Modelling progressive multiple sclerosis for new treatment strategies



Jay Roodselaar
Somerville College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy

May 2019

Abstract

Multiple sclerosis (MS) is an autoimmune, demyelinating, degenerative disease of the central nervous system. Secondary progressive (SP) MS differs from relapse-remitting (RR) MS in clinical symptoms and histopathology. As disease progresses, the proportion of B cells and plasma cells increases in the brain. Moreover, meningeal tertiary lymphoid structures (TLS) are present in a large proportion of SPMS patients and are associated with more severe pathology. Treatment is available for suppressing relapses in RRMS, but no disease modifying therapy (DMT) is available for the ongoing disease worsening in SPMS. This is, in part, due to a lack of preclinical models for SPMS. There is a need for rodent models of SPMS to investigate disease mechanisms and treatment options. This thesis describes the development of two new rodent models of SPMS, characterised by glial activation, lymphocyte infiltration, demyelination, and neuronal death behind a closed blood-brain barrier (BBB).

Focal MS-like lesions were induced by first immunising mice with MOG and CFA intradermally, followed by an intracranial injection with heat-killed mycobacterium tuberculosis in either the striatum or the piriform cortex. Mice were treated with anti-LINGO-1 or anti-CD20 antibodies 4 weeks after initiation of the lesion. Treatment effects on the different aspects of the focal lesions were evaluated by immunohistochemistry.

MS-like lesions formed in the brains independent of injection site. However, TLS were only formed in mice with lesions in the piriform cortex. Both types of lesions continued to grow over time. Anti-LINGO-1 therapy promoted oligodendrocyte survival and reduced glial activation in the brains of these mice. Treatment with anti-CD20 decreased the extent of glial activation, significantly decreased the number of B and T lymphocytes, and reduced the size of the TLS.

SPMS with and without TLS can be modelled in rodents, with lesions that continue to evolve over time behind an intact BBB. Two different treatments were shown to be effective in this model, demonstrating the potential of using anti-LINGO-1 or anti-CD20 as a DMT for SPMS.

Acknowledgements

I am forever grateful having been given the opportunity to pursue my DPhil and to have worked with so many incredible people. Firstly, I would like to thank my supervisor, professor Daniel Anthony, for providing all the support and advice throughout my time in Oxford. You have been a great supervisor to me, and a role model in the way you approach science. Thank you for having also been a great friend, and I hope we will continue to make discoveries together.

Many thanks go to Dr. Eduard Urich for the great guidance, advice, and in-depth knowledge on the different therapies I was able to work with. And thanks also to Hoffmann-La Roche, for providing the different antibodies used in this thesis. I would also like to thank Dr. Jack Miller, for his assistance, time, and guidance with the imaging experiment.

A big thank you to all members and visitors of the Anthony lab, for the support and friendships over the years. In particular, I would like to thank Yifan and Megan, for supporting me until the end. A special thank you to Inês, for being my amazing friend from the very start of my DPhil. I further would like to thank Ain, Meng, and Ralu who were always there for me for support and good company.

I would like to thank my family for their constant support and encouragement, and having faith in me, even when we are far away. If it was not for you, I would not have been where I am now.

Finally, to Leo, thank you very much for all your patience, support, faith, and making me believe that I can achieve anything.

