. Acta neuropathologica. 2013;126:353-364.

89. Pappolla M, Sambamurti K, Vidal R, et al. Evidence for lymphatic Aβ clearance in Alzheimer's transgenic mice. Neurobiology of disease. 2014;71:215-219.

90. Iliff JJ, Chen MJ, Plog BA, et al. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. Journal of Neuroscience. 2014;34(49):16180-16193.

91. Iliff JJ, Wang M, Liao Y, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. Science translational medicine. 2012;4(147):147ra111-147ra111.

92. Yang L, Kress BT, Weber HJ, et al. Evaluating glymphatic pathway function utilizing clinically relevant intrathecal infusion of CSF tracer. Journal of translational medicine. 2013;11:1-9.

93. Prinz M, Priller J, Sisodia SS, et al. Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. Nature neuroscience. 2011;14(10):1227-1235.

94. Goldmann T, Wieghofer P, Jordão MJC, et al. Origin, fate and dynamics of macrophages at central nervous system interfaces. Nature immunology. 2016;17(7):797-805.

95. Bauer J, Bradl M, Hickey WF, et al. T-cell apoptosis in inflammatory brain lesions: destruction of T cells does not depend on antigen recognition. The American journal of pathology. 1998;153(3):715-724.

96. Bechmann I, Mor G, Nilsen J, et al. FasL (CD95L, Apo1L) is expressed in the normal rat and human brain: evidence for the existence of an immunological brain barrier. Glia. 1999;27(1):62-74.

97. Meinl E, Aloisi F, Ertl B, et al. Multiple sclerosis Immunomodulatory effects of human astrocytes on T cells. Brain. 1994;117(6):1323-1332.

98. Trajkovic V, Vuckovic O, Stosic-Grujicic S, et al. Astrocyte-induced regulatory T cells mitigate CNS autoimmunity. Glia. 2004;47(2):168-179.

99. Magnus T, Schreiner B, Korn T, et al. Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: implications for immune responses and autoimmunity in the CNS. Journal of Neuroscience. 2005;25(10):2537-2546.

100. Platten M, Ho PP, Youssef S, et al. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science. 2005;310(5749):850-855.

101. Kwidzinski E, Bunse J, Aktas O, et al. Indolamine 2, 3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. The FASEB Journal. 2005;19(10):1347-1349.

102. Liu Y, Teige I, Birnir B, et al. Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. Nature medicine. 2006;12(5):518-525.

103. Perry VH. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. Journal of neuroimmunology. 1998;90(2):113-121.

104. Corcione A, Casazza S, Ferretti E, et al. Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis. Proc Natl Acad Sci U S A. 2004;101(30):11064-11069.

105. Magliozzi R, Columba-Cabezas S, Serafini B, et al. Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the meninges of mice with relapsing experimental autoimmune encephalomyelitis. Journal of Neuroimmunology. 2004;148(1):11-23.

106. Serafini B, Rosicarelli B, Magliozzi R, et al. Detection of Ectopic B-cell Follicles with Germinal Centers in the Meninges of Patients with Secondary Progressive Multiple Sclerosis. Brain Pathology. 2004;14(2):164-174.

107. Bucchieri F, Farina F, Zummo G, et al. Lymphatic vessels of the dura mater: a new discovery? J Anat. 2015;227(5):702-703.

108. Lecco V. [Probable modification of the lymphatic fissures of the walls of the venous sinuses of the dura mater]. Arch Ital Otol Rinol Laringol. 1953;64(3):287-296.

109. Mascagni P. Vasorum lymphaticorum corporis humani historia et ichnographia: Readex Microprint 1983.

110. Rennels ML, Blaumanis OR, Grady PA. Rapid solute transport throughout the brain via paravascular fluid pathways. Adv Neurol. 1990;52:431-439.

111. Rennels ML, Gregory TF, Blaumanis OR, et al. Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. Brain Res. 1985;326(1):47-63.

112. Iliff JJ, Wang M, Zeppenfeld DM, et al. Cerebral arterial pulsation drives paravascular CSF-interstitial fluid exchange in the murine brain. J Neurosci. 2013;33(46):18190-18199.

113. Kress BT, Iliff JJ, Xia M, et al. Impairment of paravascular clearance pathways in the aging brain. Ann Neurol. 2014;76(6):845-861.

114. Rangroo Thrane V, Thrane AS, Plog BA, et al. Paravascular microcirculation facilitates rapid lipid transport and astrocyte signaling in the brain. Sci Rep. 2013;3:2582.

115. Plog BA, Dashnaw ML, Hitomi E, et al. Biomarkers of traumatic injury are transported from brain to blood via the glymphatic system. J Neurosci. 2015;35(2):518-526.

116. Xie L, Kang H, Xu Q, et al. Sleep drives metabolite clearance from the adult brain. Science. 2013;342(6156):373-377.

117. Schwalbe G. Der Arachnoidalraum ein Lymphraum und sein Zusammenhang mit dem Perichorioidalraum. Centralbl med Wiss. 1969;7:465-467.

118. Zwillinger H. Die Lymphbahnen des oberen Nasalschnittes und deren Beziehungen zu den perimeningealen Lymphraumen. Arch Laryngol und Rhinol. 1912;26:66-78.

119. Hershenhouse KS, Shauly O, Gould DJ, et al. Meningeal Lymphatics: A Review and Future Directions From a Clinical Perspective. Neurosci Insights. 2019;14:1179069519889027.

120. Papadopoulos Z, Herz J, Kipnis J. Meningeal Lymphatics: From Anatomy to Central Nervous System Immune Surveillance. J Immunol. 2020;204(2):286-293.

121. Tavares GA, Louveau A. Meningeal Lymphatics: An Immune Gateway for the Central Nervous System. Cells. 2021;10(12).

122. Louveau A, Plog BA, Antila S, et al. Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. J Clin Invest. 2017;127(9):3210-3219.

123. Cohen JN, Tewalt EF, Rouhani SJ, et al. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. PloS one. 2014;9(2):e87740.

124. Rouhani SJ, Eccles JD, Riccardi P, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. Nature communications. 2015;6(1):6771.

125. Galea E, Graeber MB. Neuroinflammation: The Abused Concept. ASN Neuro. 2023;15:17590914231197523.

126. Ineichen BV, Okar SV, Proulx ST, et al. Perivascular spaces and their role in neuroinflammation. Neuron. 2022;110(21):3566-3581.

127. Ransohoff RM, Schafer D, Vincent A, et al. Neuroinflammation: Ways in Which the Immune System Affects the Brain. Neurotherapeutics. 2015;12(4):896-909.

128. Kölliker-Frers R, Udovin L, Otero-Losada M, et al. Neuroinflammation: An Integrating Overview of Reactive-Neuroimmune Cell Interactions in Health and Disease. Mediators Inflamm. 2021;2021:9999146.

129. Marchetti L, Engelhardt B. Immune cell trafficking across the blood-brain barrier in the absence and presence of neuroinflammation. Vasc Biol. 2020;2(1):H1-h18.

130. Bartholomäus I, Kawakami N, Odoardi F, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. Nature. 2009;462(7269):94-98.

131. Vajkoczy P, Laschinger M, Engelhardt B. α4-integrin-VCAM-1 binding mediates G protein–independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. The Journal of clinical investigation. 2001;108(4):557-565.

132. Haghayegh Jahromi N, Tardent H, Enzmann G, et al. A novel cervical spinal cord window preparation allows for two-photon imaging of T-cell interactions with the cervical spinal cord microvasculature during experimental autoimmune encephalomyelitis. Frontiers in immunology. 2017;8:406.

133. Kawakami N, Nägerl UV, Odoardi F, et al. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. The Journal of experimental medicine. 2005;201(11):1805.

134. Lodygin D, Odoardi F, Schläger C, et al. A combination of fluorescent NFAT and H2B sensors uncovers dynamics of T cell activation in real time during CNS autoimmunity. Nature medicine. 2013;19(6):784-790.

135. Alvermann S, Hennig C, Stüve O, et al. Immunophenotyping of cerebrospinal fluid cells in multiple sclerosis: in search of biomarkers. JAMA neurology. 2014;71(7):905-912.

136. Giunti D, Borsellino G, Benelli R, et al. Phenotypic and functional analysis of T cells homing into the CSF of subjects with inflammatory diseases of the CNS. Journal of Leucocyte Biology. 2003;73(5):584-590.

137. Laschinger M, Vajkoczy P, Engelhardt B. Encephalitogenic T cells use LFA-1 for transendothelial migration but not during capture and initial adhesion strengthening in healthy spinal cord microvessels in vivo. European journal of immunology. 2002;32(12):3598-3606.

138. Stüve O, Marra CM, Bar-Or A, et al. Altered CD4+/CD8+ T-cell ratios in cerebrospinal fluid of natalizumab-treated patients with multiple sclerosis. Archives of neurology. 2006;63(10):1383-1387.

139. Wekerle H, Linington C, Lassmann H, et al. Cellular immune reactivity within the CNS. Trends in neurosciences. 1986;9:271-277.