Contents

1. INTRODUCTION 2

1.1 AN OVERVIEW OF MULTIPLE SCLEROSIS 2

1.1.1 HISTORICAL PERSPECTIVES 2

1.1.2 PRESENTATION AND CLASSIFICATION ..... **ERROR! BOOKMARK NOT DEFINED.**

1.1.2.1 Relapse-remitting MS 4

1.1.2.2 Secondary progressive MS 4

1.1.2.3 Primary progressive MS 5

1.1.2.4 Progressive-relapsing MS 5

1.1.2.5 Neuromyelitis Optica and other diseases in the spectrum 7

1.1.2.6 Tuberculosis meningitis and other meningoencephalitis diseases 7

1.1.3 AETIOLOGY 8

1.1.4 DIAGNOSIS 10

1.1.4.1 Clinical signs 12

1.1.4.2 MRI 12

1.1.4.3 Oligoclonal bands 14

1.2 PATHOPHYSIOLOGY OF MULTIPLE SCLEROSIS 15

1.2.1 NEUROINFLAMMATION 17

1.2.1.1 Microglia/ Macrophages/ Monocytes 21

1.2.1.2 Astrocytes 23

1.2.1.3 T cells 24

1.2.1.4 B cells and plasma cells 25

1.2.2 MENINGEAL TERTIARY LYMPHOID STRUCTURES IN MS 28

1.2.3 THE BLOOD-BRAIN BARRIER 17

1.2.4 NEURODEGENERATION 36

1.2.5 DEMYELINATION 33
1.2.5.1 Oligodendrocytes 35
1.2.6 NEURONAL / AXONAL LOSS 36
1.2.7 THE ACUTE PHASE RESPONSE 38
1.2.7.1 The acute phase response after CNS injury 39
1.3 TREATMENT OF MS 43
1.3.1.1 First-line therapies 45
1.3.1.2 Second-line therapies 46
1.3.1.3 Anti-CD20 antibodies in MS 49
1.3.1.4 Other Anti-CD20 antibodies – Obinutuzumab/GA101 55
1.3.2 NEUROPROTECTIVE AND NEURORESTORATIVE AGENTS 58
1.3.2.1 Anti-LINGO-1 antibodies 60
1.3.3 LIMITATIONS AND NEW DIRECTIONS OF THERAPIES 62
1.4 ANIMAL MODELS OF MS 63
1.4.1 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS 63
1.4.2 DELAYED TYPE HYPERSENSITIVITY MODEL 66
1.4.3 FOCAL MOG MODEL 67
1.5 VIRUS-INDUCED MODELS OF MS 68
1.5.1.1 Toxin-induced models of demyelination 69
1.6 SUMMARY 70
1.7 AIMS OF THIS THESIS 71

2. METHODS 72

2.1 ANIMALS 73
2.2 REAGENTS 73
2.3 IN VIVO PROCEDURES 74

2.3.1 ANAESTHESIA 74
2.3.2 IMMUNISATION 74
2.3.3 STEREOTAXIC SURGERY 75
2.3.4 ADMINISTRATION OF DRUGS 75
2.4 BEHAVIOURAL TESTING 76
2.4.1 INVERTED SCREEN 76
2.4.2 FIXED RODS 76
2.5 MRI 77
2.6 TISSUE SAMPLE COLLECTION AND PREPARATION 77
2.7 BLOOD ANALYSIS 78
2.8 HISTOLOGY 78
2.8.1 GELATINISING SLIDES 78
2.8.2 SECTIONING OF TISSUES 79
2.8.3 IMMUNOHISTOCHEMISTRY 79
2.8.4 COUNTERSTAINING AND MOUNTING 80
2.8.5 QUANTIFICATION AND ANALYSIS OF THE HISTOLOGY 81
2.9 MOLECULAR BIOLOGY 82
2.9.1 MRNA EXTRACTION 82
2.9.2 CDNA CONVERSION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION 83
2.10 FLOW CYTOMETRY 84
2.10.1 CELL ISOLATION PROCEDURE 84
2.10.2 CELL STAINING PROCEDURE 84
2.10.3 GATING STRATEGY 85
2.11 STATISTICAL ANALYSIS 85