140. Hickey W, Hsu B, Kimura H. T-lymphocyte entry into the central nervous system. Journal of neuroscience research. 1991;28(2):254-260.

141. Greter M, Heppner FL, Lemos MP, et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nature medicine. 2005;11(3):328-334.

142. Schläger C, Körner H, Krueger M, et al. Effector T-cell trafficking between the leptomeninges and the cerebrospinal fluid. Nature. 2016;530(7590):349-353.

143. Liebner S, Dijkhuizen RM, Reiss Y, et al. Functional morphology of the blood–brain barrier in health and disease. Acta neuropathologica. 2018;135:311-336.

144. Mapunda JA, Tibar H, Regragui W, et al. How Does the Immune System Enter the Brain? Front Immunol. 2022;13:805657.

145. Sathiyanadan K, Coisne C, Enzmann G, et al. PSGL-1 and E/P-selectins are essential for T-cell rolling in inflamed CNS microvessels but dispensable for initiation of EAE. European journal of immunology. 2014;44(8):2287-2294.

146. Battistini L, Piccio L, Rossi B, et al. CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. Blood. 2003;101(12):4775-4782.

147. Piccio L, Rossi B, Scarpini E, et al. Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric Gi-linked receptors. The journal of immunology. 2002;168(4):1940-1949.

148. Minten C, Alt C, Gentner M, et al. DARC shuttles inflammatory chemokines across the blood–brain barrier during autoimmune central nervous system inflammation. Brain. 2014;137(5):1454-1469.

149. Steiner O, Coisne C, Cecchelli R, et al. Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood–brain barrier endothelium. The Journal of Immunology. 2010;185(8):4846-4855.

150. Steffen BJ, Butcher EC, Engelhardt B. Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune

encephalomyelitis in the central nervous system in the SJL/J mouse. The American journal of pathology. 1994;145(1):189.

151. Greenwood J, Wang Y, Calder V. Lymphocyte adhesion and transendothelial migration in the central nervous system: the role of LFA-1, ICAM-1, VLA-4 and VCAM-1. off. Immunology. 1995;86(3):408.

152. McCormack PL. Natalizumab: a review of its use in the management of relapsing-remitting multiple sclerosis. Drugs. 2013;73(13):1463-1481.

153. Abadier M, Haghayegh Jahromi N, Cardoso Alves L, et al. Cell surface levels of endothelial ICAM-1 influence the transcellular or paracellular T-cell diapedesis across the blood–brain barrier. European journal of immunology. 2015;45(4):1043-1058.

154. Owens T, Bechmann I, Engelhardt B. Perivascular spaces and the two steps to neuroinflammation. Journal of Neuropathology & Experimental Neurology. 2008;67(12):1113-1121.

155. Hannocks M-J, Pizzo ME, Huppert J, et al. Molecular characterization of perivascular drainage pathways in the murine brain. Journal of Cerebral Blood Flow & Metabolism. 2018;38(4):669-686.

156. Sixt M, Engelhardt B, Pausch F, et al. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood–brain barrier in experimental autoimmune encephalomyelitis. The Journal of cell biology. 2001;153(5):933.

157. Agrawal S, Anderson P, Durbeej M, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. The Journal of experimental medicine. 2006;203(4):1007.

158. Song J, Wu C, Korpos E, et al. Focal MMP-2 and MMP-9 activity at the blood-brain barrier promotes chemokine-induced leukocyte migration. Cell reports. 2015;10(7):1040-1054.

159. Antel JP, Owens T. Immune regulation and CNS autoimmune disease. J Neuroimmunol. 1999;100(1-2):181-189.

160. Anthony I, Crawford D, Bell J. B lymphocytes in the normal brain: contrasts with HIV-associated lymphoid infiltrates and lymphomas. Brain. 2003;126(5):1058-1067.

161. Kowarik M, Grummel V, Wemlinger S, et al. Immune cell subtyping in the cerebrospinal fluid of patients with neurological diseases. Journal of neurology. 2014;261:130-143.

162. Machado-Santos J, Saji E, Tröscher AR, et al. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. Brain. 2018;141(7):2066-2082.

163. Alter A, Duddy M, Hebert S, et al. Determinants of human B cell migration across brain endothelial cells. The Journal of Immunology. 2003;170(9):4497-4505.

164. Michel L, Grasmuck C, Charabati M, et al. Activated leukocyte cell adhesion molecule regulates B lymphocyte migration across central nervous system barriers. Science translational medicine. 2019;11(518):eaaw0475.

165. Bellingacci L, Canonichesi J, Mancini A, et al. Cytokines, synaptic plasticity and network dynamics: a matter of balance. Neural Regen Res. 2023;18(12):2569-2572.

166. Zipp F, Bittner S, Schafer DP. Cytokines as emerging regulators of central nervous system synapses. Immunity. 2023;56(5):914-925.

167. Salvador AF, de Lima KA, Kipnis J. Neuromodulation by the immune system: a focus on cytokines. Nature Reviews Immunology. 2021;21(8):526-541.

168. Zengeler KE, Lukens JR. Innate immunity at the crossroads of healthy brain maturation and neurodevelopmental disorders. Nature Reviews Immunology. 2021;21(7):454-468.

169. Kent SA, Miron VE. Microglia regulation of central nervous system myelin health and regeneration. Nature Reviews Immunology. 2024;24(1):49-63.

170. Li Q, Barres BA. Microglia and macrophages in brain homeostasis and disease. Nature Reviews Immunology. 2018;18(4):225-242.

171. Stevens B, Allen NJ, Vazquez LE, et al. The Classical Complement Cascade Mediates CNS Synapse Elimination. Cell. 2007;131(6):1164-1178.

172. Stevens B, Johnson MB. The complement cascade repurposed in the brain. Nature Reviews Immunology. 2021;21(10):624-625.

173. Schiller M, Ben-Shaanan TL, Rolls A. Neuronal regulation of immunity: why, how and where? Nature Reviews Immunology. 2021;21(1):20-36.

174. Huh JR, Veiga-Fernandes H. Neuroimmune circuits in inter-organ communication. Nature Reviews Immunology. 2020;20(4):217-228.

175. Haykin H, Rolls A. The neuroimmune response during stress: A physiological perspective. Immunity. 2021;54(9):1933-1947.

176. Ménard C, Pfau ML, Hodes GE, et al. Immune and Neuroendocrine Mechanisms of Stress Vulnerability and Resilience. Neuropsychopharmacology. 2017;42(1):62-80.

177. Iadecola C, Buckwalter MS, Anrather J. Immune responses to stroke: mechanisms, modulation, and therapeutic potential. J Clin Invest. 2020;130(6):2777-2788.

178. Attfield KE, Jensen LT, Kaufmann M, et al. The immunology of multiple sclerosis. Nature Reviews Immunology. 2022;22(12):734-750.

179. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Nature Reviews Immunology. 2015;15(9):545-558.

180. Tansey MG, Wallings RL, Houser MC, et al. Inflammation and immune dysfunction in Parkinson disease. Nature Reviews Immunology. 2022;22(11):657-673.

181. Shi Y, Holtzman DM. Interplay between innate immunity and Alzheimer disease: APOE and TREM2 in the spotlight. Nature Reviews Immunology. 2018;18(12):759-772.

182. Baral P, Udit S, Chiu IM. Pain and immunity: implications for host defence. Nature Reviews Immunology. 2019;19(7):433-447.

183. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nature Reviews Immunology. 2016;16(1):22-34.

184. Poletti S, Mazza MG, Benedetti F. Inflammatory mediators in major depression and bipolar disorder. Translational Psychiatry. 2024;14(1):247.

185. Prüss H. Autoantibodies in neurological disease. Nature Reviews Immunology. 2021;21(12):798-813.

186. Tunkel AR, Glaser CA, Bloch KC, et al. The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. Clinical infectious diseases. 2008:303-327.

187. Venkatesan A, Tunkel AR, Bloch KC, et al. Case definitions, diagnostic algorithms, and priorities in encephalitis: consensus statement of the international encephalitis consortium. Clin Infect Dis. 2013;57(8):1114-1128.

188. Granerod J, Ambrose HE, Davies NW, et al. Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study. The Lancet infectious diseases. 2010;10(12):835-844.

189. Ball R, Halsey N, Braun MM, et al. Development of case definitions for acute encephalopathy, encephalitis, and multiple sclerosis reports to the Vaccine Adverse Event Reporting System. Journal of clinical epidemiology. 2002;55(8):819-824.

190. Glaser C, Honarmand S, Anderson L, et al. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clinical Infectious Diseases. 2006;43(12):1565-1577.

191. Sejvar JJ, Kohl KS, Bilynsky R, et al. Encephalitis, myelitis, and acute disseminated encephalomyelitis (ADEM): case definitions and guidelines for collection, analysis, and presentation of immunization safety data. Vaccine. 2007;25(31):5771-5792.

192. Elbers JM, Bitnun A, Richardson SE, et al. A 12-Year Prospective Study of Childhood Herpes Simplex Encephalitis: Is There a Broader Spectrum of Disease? Pediatrics. 2007;119(2):e399-e407.

193. Jmor F, Emsley HC, Fischer M, et al. The incidence of acute encephalitis syndrome in Western industrialised and tropical countries. Virol J. 2008;5:134.

194. Bloch KC, Glaser C. Diagnostic approaches for patients with suspected encephalitis. Current infectious disease reports. 2007;9(4):315-322.

195. Gable MS, Sheriff H, Dalmau J, et al. The frequency of autoimmune N-methyl-D-aspartate receptor encephalitis surpasses that of individual viral etiologies in young individuals enrolled in the California Encephalitis Project. Clinical Infectious Diseases. 2012;54(7):899-904.

196. Britton PN, Eastwood K, Paterson B, et al. Consensus guidelines for the investigation and management of encephalitis in adults and children in A ustralia and N ew Z ealand. Internal medicine journal. 2015;45(5):563-576.

197. Vora NM, Holman RC, Mehal JM, et al. Burden of encephalitis-associated hospitalizations in the United States, 1998-2010. Neurology. 2014;82(5):443-451.

198. Dubey D, Pittock SJ, Kelly CR, et al. Autoimmune encephalitis epidemiology and a comparison to infectious encephalitis. Ann Neurol. 2018;83(1):166-177.

199. Huang CN, Tian XB, Jiang SM, et al. Comparisons Between Infectious and Autoimmune Encephalitis: Clinical Signs, Biochemistry, Blood Counts, and Imaging Findings. Neuropsychiatr Dis Treat. 2020;16:2649-2660.

200. Segal Y, Rotschild O, Mina Y, et al. Epidemiology and Characteristics of Autoimmune Encephalitis and Comparison to Infectious Causes – Experience from a Large Tertiary Center (P7-14.001). Neurology. 2024;102(17_supplement_1):2779.

201. Graus F, Titulaer MJ, Balu R, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol. 2016;15(4):391-404.

202. Uy CE, Binks S, Irani SR. Autoimmune encephalitis: clinical spectrum and management. Pract Neurol. 2021;21(5):412-423.

203. Varley J, Taylor J, Irani SR. Autoantibody-mediated diseases of the CNS: Structure, dysfunction and therapy. Neuropharmacology. 2018;132:71-82.

204. Sabater L, Gaig C, Gelpi E, et al. A novel non-rapid-eye movement and rapid-eye-movement parasomnia with sleep breathing disorder associated with antibodies to IgLON5: a case series, characterisation of the antigen, and post-mortem study. The Lancet Neurology. 2014;13(6):575-586.

205. Lai M, Hughes EG, Peng X, et al. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. Annals of neurology. 2009;65(4):424-434.

206. Dalmau J, Lancaster E, Martinez-Hernandez E, et al. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. The Lancet Neurology. 2011;10(1):63-74.

207. Lai M, Huijbers MG, Lancaster E, et al. Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. The Lancet Neurology. 2010;9(8):776-785.

208. Irani SR, Alexander S, Waters P, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain. 2010;133(9):2734-2748.

209. Lancaster E, Huijbers MG, Bar V, et al. Investigations of caspr2, an autoantigen of encephalitis and neuromyotonia. Ann Neurol. 2011;69(2):303-311.

210. Brilot F, Dale RC, Selter RC, et al. Antibodies to native myelin oligodendrocyte glycoprotein in children with inflammatory demyelinating central nervous system disease.

Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society. 2009;66(6):833-842.

211. McKeon A, Lennon V, Lotze T, et al. CNS aquaporin-4 autoimmunity in children. Neurology. 2008;71(2):93-100.

212. Petit-Pedrol M, Armangue T, Peng X, et al. Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies. The lancet neurology. 2014;13(3):276-286.

213. Lancaster E, Lai M, Peng X, et al. Antibodies to the GABAB receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. The Lancet Neurology. 2010;9(1):67-76.

214. Boronat A, Gelfand JM, Gresa-Arribas N, et al. Encephalitis and antibodies to dipeptidyl-peptidase–like protein-6, a subunit of Kv4. 2 potassium channels. Annals of neurology. 2013;73(1):120-128.

215. Malter MP, Helmstaedter C, Urbach H, et al. Antibodies to glutamic acid decarboxylase define a form of limbic encephalitis. Annals of neurology. 2010;67(4):470-478.

216. Gresa-Arribas N, Planagumà J, Petit-Pedrol M, et al. Human neurexin-3α antibodies associate with encephalitis and alter synapse development. Neurology. 2016;86(24):2235-2242.

217. Lancaster E, Martinez-Hernandez E, Titulaer M, et al. Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome. Neurology. 2011;77(18):1698-1701.

218. Dale RC, Merheb V, Pillai S, et al. Antibodies to surface dopamine-2 receptor in autoimmune movement and psychiatric disorders. Brain. 2012;135(11):3453-3468.

219. Hutchinson M, Waters P, McHugh J, et al. Progressive encephalomyelitis, rigidity, and myoclonus: a novel glycine receptor antibody. Neurology. 2008;71(16):1291-1292.

220. Dalmau J, Geis C, Graus F. Autoantibodies to Synaptic Receptors and Neuronal Cell Surface Proteins in Autoimmune Diseases of the Central Nervous System. Physiol Rev. 2017;97(2):839-887.

221. Leypoldt F, Armangue T, Dalmau J. Autoimmune encephalopathies. Ann N Y Acad Sci. 2015;1338(1):94-114.

222. Sun B, Ramberger M, O'Connor KC, et al. The B cell immunobiology that underlies CNS autoantibody-mediated diseases. Nature Reviews Neurology. 2020;16(9):481-492.

223. Shin Y-W, Lee S-T, Park K-I, et al. Treatment strategies for autoimmune encephalitis. Therapeutic Advances in Neurological Disorders. 2018;11:1756285617722347.

224. Valentino P, Marnetto F, Granieri L, et al. Aquaporin-4 antibody titration in NMO patients treated with rituximab: A retrospective study. Neurol Neuroimmunol Neuroinflamm. 2017;4(2):e317.

225. Kim S-H, Kim W, Li XF, et al. Repeated Treatment With Rituximab Based on the Assessment of Peripheral Circulating Memory B Cells in Patients With Relapsing Neuromyelitis Optica Over 2 Years. Archives of Neurology. 2011;68(11):1412-1420.

226. Pellkofer HL, Krumbholz M, Berthele A, et al. Long-term follow-up of patients with neuromyelitis optica after repeated therapy with rituximab. Neurology. 2011;76(15):1310-1315.

227. Thompson J, Bi M, Murchison AG, et al. The importance of early immunotherapy in patients with faciobrachial dystonic seizures. Brain. 2018;141(2):348-356.

228. de Bruijn MA, van Sonderen A, van Coevorden-Hameete MH, et al. Evaluation of seizure treatment in anti-LGI1, anti-NMDAR, and anti-GABABR encephalitis. Neurology. 2019;92(19):e2185-e2196.

229. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. Br J Pharmacol. 2006;148(3):245-254.

230. Wiseman AC. Immunosuppressive Medications. Clin J Am Soc Nephrol. 2016;11(2):332-343.

231. Franco LM, Gadkari M, Howe KN, et al. Immune regulation by glucocorticoids can be linked to cell type–dependent transcriptional responses. Journal of Experimental Medicine. 2019;216(2):384-406.

232. Lee J, Machin M, Russell KE, et al. Corticosteroid modulation of immunoglobulin expression and B-cell function in COPD. The FASEB Journal. 2016;30(5):2014-2026.

233. Liberman AC, Budziñski ML, Sokn C, et al. Regulatory and Mechanistic Actions of Glucocorticoids on T and Inflammatory Cells. Frontiers in Endocrinology. 2018;9.

234. Cousins DJ, McDonald J, Lee TH. Therapeutic approaches for control of transcription factors in allergic disease. J Allergy Clin Immunol. 2008;121(4):803-809; quiz 810-801.

235. Brito-Zerón P, Kostov B, Bosch X, et al. Therapeutic approach to IgG4-related disease: A systematic review. Medicine (Baltimore). 2016;95(26):e4002.

236. Jacob S, Rajabally YA. Current proposed mechanisms of action of intravenous immunoglobulins in inflammatory neuropathies. Curr Neuropharmacol. 2009;7(4):337-342.

237. Hansen RJ, Balthasar JP. Intravenous immunoglobulin mediates an increase in anti-platelet antibody clearance via the FcRn receptor. Thromb Haemost. 2002;88(6):898-899.

238. Reeves HM, Winters JL. The mechanisms of action of plasma exchange. Br J Haematol. 2014;164(3):342-351.

239. Dogan Onugoren M, Golombeck KS, Bien C, et al. Immunoadsorption therapy in autoimmune encephalitides. Neurol Neuroimmunol Neuroinflamm. 2016;3(2):e207.

240. Lee WJ, Lee ST, Byun JI, et al. Rituximab treatment for autoimmune limbic encephalitis in an institutional cohort. Neurology. 2016;86(18):1683-1691.

241. Titulaer MJ, McCracken L, Gabilondo I, et al. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. Lancet Neurol. 2013;12(2):157-165.