3. THE DEVELOPMENT OF A NEW FOCAL MODEL OF MULTIPLE SCLEROSIS IN MICE....86

3.1 INTRODUCTION AND AIMS....87

3.2 METHODS....90

3.2.1 INDUCTION OF FOCAL EAE IN MICE....90

3.2.2 INDUCTION OF THE NEW FOCAL DTH-MOG MODEL....90

3.2.3 TREATMENT WITH ANTI-LINGO-1....91

3.2.4 TREATMENT WITH ANTI-CD20....91

3.3 RESULTS....92

3.3.1 MODEL DEVELOPMENT....92

3.3.1.1 The focal MOG model does not induce MS-like lesions in mice....92

3.3.1.2 MOG protein does not change the focal MS-like lesions....92

3.3.1.3 Intracranial TB generates MS-like lesion in MOG-immunised mice....95

3.3.2 TREATMENT WITH ANTI-LINGO1....95

3.3.2.1 Anti-Lingo-1 increased mRNA expression levels of myelin proteins in the DTH-MOG lesion....97

3.3.2.2 Anti-LINGO-1 increases the number of oligodendrocyte precursor cells....99

3.3.2.3 Anti-LINGO-1 treatment reduces neuroinflammation in the DTH-MOG model. 101

3.3.3 TREATMENT WITH ANTI-CD20....103

3.3.3.1 Different Fc variants of GA101 deplete peripheral B cells equally....103

3.3.3.2 PGLALA reduced microglial and astrocyte activation....106

3.3.3.3 PGLALA significantly reduces B and T cells in the brain....106

3.3.3.4 PGLALA significantly depletes splenic B cells and liver neutrophils....109

3.4 DISCUSSION....111

3.5 CONCLUSION FROM THIS CHAPTER....118

4. MODELLING TERTIARY LYMPHOID STRUCTURES IN A MOUSE MODEL OF MULTIPLE SCLEROSIS. 119

4.1 INTRODUCTION AND AIMS 120

4.2 METHODS 123

4.2.1 INDUCTION OF THE DTH-TLS MODEL 123

4.2.2 CHARACTERISATION OF THE DTH-TLS MODEL 123

4.2.3 TREATMENT WITH ANTI-CD20 123

4.3 RESULTS 124

4.3.1 MODEL CHARACTERISATION 124

4.3.1.1 The DTH-TLS model does not cause weight loss, or loss of motor function. 124

4.3.1.2 Chronically activated microglia and astrocytes spread out over time 127

4.3.1.3 B and T cells increase in number over time and form TLS-like structures in the meninges in the DTH-TLS model. 131

4.3.1.4 Proliferating B cells were present in meningeal TLS in the DTH-TLS model. 133

4.3.1.5 Worsening demyelination in the olfactory tract of mice with the DTH-TLS model. 134

4.3.1.6 No blood-brain barrier breakdown detected with gadolinium enhanced MRI in the DTH-TLS model on day 100. 136

4.3.2 TYPE I VS TYPE II ANTI-CD20 THERAPY 138

4.3.2.1 Anti-CD20 significantly depletes peripheral B cells. 138

4.3.2.2 Anti-CD20 treatment reduces microglial and astrocyte activation in DTH-TLS lesions. 140

4.3.2.3 Anti-CD20 therapy significantly reduces the number of B and T cells in the brain. 142

4.3.2.4 GA101 protects neurons in the DTH-TLS model. 144

4.4 DISCUSSION 146

4.5 CONCLUSION FROM THIS CHAPTER 152

5. THE EFFECT OF ANTI-CD20 ON THE ACUTE PHASE RESPONSE AFTER ACUTE BRAIN INFLAMMATION 153

5.1 INTRODUCTION AND AIMS 154

5.2 METHODS 156

5.2.1 TREATMENT WITH ANTI-CD20 156

5.2.2 INTRACRANIAL INJECTION OF IL-1B 156

5.2.3 TISSUE COLLECTION 156

5.3 RESULTS 157

5.3.1 A SINGLE DOSE OF GA101 DEPLETES PERIPHERAL B CELLS. 157

5.3.2 GA101 REDUCES THE NUMBER OF LIVER NEUTROPHILS, BUT NOT KUPFFER CELLS, ASSOCIATED WITH THE HEPATIC ACUTE PHASE RESPONSE AFTER ACUTE BRAIN INFLAMMATION. 159

5.3.3 GA101 AFFECTS IL1B MRNA EXPRESSION IN THE LIVER AFTER ACUTE BRAIN INFLAMMATION. 161

5.3.4 ANTI-CD20 DOES NOT AFFECT NEUTROPHIL RECRUITMENT TO THE BRAIN IN AN ACUTE BRAIN INFLAMMATION MODEL 164

5.3.5 ANTI-CD20 DOES NOT AFFECT MICROGLIAL ACTIVATION IN AN ACUTE BRAIN INFLAMMATION MODEL. 166

5.4 DISCUSSION 168

5.5 CONCLUSIONS FROM THIS CHAPTER 172

6. GENERAL DISCUSSION AND FUTURE DIRECTIONS. 173

6.1 CONCLUDING REMARKS 184

6.2 BIBLIOGRAPHY 186

List of Figures

Figure 1.1 Forms of multiple sclerosis..... 6

Figure 1.2 Types of cortical lesions..... 16

Figure 1.3 Tertiary lymphoid structure..... 30

Figure 1.4 The acute phase response after brain injury. 41

Figure 1.5 The relative efficacy and risk of different MS therapies. 44

Figure 1.6 Mechanisms of action of anti-CD20 antibodies..... 50

Figure 1.7 CD20 binding sites..... 54

Figure 3.1 The adapted focal EAE model does not induce MS-like lesions in mice. ..... **Error! Bookmark not defined.**

Figure 3.2 A new focal model for MS. ..... **Error! Bookmark not defined.**

Figure 3.3 Anti-Lingo-1 treatment increases MBP expression in the MOG-DTH model. ..... **Error! Bookmark not defined.**