242. Kosmidis ML, Dalakas MC. Practical considerations on the use of rituximab in autoimmune neurological disorders. Ther Adv Neurol Disord. 2010;3(2):93-105.

243. Damato V, Evoli A, Iorio R. Efficacy and Safety of Rituximab Therapy in Neuromyelitis Optica Spectrum Disorders: A Systematic Review and Meta-analysis. JAMA Neurol. 2016;73(11):1342-1348.

244. Hachiya Y, Uruha A, Kasai-Yoshida E, et al. Rituximab ameliorates anti-N-methyl-D-aspartate receptor encephalitis by removal of short-lived plasmablasts. Journal of neuroimmunology. 2013;265(1-2):128-130.

245. van Sonderen A, Ariño H, Petit-Pedrol M, et al. The clinical spectrum of Caspr2 antibody-associated disease. Neurology. 2016;87(5):521-528.

246. Irani SR, Pettingill P, Kleopa KA, et al. Morvan syndrome: clinical and serological observations in 29 cases. Ann Neurol. 2012;72(2):241-255.

247. Dalakas MC. Invited article: inhibition of B cell functions: implications for neurology. Neurology. 2008;70(23):2252-2260.

248. Chamberlain N, Massad C, Oe T, et al. Rituximab does not reset defective early B cell tolerance checkpoints. The Journal of clinical investigation. 2016;126(1):282-287.

249. Monson NL, Cravens PD, Frohman EM, et al. Effect of rituximab on the peripheral blood and cerebrospinal fluid B cells in patients with primary progressive multiple sclerosis. Archives of neurology. 2005;62(2):258-264.

250. Martin F, Chan AC. B cell immunobiology in disease: evolving concepts from the clinic. Annu Rev Immunol. 2006;24(1):467-496.

251. Lebrun C, Cohen M, Rosenthal-Allieri MA, et al. Only follow-up of memory B cells helps monitor rituximab administration to patients with neuromyelitis optica spectrum disorders. Neurology and Therapy. 2018;7:373-383.

252. Petereit H, Rubbert-Roth A. Rituximab levels in cerebrospinal fluid of patients with neurological autoimmune disorders. Multiple Sclerosis Journal. 2009;15(2):189-192.

253. Jiang R, Fichtner ML, Hoehn KB, et al. Single-cell repertoire tracing identifies rituximab-resistant B cells during myasthenia gravis relapses. JCI Insight. 2020;5(14).

254. Bashford-Rogers R, Bergamaschi L, McKinney E, et al. Analysis of the B cell receptor repertoire in six immune-mediated diseases. Nature. 2019;574(7776):122-126.

255. Cupps T, Edgar L, Fauci A. Suppression of human B lymphocyte function by cyclophosphamide. Journal of immunology (Baltimore, Md: 1950). 1982;128(6):2453-2457.

256. Hoyer BF, Moser K, Hauser AE, et al. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. Arthritis Res Ther. 2004;6:1-35.

257. Kong S-S, Chen Y-J, Su IC, et al. Immunotherapy for anti-NMDA receptor encephalitis: Experience from a single center in Taiwan. Pediatrics & Neonatology. 2019;60(4):417-422.

258. Kessler RA, Mealy MA, Levy M. Treatment of neuromyelitis optica spectrum disorder: acute, preventive, and symptomatic. Current treatment options in neurology. 2016;18:1-15.

259. Stellmann J-P, Krumbholz M, Friede T, et al. Immunotherapies in neuromyelitis optica spectrum disorder: efficacy and predictors of response. Journal of Neurology, Neurosurgery & Psychiatry. 2017;88(8):639-647.

260. Meister S, Schubert U, Neubert K, et al. Extensive immunoglobulin production sensitizes myeloma cells for proteasome inhibition. Cancer research. 2007;67(4):1783-1792.

261. Scheibe F, Prüss H, Mengel AM, et al. Bortezomib for treatment of therapy-refractory anti-NMDA receptor encephalitis. Neurology. 2017;88(4):366-370.

262. Shin YW, Lee ST, Kim TJ, et al. Bortezomib treatment for severe refractory anti-NMDA receptor encephalitis. Annals of Clinical and Translational Neurology. 2018;5(5):598-605.

263. Zhang C, Tian D-C, Yang C-S, et al. Safety and efficacy of bortezomib in patients with highly relapsing neuromyelitis optica spectrum disorder. JAMA neurology. 2017;74(8):1010-1012.

264. Mihara M, Kasutani K, Okazaki M, et al. Tocilizumab inhibits signal transduction mediated by both mIL-6R and sIL-6R, but not by the receptors of other members of IL-6 cytokine family. International immunopharmacology. 2005;5(12):1731-1740.

265. Kang S, Tanaka T, Kishimoto T. Therapeutic uses of anti-interleukin-6 receptor antibody. International immunology. 2015;27(1):21-29.

266. Ringelstein M, Ayzenberg I, Harmel J, et al. Long-term therapy with interleukin 6 receptor blockade in highly active neuromyelitis optica spectrum disorder. JAMA neurology. 2015;72(7):756-763.

267. Araki M, Matsuoka T, Miyamoto K, et al. Efficacy of the anti–IL-6 receptor antibody tocilizumab in neuromyelitis optica: a pilot study. Neurology. 2014;82(15):1302-1306.

268. Krogias C, Hoepner R, Müller A, et al. Successful treatment of anti-Caspr2 syndrome by interleukin 6 receptor blockade through tocilizumab. JAMA neurology. 2013;70(8):1056-1059.

269. Lee W-J, Lee S-T, Moon J, et al. Tocilizumab in autoimmune encephalitis refractory to rituximab: an institutional cohort study. Neurotherapeutics. 2016;13(4):824-832.

270. Liao W, Lin J-X, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013;38(1):13-25.

271. Klatzmann D, Abbas AK. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. Nature Reviews Immunology. 2015;15(5):283-294.

272. Yu A, Zhu L, Altman NH, et al. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. Immunity. 2009;30(2):204-217.

273. Yu A, Snowhite I, Vendrame F, et al. Selective IL-2 responsiveness of regulatory T cells through multiple intrinsic mechanisms supports the use of low-dose IL-2 therapy in type 1 diabetes. Diabetes. 2015;64(6):2172-2183.

274. Lim J-A, Lee S-T, Moon J, et al. New feasible treatment for refractory autoimmune encephalitis: low-dose interleukin-2. Journal of Neuroimmunology. 2016;299:107-111.

275. Ariño H, Armangué T, Petit-Pedrol M, et al. Anti-LGI1–associated cognitive impairment: presentation and long-term outcome. Neurology. 2016;87(8):759-765.

276. van Sonderen A, Thijs RD, Coenders EC, et al. Anti-LGI1 encephalitis: clinical syndrome and long-term follow-up. Neurology. 2016;87(14):1449-1456.

277. Gabilondo I, Saiz A, Galán L, et al. Analysis of relapses in anti-NMDAR encephalitis. Neurology. 2011;77(10):996-999.

278. Nosadini M, Mohammad SS, Ramanathan S, et al. Immune therapy in autoimmune encephalitis: a systematic review. Expert review of neurotherapeutics. 2015;15(12):1391-1419.

279. Waters P, Pettingill P, Lang B. Chapter 9 - Detection methods for neural autoantibodies. In Pittock SJ, Vincent A, (Eds). Handbook of Clinical Neurology: Elsevier 2016:147-163.

280. Ramanathan S, Al-Diwani A, Waters P, et al. The autoantibody-mediated encephalitides: from clinical observations to molecular pathogenesis. Journal of Neurology. 2021;268(5):1689-1707.

281. Gill AJ, Venkatesan A. Pathogenic mechanisms in neuronal surface autoantibody-mediated encephalitis. Journal of Neuroimmunology. 2022;368:577867.

282. Diamond B, Honig G, Mader S, et al. Brain-reactive antibodies and disease. Annu Rev Immunol. 2013;31:345-385.

283. Nagele EP, Han M, Acharya NK, et al. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. PloS one. 2013;8(4):e60726.

284. Levin EC, Acharya NK, Han M, et al. Brain-reactive autoantibodies are nearly ubiquitous in human sera and may be linked to pathology in the context of blood–brain barrier breakdown. Brain research. 2010;1345:221-232.

285. Brown JW, Martin PJ, Thorpe JW, et al. Long-term remission with rituximab in refractory leucine-rich glioma inactivated 1 antibody encephalitis. J Neuroimmunol. 2014;271(1-2):66-68.

286. Irani SR, Gelfand JM, Bettcher BM, et al. Effect of rituximab in patients with leucine-rich, glioma-inactivated 1 antibody–associated encephalopathy. JAMA neurology. 2014;71(7):896-900.

287. Gresa-Arribas N, Titulaer MJ, Torrents A, et al. Antibody titres at diagnosis and during follow-up of anti-NMDA receptor encephalitis: a retrospective study. The Lancet Neurology. 2014;13(2):167-177.

288. Waters P, Jarius S, Littleton E, et al. Aquaporin-4 antibodies in neuromyelitis optica and longitudinally extensive transverse myelitis. Arch Neurol. 2008;65(7):913-919.

289. Witebsky E, Rose NR, Terplan K, et al. CHRONIC THYROIDITIS AND AUTOIMMUNIZATION. Journal of the American Medical Association. 1957;164(13):1439-1447.

290. Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today. 1993;14(9):426-430.

291. Vincent A, Irani SR. Caspr2 antibodies in patients with thymomas. J Thorac Oncol. 2010;5(10 Suppl 4):S277-280.

292. Lai M, Huijbers MG, Lancaster E, et al. Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. Lancet Neurol. 2010;9(8):776-785.

293. Boyko M, Au KLK, Casault C, et al. Systematic review of the clinical spectrum of CASPR2 antibody syndrome. J Neurol. 2020;267(4):1137-1146.

294. Hu W, Wang E, Fang H, et al. Clinical spectrum of contactin-associated protein 2 autoimmune encephalitis in children. Front Neurosci. 2023;17:1106214.

295. Syrbe S, Stettner GM, Bally J, et al. CASPR2 autoimmunity in children expanding to mild encephalopathy with hypertension. Neurology. 2020;94(22):e2290-e2301.

296. Muñiz-Castrillo S, Joubert B, Elsensohn MH, et al. Anti-CASPR2 clinical phenotypes correlate with HLA and immunological features. J Neurol Neurosurg Psychiatry. 2020;91(10):1076-1084.

297. Gadoth A, Pittock SJ, Dubey D, et al. Expanded phenotypes and outcomes among 256 LGI1/CASPR2-IgG–positive patients. Annals of Neurology. 2017;82(1):79-92.

298. Klein CJ, Lennon VA, Aston PA, et al. Insights from LGI1 and CASPR2 potassium channel complex autoantibody subtyping. JAMA Neurol. 2013;70(2):229-234.

299. Varley JA, Strippel C, Handel A, et al. Autoimmune encephalitis: recent clinical and biological advances. J Neurol. 2023;270(8):4118-4131.

300. Gövert F, Abrante L, Becktepe J, et al. Distinct movement disorders in contactin-associated-protein-like-2 antibody-associated autoimmune encephalitis. Brain. 2023;146(2):657-667.

301. Garrido Sanabria ER, Zahid A, Britton J, et al. CASPR2-IgG-associated autoimmune seizures. Epilepsia. 2022;63(3):709-722.

302. Ramanathan S, Tseng M, Davies AJ, et al. Leucine-Rich Glioma-Inactivated 1 versus Contactin-Associated Protein-like 2 Antibody Neuropathic Pain: Clinical and Biological Comparisons. Ann Neurol. 2021;90(4):683-690.

303. Greguletz P, Plötz M, Baade-Büttner C, et al. Different pain phenotypes are associated with anti-Caspr2 autoantibodies. J Neurol. 2024;271(5):2736-2744.

304. Körtvelyessy P, Bauer J, Stoppel CM, et al. Complement-associated neuronal loss in a patient with CASPR2 antibody-associated encephalitis. Neurol Neuroimmunol Neuroinflamm. 2015;2(2):e75.

305. Ünverengil G, Vanli Yavuz EN, Tüzün E, et al. Brain Infiltration of Immune Cells in CASPR2-Antibody Associated Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis. Noro Psikiyatr Ars. 2016;53(4):344-347.

306. Binks S, Varley J, Lee W, et al. Distinct HLA associations of LGI1 and CASPR2-antibody diseases. Brain. 2018;141(8):2263-2271.

307. Kusner LL, Losen M, Vincent A, et al. Guidelines for pre-clinical assessment of the acetylcholine receptor--specific passive transfer myasthenia gravis model-Recommendations for methods and experimental designs. Exp Neurol. 2015;270:3-10.

308. Zou Y, Zhang WF, Liu HY, et al. Structure and function of the contactin-associated protein family in myelinated axons and their relationship with nerve diseases. Neural Regen Res. 2017;12(9):1551-1558.

309. Poliak S, Gollan L, Martinez R, et al. Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. Neuron. 1999;24(4):1037-1047.

310. Graf ER, Zhang X, Jin SX, et al. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell. 2004;119(7):1013-1026.

311. Baumgartner S, Littleton JT, Broadie K, et al. A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. Cell. 1996;87(6):1059-1068.

312. Missler M, Südhof TC. Neurexins: three genes and 1001 products. Trends Genet. 1998;14(1):20-26.

313. Dean C, Dresbach T. Neuroligins and neurexins: linking cell adhesion, synapse formation and cognitive function. Trends in Neurosciences. 2006;29(1):21-29.

314. Joubert B, Saint-Martin M, Noraz N, et al. Characterization of a Subtype of Autoimmune Encephalitis With Anti–Contactin-Associated Protein-like 2 Antibodies in the Cerebrospinal Fluid, Prominent Limbic Symptoms, and Seizures. JAMA Neurology. 2016;73(9):1115-1124.

315. Patterson KR, Dalmau J, Lancaster E. Mechanisms of Caspr2 antibodies in autoimmune encephalitis and neuromyotonia. Ann Neurol. 2018;83(1):40-51.

316. Saint-Martin M, Pieters A, Déchelotte B, et al. Impact of anti-CASPR2 autoantibodies from patients with autoimmune encephalitis on CASPR2/TAG-1 interaction and Kv1 expression. J Autoimmun. 2019;103:102284.

317. Giannoccaro MP, Menassa DA, Jacobson L, et al. Behaviour and neuropathology in mice injected with human contactin-associated protein 2 antibodies. Brain. 2019;142(7):2000-2012.

318. Fernandes D, Santos SD, Coutinho E, et al. Disrupted AMPA Receptor Function upon Genetic- or Antibody-Mediated Loss of Autism-Associated CASPR2. Cereb Cortex. 2019;29(12):4919-4931.

319. Joubert B, Petit-Pedrol M, Planagumà J, et al. Human CASPR2 Antibodies Reversibly Alter Memory and the CASPR2 Protein Complex. Ann Neurol. 2022;91(6):801-813.

320. Romoli M, Krashia P, Sen A, et al. Hippocampal epileptogenesis in autoimmune encephalitis. Ann Clin Transl Neurol. 2019;6(11):2261-2269.

321. Kirschstein T, Sadkiewicz E, Hund-Göschel G, et al. Stereotactically Injected Kv1.2 and CASPR2 Antisera Cause Differential Effects on CA1 Synaptic and Cellular Excitability, but Both Enhance the Vulnerability to Pro-epileptic Conditions. Front Synaptic Neurosci. 2020;12:13.

322. Poliak S, Gollan L, Salomon D, et al. Localization of Caspr2 in myelinated nerves depends on axon-glia interactions and the generation of barriers along the axon. J Neurosci. 2001;21(19):7568-7575.

323. Arroyo EJ, Xu T, Poliak S, et al. Internodal specializations of myelinated axons in the central nervous system. Cell Tissue Res. 2001;305(1):53-66.

324. Scherer SS, Arroyo EJ. Recent progress on the molecular organization of myelinated axons. J Peripher Nerv Syst. 2002;7(1):1-12.

325. Rasband MN, Park EW, Zhen D, et al. Clustering of neuronal potassium channels is independent of their interaction with PSD-95. J Cell Biol. 2002;159(4):663-672.

326. Denisenko-Nehrbass N, Oguievetskaia K, Goutebroze L, et al. Protein 4.1B associates with both Caspr/paranodin and Caspr2 at paranodes and juxtaparanodes of myelinated fibres. Eur J Neurosci. 2003;17(2):411-416.

327. Spiegel I, Salomon D, Erne B, et al. Caspr3 and caspr4, two novel members of the caspr family are expressed in the nervous system and interact with PDZ domains. Mol Cell Neurosci. 2002;20(2):283-297.

328. Poliak S, Salomon D, Elhanany H, et al. Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. J Cell Biol. 2003;162(6):1149-1160.

329. Traka M, Goutebroze L, Denisenko N, et al. Association of TAG-1 with Caspr2 is essential for the molecular organization of juxtaparanodal regions of myelinated fibers. J Cell Biol. 2003;162(6):1161-1172.

330. Nakabayashi K, Scherer SW. The Human Contactin-Associated Protein-like 2 Gene (CNTNAP2) Spans over 2 Mb of DNA at Chromosome 7q35. Genomics. 2001;73(1):108-112.

331. Saint-Martin M, Joubert B, Pellier-Monnin V, et al. Contactin-associated protein-like 2, a protein of the neurexin family involved in several human diseases. European Journal of Neuroscience. 2018;48(3):1906-1923.

332. Rodenas-Cuadrado P, Ho J, Vernes SC. Shining a light on CNTNAP2: complex functions to complex disorders. Eur J Hum Genet. 2014;22(2):171-178.

333. Zhao Y, Liu X, Sun H, et al. Contactin-associated protein-like 2 expression in SH-SY5Y cells is upregulated by a FOXP2 mutant with a shortened poly-glutamine tract. Mol Med Rep. 2015;12(6):8162-8168.

334. Rubio-Marrero EN, Vincelli G, Jeffries CM, et al. Structural Characterization of the Extracellular Domain of CASPR2 and Insights into Its Association with the Novel Ligand Contactin1. J Biol Chem. 2016;291(11):5788-5802.

335. Lu Z, Reddy MV, Liu J, et al. Molecular Architecture of Contactin-associated Protein-like 2 (CNTNAP2) and Its Interaction with Contactin 2 (CNTN2). J Biol Chem. 2016;291(46):24133-24147.

336. Gordon A, Salomon D, Barak N, et al. Expression of Cntnap2 (Caspr2) in multiple levels of sensory systems. Mol Cell Neurosci. 2016;70:42-53.

337. Pinatel D, Hivert B, Saint-Martin M, et al. The Kv1-associated molecules TAG-1 and Caspr2 are selectively targeted to the axon initial segment in hippocampal neurons. J Cell Sci. 2017;130(13):2209-2220.

338. Pinatel D, Hivert B, Boucraut J, et al. Inhibitory axons are targeted in hippocampal cell culture by anti-Caspr2 autoantibodies associated with limbic encephalitis. Front Cell Neurosci. 2015;9:265.

339. Varea O, Martin-de-Saavedra MD, Kopeikina KJ, et al. Synaptic abnormalities and cytoplasmic glutamate receptor aggregates in contactin associated protein-like 2/Caspr2 knockout neurons. Proceedings of the National Academy of Sciences. 2015;112(19):6176-6181.

340. Inda MC, DeFelipe J, Muñoz A. Voltage-gated ion channels in the axon initial segment of human cortical pyramidal cells and their relationship with chandelier cells. Proc Natl Acad Sci U S A. 2006;103(8):2920-2925.

341. Ogawa Y, Horresh I, Trimmer JS, et al. Postsynaptic density-93 clusters Kv1 channels at axon initial segments independently of Caspr2. J Neurosci. 2008;28(22):5731-5739.

342. Scott BH, Leccese PA, Saleem KS, et al. Intrinsic Connections of the Core Auditory Cortical Regions and Rostral Supratemporal Plane in the Macaque Monkey. Cereb Cortex. 2017;27(1):809-840.

343. Scott R, Sánchez-Aguilera A, van Elst K, et al. Loss of Cntnap2 Causes Axonal Excitability Deficits, Developmental Delay in Cortical Myelination, and Abnormal Stereotyped Motor Behavior. Cereb Cortex. 2019;29(2):586-597.

344. Bridi MS, Park SM, Huang S. Developmental Disruption of GABA_AR-Meditated Inhibition in Cntnap2 KO Mice. eneuro. 2017;4(5):ENEURO.0162-0117.2017.

345. Jurgensen S, Castillo PE. Selective Dysregulation of Hippocampal Inhibition in the Mouse Lacking Autism Candidate Gene CNTNAP2. J Neurosci. 2015;35(43):14681-14687.

346. Peñagarikano O, Abrahams BS, Herman EI, et al. Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. Cell. 2011;147(1):235-246.

347. Gdalyahu A, Lazaro M, Penagarikano O, et al. The Autism Related Protein Contactin-Associated Protein-Like 2 (CNTNAP2) Stabilizes New Spines: An In Vivo Mouse Study. PLoS One. 2015;10(5):e0125633.

348. Anderson GR, Galfin T, Xu W, et al. Candidate autism gene screen identifies critical role for cell-adhesion molecule CASPR2 in dendritic arborization and spine development. Proc Natl Acad Sci U S A. 2012;109(44):18120-18125.

349. Argent L, Winter F, Prickett I, et al. Caspr2 interacts with type 1 inositol 1,4,5-trisphosphate receptor in the developing cerebellum and regulates Purkinje cell morphology. J Biol Chem. 2020;295(36):12716-12726.

350. Angum F, Khan T, Kaler J, et al. The Prevalence of Autoimmune Disorders in Women: A Narrative Review. Cureus. 2020;12(5):e8094.

351. Townsend LB, Smith SL. Genotype- and sex-dependent effects of altered Cntnap2 expression on the function of visual cortical areas. J Neurodev Disord. 2017;9:2.

352. Brimberg L, Mader S, Jeganathan V, et al. Caspr2-reactive antibody cloned from a mother of an ASD child mediates an ASD-like phenotype in mice. Mol Psychiatry. 2016;21(12):1663-1671.

353. Hoffman EJ, Turner KJ, Fernandez JM, et al. Estrogens Suppress a Behavioral Phenotype in Zebrafish Mutants of the Autism Risk Gene, CNTNAP2. Neuron. 2016;89(4):725-733.

354. Jones BG, Penkert RR, Xu B, et al. Binding of estrogen receptors to switch sites and regulatory elements in the immunoglobulin heavy chain locus of activated B cells suggests a direct influence of estrogen on antibody expression. Molecular Immunology. 2016;77:97-102.

355. Murphy K, Weaver C. Janeway's Immunobiology: W.W. Norton 2016.

356. Riedel S. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent). 2005;18(1):21-25.

357. Behring Ev. Ueber das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. 2013.

358. Behring Ev. Untersuchungen über das Zustandekommen der Diphtherie-Immunität bei Thieren. 2013.

359. Ehrlich P. Die Seitenkettentheorie und ihre Gegner. Muenchener medizinische Wochenschrift. 1901:2123-2124.

360. Ehrlich P. Die Schutzstoffe des Blutes. Deutsche medizinische Wochenschrift. 1901;27(50):865-867, 888-891 and 913-916.

361. Porter RR. The hydrolysis of rabbit y-globulin and antibodies with crystalline papain. Biochem J. 1959;73(1):119-126.

362. Edelman GM, Cunningham BA, Gall WE, et al. The covalent structure of an entire gammaG immunoglobulin molecule. Proc Natl Acad Sci U S A. 1969;63(1):78-85.

363. Delves PJ, Martin SJ, Burton DR, et al. Roitt's Essential Immunology: Wiley 2011.

364. Lu LL, Suscovich TJ, Fortune SM, et al. Beyond binding: antibody effector functions in infectious diseases. Nat Rev Immunol. 2018;18(1):46-61.

365. Damelang T, Brinkhaus M, van Osch TLJ, et al. Impact of structural modifications of IgG antibodies on effector functions. Front Immunol. 2023;14:1304365.

366. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S41-52.

367. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. Front Immunol. 2014;5:520.

368. Ferrante A, Beard LJ, Feldman RG. IgG subclass distribution of antibodies to bacterial and viral antigens. Pediatr Infect Dis J. 1990;9(8 Suppl):S16-24.

369. Jefferis R, Kumararatne DS. Selective IgG subclass deficiency: quantification and clinical relevance. Clin Exp Immunol. 1990;81(3):357-367.

370. Hammarström L, Smith CI. IgG2 deficiency in a healthy blood donor. Concomitant lack of IgG2, IgA and IgE immunoglobulins and specific anti-carbohydrate antibodies. Clin Exp Immunol. 1983;51(3):600-604.

371. Kuijpers TW, Weening RS, Out TA. IgG subclass deficiencies and recurrent pyogenic infections, unresponsiveness against bacterial polysaccharide antigens. Allergol Immunopathol (Madr). 1992;20(1):28-34.

372. Aalberse RC, Stapel SO, Schuurman J, et al. Immunoglobulin G4: an odd antibody. Clin Exp Allergy. 2009;39(4):469-477.

373. Nouri-Aria KT, Wachholz PA, Francis JN, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J Immunol. 2004;172(5):3252-3259.

374. Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. J Immunol. 1983;130(2):722-726.

375. Aalberse RC, Dieges PH, Knul-Bretlova V, et al. IgG4 as a blocking antibody. Clin Rev Allergy. 1983;1(2):289-302.

376. Jutel M, Akdis CA. Immunological mechanisms of allergen-specific immunotherapy. Allergy. 2011;66(6):725-732.

377. Kurniawan A, Yazdanbakhsh M, van Ree R, et al. Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human filariasis. J Immunol. 1993;150(9):3941-3950.

378. Sah RP, Chari ST. Serologic issues in IgG4-related systemic disease and autoimmune pancreatitis. Curr Opin Rheumatol. 2011;23(1):108-113.

379. Yamamoto M, Tabeya T, Naishiro Y, et al. Value of serum IgG4 in the diagnosis of IgG4-related disease and in differentiation from rheumatic diseases and other diseases. Mod Rheumatol. 2012;22(3):419-425.

380. Mahajan VS, Mattoo H, Deshpande V, et al. IgG4-related disease. Annu Rev Pathol. 2014;9:315-347.

381. Kaunitz JD. Development of Monoclonal Antibodies: The Dawn of mAb Rule. Dig Dis Sci. 2017;62(4):831-832.

382. Askonas BA, Williamson AR, Wright BE. Selection of a single antibody-forming cell clone and its propagation in syngeneic mice. Proc Natl Acad Sci U S A. 1970;67(3):1398-1403.

383. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256(5517):495-497.

384. Liu JK. The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Ann Med Surg (Lond). 2014;3(4):113-116.

385. Smith SL. Ten years of Orthoclone OKT3 (muromonab-CD3): a review. J Transpl Coord. 1996;6(3):109-119; quiz 120-101.

386. Yam-Puc JC, Zhang L, Zhang Y, et al. Role of B-cell receptors for B-cell development and antigen-induced differentiation. F1000Res. 2018;7:429.

387. Vale AM, Kearney JF, Nobrega A, et al. Chapter 7 - Development and Function of B Cell Subsets. In Alt FW, Honjo T, Radbruch A, et al., (Eds). Molecular Biology of B Cells (Second Edition). London: Academic Press 2015:99-119.

388. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19:595-621.

389. Cooper MD, Alder MN. The evolution of adaptive immune systems. Cell. 2006;124(4):815-822.

390. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood. 2008;112(5):1570-1580.

391. Tanabe S, Yamashita T. B lymphocytes: Crucial contributors to brain development and neurological diseases. Neuroscience Research. 2019;139:37-41.

392. Korin B, Ben-Shaanan TL, Schiller M, et al. High-dimensional, single-cell characterization of the brain's immune compartment. Nature Neuroscience. 2017;20(9):1300-1309.

393. Schafflick D, Wolbert J, Heming M, et al. Single-cell profiling of CNS border compartment leukocytes reveals that B cells and their progenitors reside in non-diseased meninges. Nat Neurosci. 2021;24(9):1225-1234.

394. Kleine TO, Benes L. Immune surveillance of the human central nervous system (CNS): different migration pathways of immune cells through the blood-brain barrier and blood-cerebrospinal fluid barrier in healthy persons. Cytometry A. 2006;69(3):147-151.

395. Wilson R, Makuch M, Kienzler AK, et al. Condition-dependent generation of aquaporin-4 antibodies from circulating B cells in neuromyelitis optica. Brain. 2018;141(4):1063-1074.

396. Ramberger M, Berretta A, Tan JMM, et al. Distinctive binding properties of human monoclonal LGI1 autoantibodies determine pathogenic mechanisms. Brain. 2020;143(6):1731-1745.

397. Winklmeier S, Schlüter M, Spadaro M, et al. Identification of circulating MOG-specific B cells in patients with MOG antibodies. Neurol Neuroimmunol Neuroinflamm. 2019;6(6):625.

398. Makuch M, Wilson R, Al-Diwani A, et al. N-methyl-D-aspartate receptor antibody production from germinal center reactions: Therapeutic implications. Ann Neurol. 2018;83(3):553-561.

399. Kornau HC, Kreye J, Stumpf A, et al. Human Cerebrospinal Fluid Monoclonal LGI1 Autoantibodies Increase Neuronal Excitability. Ann Neurol. 2020;87(3):405-418.

400. Kreye J, Wenke NK, Chayka M, et al. Human cerebrospinal fluid monoclonal N-methyl-D-aspartate receptor autoantibodies are sufficient for encephalitis pathogenesis. Brain. 2016;139(Pt 10):2641-2652.

401. Theorell J, Harrison R, Williams R, et al. Ultrahigh frequencies of peripherally matured LGI1- and CASPR2-reactive B cells characterize the cerebrospinal fluid in autoimmune encephalitis. Proc Natl Acad Sci U S A. 2024;121(7):e2311049121.

402. Cooper MD, Peterson RD, Good RA. DELINEATION OF THE THYMIC AND BURSAL LYMPHOID SYSTEMS IN THE CHICKEN. Nature. 1965;205:143-146.

403. Cooper MD, Raymond DA, Peterson RD, et al. The functions of the thymus system and the bursa system in the chicken. J Exp Med. 1966;123(1):75-102.

404. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J Exp Med. 1968;128(4):801-820.

405. Mitchell GF, Miller JF. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J Exp Med. 1968;128(4):821-837.

406. Binks SNM, Klein CJ, Waters P, et al. LGI1, CASPR2 and related antibodies: a molecular evolution of the phenotypes. J Neurol Neurosurg Psychiatry. 2018;89(5):526-534.

407. Damato V, Theorell J, Al-Diwani A, et al. Rituximab abrogates aquaporin-4-specific germinal center activity in patients with neuromyelitis optica spectrum disorders. Proc Natl Acad Sci U S A. 2022;119(24):e2121804119.

408. Jiang Y, Tan C, Li T, et al. Phenotypic Spectrum of CASPR2 and LGI1 Antibodies Associated Neurological Disorders in Children. Front Pediatr. 2022;10:815976.

409. Seery N, Butzkueven H, O'Brien TJ, et al. Contemporary advances in antibody-mediated encephalitis: anti-LGI1 and anti-Caspr2 antibody (Ab)-mediated encephalitides. Autoimmun Rev. 2022;21(5):103074.

410. van Sonderen A, Petit-Pedrol M, Dalmau J, et al. The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. Nature Reviews Neurology. 2017;13(5):290-301.

411. Joubert B, Gobert F, Thomas L, et al. Autoimmune episodic ataxia in patients with anti-CASPR2 antibody-associated encephalitis. Neurol Neuroimmunol Neuroinflamm. 2017;4(4):e371.

412. Wang J, Qiu Z, Li D, et al. Anti-contactin-associated protein-like 2 antibody-associated cerebellar ataxia: A case report and literature review. J Neuroimmunol. 2021;353:577515.

413. Dawes JM, Weir GA, Middleton SJ, et al. Immune or Genetic-Mediated Disruption of CASPR2 Causes Pain Hypersensitivity Due to Enhanced Primary Afferent Excitability. Neuron. 2018;97(4):806-822.e810.

414. Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res. 1988;31(1):47-59.

415. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process. 2012;13(2):93-110.

416. Sharma S, Rakoczy S, Brown-Borg H. Assessment of spatial memory in mice. Life Sci. 2010;87(17-18):521-536.

417. Coutinho E, Menassa DA, Jacobson L, et al. Persistent microglial activation and synaptic loss with behavioral abnormalities in mouse offspring exposed to CASPR2-antibodies in utero. Acta Neuropathol. 2017;134(4):567-583.

418. Bien CG, Mirzadjanova Z, Baumgartner C, et al. Anti-contactin-associated protein-2 encephalitis: relevance of antibody titres, presentation and outcome. European Journal of Neurology. 2017;24(1):175-186.

419. Sarvas HO, Seppälä IJT, Tähtinen T, et al. Mouse IgG antibodies have subclass associated affinity differences. Molecular Immunology. 1983;20(3):239-246.

420. Klein-Schneegans AS, Kuntz L, Fonteneau P, et al. Serum concentrations of IgM, IgG1, IgG2b, IgG3 and IgA in C57BL/6 mice and their congenics at the lpr (lymphoproliferation) locus. J Autoimmun. 1989;2(6):869-875.

421. Kelly MJ, Grant E, Murchison AG, et al. Magnetic Resonance Imaging Characteristics of LGI1-Antibody and CASPR2-Antibody Encephalitis. JAMA Neurol. 2024;81(5):525-533.

422. Petit-Pedrol M, Sell J, Planagumà J, et al. LGI1 antibodies alter Kv1.1 and AMPA receptors changing synaptic excitability, plasticity and memory. Brain. 2018;141(11):3144-3159.

423. Bird CM, Burgess N. The hippocampus and memory: insights from spatial processing. Nat Rev Neurosci. 2008;9(3):182-194.

424. Bannerman DM, Matthews P, Deacon RM, et al. Medial septal lesions mimic effects of both selective dorsal and ventral hippocampal lesions. Behav Neurosci. 2004;118(5):1033-1041.

425. Bannerman DM, Deacon RM, Offen S, et al. Double dissociation of function within the hippocampus: spatial memory and hyponeophagia. Behav Neurosci. 2002;116(5):884-901.

426. Ghasemi M, Navidhamidi M, Rezaei F, et al. Anxiety and hippocampal neuronal activity: Relationship and potential mechanisms. Cogn Affect Behav Neurosci. 2022;22(3):431-449.

427. Bergman I, Burckart GJ, Pohl CR, et al. Pharmacokinetics of IgG and IgM anti-ganglioside antibodies in rats and monkeys after intrathecal administration. J Pharmacol Exp Ther. 1998;284(1):111-115.

428. Noguchi Y, Kato M, Ozeki K, et al. Pharmacokinetics of an intracerebroventricularly administered antibody in rats. MAbs. 2017;9(7):1210-1215.

429. Zhang Y, Pardridge WM. Mediated efflux of IgG molecules from brain to blood across the blood-brain barrier. J Neuroimmunol. 2001;114(1-2):168-172.

430. Cooper PR, Ciambrone GJ, Kliwinski CM, et al. Efflux of monoclonal antibodies from rat brain by neonatal Fc receptor, FcRn. Brain Res. 2013;1534:13-21.

431. Shivaram S, Nagappa M, Seshagiri DV, et al. P-AD003. Autonomic dysfunction in CASPR2 antibody associated neurological disease. Clinical Neurophysiology. 2021;132(8):e95.

432. Gilbert E, Binks S, Damato V, et al. The gut microbiome associated with LGI1- and CASPR2-antibody encephalitis. medRxiv. 2024:2024.2004.2025.24305899.

433. Barone FC, Deegan JF, Price WJ, et al. Cold-restraint stress increases rat fecal pellet output and colonic transit. Am J Physiol. 1990;258(3 Pt 1):G329-337.

434. Benoit J, Muñiz-Castrillo S, Vogrig A, et al. Early-Stage Contactin-Associated Protein-like 2 Limbic Encephalitis: Clues for Diagnosis. Neurol Neuroimmunol Neuroinflamm. 2023;10(1).

435. Zou A, Ramanathan S, Dale RC, et al. Single-cell approaches to investigate B cells and antibodies in autoimmune neurological disorders. Cell Mol Immunol. 2021;18(2):294-306.

436. Pedrioli A, Oxenius A. Single B cell technologies for monoclonal antibody discovery. Trends Immunol. 2021;42(12):1143-1158.

437. Tiller T, Meffre E, Yurasov S, et al. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. J Immunol Methods. 2008;329(1-2):112-124.

438. Cotzomi E, Stathopoulos P, Lee CS, et al. Early B cell tolerance defects in neuromyelitis optica favour anti-AQP4 autoantibody production. Brain. 2019;142(6):1598-1615.

439. Ceanga M, Rahmati V, Haselmann H, et al. Human NMDAR autoantibodies disrupt excitatory-inhibitory balance, leading to hippocampal network hypersynchrony. Cell Rep. 2023;42(10):113166.

440. Kreye J, Wright SK, van Casteren A, et al. Encephalitis patient-derived monoclonal GABAA receptor antibodies cause epileptic seizures. J Exp Med. 2021;218(11).

441. Wardemann H, Yurasov S, Schaefer A, et al. Predominant autoantibody production by early human B cell precursors. Science. 2003;301(5638):1374-1377.

442. Padamsey Z, McGuinness L, Bardo SJ, et al. Activity-Dependent Exocytosis of Lysosomes Regulates the Structural Plasticity of Dendritic Spines. Neuron. 2017;93(1):132-146.

443. Patil SS, Sunyer B, Höger H, et al. Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. Behav Brain Res. 2009;198(1):58-68.

444. Brooks SP, Pask T, Jones L, et al. Behavioural profiles of inbred mouse strains used as transgenic backgrounds. II: cognitive tests. Genes, Brain and Behavior. 2005;4(5):307-317.

445. Kraeuter AK, Guest PC, Sarnyai Z. The Y-Maze for Assessment of Spatial Working and Reference Memory in Mice. Methods Mol Biol. 2019;1916:105-111.

446. Olsen AL, Lai Y, Dalmau J, et al. Caspr2 autoantibodies target multiple epitopes. Neurol Neuroimmunol Neuroinflamm. 2015;2(4):e127.

447. Chakraborty S, Zawieja SD, Wang W, et al. Lipopolysaccharide modulates neutrophil recruitment and macrophage polarization on lymphatic vessels and impairs lymphatic function in rat mesentery. Am J Physiol Heart Circ Physiol. 2015;309(12):H2042-2057.

448. Zhou H, Andonegui G, Wong CH, et al. Role of endothelial TLR4 for neutrophil recruitment into central nervous system microvessels in systemic inflammation. J Immunol. 2009;183(8):5244-5250.

449. Wiedholz LM, Owens WA, Horton RE, et al. Mice lacking the AMPA GluR1 receptor exhibit striatal hyperdopaminergia and 'schizophrenia-related' behaviors. Mol Psychiatry. 2008;13(6):631-640.

450. Pierson ER, Wagner CA, Goverman JM. The contribution of neutrophils to CNS autoimmunity. Clin Immunol. 2018;189:23-28.

451. Rumble JM, Huber AK, Krishnamoorthy G, et al. Neutrophil-related factors as biomarkers in EAE and MS. J Exp Med. 2015;212(1):23-35.

452. Christy AL, Walker ME, Hessner MJ, et al. Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE. J Autoimmun. 2013;42:50-61.

453. Bernardes-Silva M, Anthony DC, Issekutz AC, et al. Recruitment of neutrophils across the blood-brain barrier: the role of E- and P-selectins. J Cereb Blood Flow Metab. 2001;21(9):1115-1124.

454. Neumann J, Riek-Burchardt M, Herz J, et al. Very-late-antigen-4 (VLA-4)-mediated brain invasion by neutrophils leads to interactions with microglia, increased ischemic injury and impaired behavior in experimental stroke. Acta Neuropathol. 2015;129(2):259-277.

455. Zenaro E, Pietronigro E, Della Bianca V, et al. Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. Nat Med. 2015;21(8):880-886.

456. Demirci S, Demirci S, Kutluhan S, et al. The clinical significance of the neutrophil-to-lymphocyte ratio in multiple sclerosis. Int J Neurosci. 2016;126(8):700-706.

457. Naegele M, Tillack K, Reinhardt S, et al. Neutrophils in multiple sclerosis are characterized by a primed phenotype. J Neuroimmunol. 2012;242(1-2):60-71.

458. Summers C, Rankin SM, Condliffe AM, et al. Neutrophil kinetics in health and disease. Trends Immunol. 2010;31(8):318-324.

459. Luo XM, Maarschalk E, O'Connell RM, et al. Engineering human hematopoietic stem/progenitor cells to produce a broadly neutralizing anti-HIV antibody after in vitro maturation to human B lymphocytes. Blood. 2009;113(7):1422-1431.

460. Carlring J, Altaher HM, Clark S, et al. CD154-CD40 interactions in the control of murine B cell hematopoiesis. J Leukoc Biol. 2011;89(5):697-706.

461. Su K-Y, Nojima T, Kuraoka M, et al. An in vitro culture system for studying the human B cell repertoire (P1459). The Journal of Immunology. 2013;190(1_Supplement):174.112-174.112.

462. Su KY, Watanabe A, Yeh CH, et al. Efficient Culture of Human Naive and Memory B Cells for Use as APCs. J Immunol. 2016;197(10):4163-4176.

463. DeLaura I, Schroder PM, Yoon J, et al. A novel method for in vitro culture and expansion of nonhuman primate B cells. J Immunol Methods. 2022;511:113363.

464. Song S, Manook M, Kwun J, et al. Allo-Specific Humoral Responses: New Methods for Screening Donor-Specific Antibody and Characterization of HLA-Specific Memory B Cells. Front Immunol. 2021;12:705140.

465. Cree BA, Lamb S, Morgan K, et al. An open label study of the effects of rituximab in neuromyelitis optica. Neurology. 2005;64(7):1270-1272.

466. Irani SR, Gelfand JM, Bettcher BM, et al. Effect of rituximab in patients with leucine-rich, glioma-inactivated 1 antibody-associated encephalopathy. JAMA Neurol. 2014;71(7):896-900.

467. Tea F, Lopez JA, Ramanathan S, et al. Characterization of the human myelin oligodendrocyte glycoprotein antibody response in demyelination. Acta Neuropathol Commun. 2019;7(1):145.

468. Mayer MC, Breithaupt C, Reindl M, et al. Distinction and temporal stability of conformational epitopes on myelin oligodendrocyte glycoprotein recognized by patients with different inflammatory central nervous system diseases. J Immunol. 2013;191(7):3594-3604.

469. Takata K, Stathopoulos P, Cao M, et al. Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients. JCI Insight. 2019;4(12).

470. Sabatino JJ, Pröbstel A-K, Zamvil SS. B cells in autoimmune and neurodegenerative central nervous system diseases. Nature Reviews Neuroscience. 2019;20(12):728-745.

471. Fichtner ML, Hoehn KB, Ford EE, et al. Reemergence of pathogenic, autoantibody-producing B cell clones in myasthenia gravis following B cell depletion therapy. Acta Neuropathol Commun. 2022;10(1):154.

472. Rose N, Holdermann S, Callegari I, et al. Receptor clustering and pathogenic complement activation in myasthenia gravis depend on synergy between antibodies with multiple subunit specificities. Acta Neuropathol. 2022;144(5):1005-1025.

473. Whittle JR, Wheatley AK, Wu L, et al. Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. J Virol. 2014;88(8):4047-4057.

474. Mathew NR, Jayanthan JK, Smirnov IV, et al. Single-cell BCR and transcriptome analysis after influenza infection reveals spatiotemporal dynamics of antigen-specific B cells. Cell Rep. 2021;35(12):109286.

475. Velounias RL, Tull TJ. Human B-cell subset identification and changes in inflammatory diseases. Clin Exp Immunol. 2022;210(3):201-216.

476. Aranow C, Diamond B, Mackay M. 50 - Systemic lupus erythematosus. In Rich RR, Fleisher TA, Shearer WT, et al., (Eds). Clinical Immunology (Fourth Edition). London: Elsevier 2013:603-620.

477. Spadaro M, Winklmeier S, Beltrán E, et al. Pathogenicity of human antibodies against myelin oligodendrocyte glycoprotein. Ann Neurol. 2018;84(2):315-328.