Figure 3.4 Increased proliferation of oligodendrocyte precursor cells.**Error! Bookmark not defined.**

Figure 3.5 Anti-LINGO-1 treatment reduces astrocyte and microglial activation in the DTH-MOG model.. ..... **Error! Bookmark not defined.**

Figure 3.6 Peripheral effects of the different Fc variants of GA101..**Error! Bookmark not defined.**

Figure 3.7 Anti-CD20 reduced microglial and astrocyte activation.**Error! Bookmark not defined.**

Figure 3.8 PGLALA significantly reduces brain lymphocytes.**Error! Bookmark not defined.**

Figure 3.9 PGLALA depletes splenic B cells and liver neutrophils.**Error! Bookmark not defined.**

Figure 3.10 A simplified overview of the effects of LINGO-1 on CNS cell types.....**Error! Bookmark not defined.**

Figure 4.1 The DTH-TLS model caused transient weight loss, but no impairment in motor function. 126

Figure 4.2 Spreading microglial activation in the DTH-TLS model..... 128

Figure 4.3 Spreading astrocyte activation in the DTH-TLS model. 130

Figure 4.4 B and T cells increase in number over time and form TLS-like structures in the meninges..... 132

Figure 4.5 Proliferating B cells are present in meningeal TLS in the DTH-TLS model. 133

Figure 4.6 Progressing demyelination in the DTH-TLS model.. 135

Figure 4.7 No blood-brain barrier breakdown observed with gadolinium enhanced MRI in the DTH-TLS model on day 100..... 137

Figure 4.8 Anti CD20 depletes peripheral B cells in the DTH-TLS model. 139

Figure 4.9 Anti-CD20 therapy reduces glial activation in DTH-TLS lesion. 141

Figure 4.10 Anti-CD20 reduces the number of brain B and T cells..... 143

Figure 4.11 GA101 protects neurons in the DTH-TLS model.. 145

Figure 5.1 Treatment paradigm. 156

Figure 5.2 A single dose of GA101 significantly depletes peripheral B cells..... 158

Figure 5.3 GA101 reduces the number of liver neutrophils, but not Kupffer cells, associated with the hepatic acute phase response after acute brain inflammation. 160

Figure 5.4 GA101 affects IL1B mRNA expression in the liver after acute brain inflammation. 163

Figure 5.5 Anti-CD20 does not affect neutrophil recruitment in an acute brain inflammation model. 165

Figure 5.6 Anti-CD20 does not affect microglial activation in an acute brain inflammation model. 167

List of Tables

Table 1-1 The Schumacher criteria. 10

Table 2.1 Primary antibodies used for immunohistochemistry. 80

Table 2.2 Primers and sequences 83

Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
AHSCT	Autologous Haemopoietic Stem-Cell Transplantation
APC	Antigen Presenting Cell
APP	Acute Phase Protein
APR	Acute Phase Response
ARR	Annual Relapse Rate
AQP4	Aquaporin-4
BAFF	B-cell Activating Factor
BBB	Blood-Brain Barrier
BBB-EC	Blood-Brain Barrier Endothelial Cell
BCG	Bacillus Calmette-Guérin
BCR	B Cell Receptor
BDNF	Brain-Derived Neurotrophic Factor
BTK	Bruton's Tyrosine Kinase
CCL	Chemokine (C-C motif) Ligand
CD	Cluster of Differentiation
CDC	Complement-Dependent cytotoxicity
CDMS	Clinically Definite Multiple Sclerosis
CXCL	Chemokine (C-X-C motif) Ligand
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
CRC	CXCL12-expressing Reticular Cells
CrEAE	Chronic Relapse-remitting EAE
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid

DIS	Dissemination in Space
DIT	Dissemination in Time
DMF	Dimethyl Fumarate
DMT	Disease Modifying Therapy
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
EDSS	Expanded Disability Status Scale
EV	Extracellular Vesicle
FcγR	Fcγ Receptor
FDA	Food and Drug Administration
FDC	Follicular Dendritic Cell
FRC	Fibroblastic Reticular Cell
FTY720	Fingolimod
GA	Glatiramer Acetate
Gd	Gadolinium
GFAP	Glial Fibrillary Acidic Protein
GM	Grey matter
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HEV	High Endothelial Venule
Iba-1	Ionized calcium-Binding Adapter molecule 1
ICAM	Intercellular Adhesion Molecule
IFNβ	Interferon β
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin

LA	Lipoic Acid
LAM	Leukocyte Adhesion Molecule
LINGO-1	LRR and Ig domain-containing Nogo Receptor-Interacting protein
LT	Lymphotoxin
mAb	Monoclonal Antibody
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MHV	Mouse Hepatitis Virus
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NAWM	Normal Appearing White Matter
NGF	Nerve Growth Factor
NgR1	Nogo-66 Receptor
NMO	Neuromyelitis Optica
NMOSD	Neuromyelitis Optica Spectrum Disorders
OBZ	Obinutuzumab
OCB	Oligoclonal Band
OCR	Ocrelizumab
OFA	Ofatumumab
OPC	Oligodendrocyte Progenitor Cell
PDGF	Platelet-Derived Growth Factor
PLP	Proteolipid Protein
PML	Multifocal Leukoencephalopathy
PPMS	Primary Progressive Multiple Sclerosis

PTx	Pertussis Toxin
ROS	Reactive Oxygen Species
RPMS	Relapsing-Progressing Multiple Sclerosis
RRMS	Relapse-Remitting Multiple Sclerosis
RTX	Rituximab
SAA	Serum Amyloid A
S1P	Sphingosine-1-Phosphate
SPMS	Secondary Progressive Multiple Sclerosis
TB	Mycobacterium Tuberculosis
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TLS	Tertiary Lymphoid Structure
TMEV	Theiler's Virus
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
UBX	Ublituximab
VCAM	Vascular Cell Adhesion Molecule
VEP	Visual Evoked Potentials
WM	White Matter

1. Introduction

1.1 An overview of multiple sclerosis

Multiple sclerosis (MS) is an inflammatory, autoimmune, demyelinating disease that affects the central nervous system (CNS) (Compston and Coles 2008). MS can have a wide range of neurological symptoms, including motor deficits and autonomic disturbances, but also psychiatric effects, depending on where the disease manifests topographically. The disease affects over 2 million people worldwide and its cause is still unknown (Group 2017). MS is characterised by inflammatory plaques in the brain, spinal cord, and optic nerve, displaying immune cell infiltration, demyelination, and neurodegeneration.

1.1.1 Historical perspectives

Robert Carswell (1793–1857) and Jean Cruveilhier (1791 –1874) independently described the brain plaques seen in MS in the first half of the nineteenth century, however, French neurologist Jean-Martin Charcot (1791-1874) was the first to recognise multiple sclerosis as a distinct disease in 1868. He attributed clinical signs seen in MS to the CNS plaques: the combination of nystagmus, intention tremor, and scanning speech are now known as Charcot's triad. Later on, Charcot described more characteristics of MS, including demyelination and axonal loss (Charcot and Ball 1868, Compston 1988, Poser and Brinar 2004, Murray 2005). Charcot's description allowed other clinicians to recognise and study MS more easily, which has led to better characterisation and diagnosis of the disease, which is still evolving. It was not until 1995, that a standardised nomenclature was introduced. To facilitate mutual understanding between clinical and research communities, an international survey of clinicians produced standardised definitions for the most common subtypes of MS. Based on clinical symptoms, MS was divided into four main subtypes: relapse-remitting (RR) MS, secondary progressive (SP) MS, primary progressive (PP) MS, and relapsing-progressive (RP) MS. These

new definitions for subtypes have since made it easier to design clinical trials and to predict which patients may benefit from which treatments (Lublin and Reingold 1996).

1.1.2 Prevalence and demographics

The prevalence of MS varies around the world – it is highest in Northern Europe, Southern Australia, and the middle part of Northern America, and is lower in areas around the equator (Noseworthy et al. 2000, Dendrou, Fugger, and Friese 2015). Women are more likely to develop MS than men, and individuals with a relative who has MS have an increased risk of developing MS (Goodin 2014). The prevalence in women has increased from 2:1 in the 1950s to almost 3:1 in the 2010s. However, the cause of this increasing imbalance is unknown. Currently, more patients are diagnosed with MS in general, which may reflect better diagnostics, but may also be owing to several risk factors, including smoking and obesity (Koch-Henriksen and Sørensen 2010).

1.1.3 Presentation and classification

MS Patients typically start presenting with symptoms between the ages of 20 and 40, although the disease can manifest at any point in life. Furthermore, some people with pathological MS (based on imaging) never experience any clinical symptoms (Goodin 2014, Engell 1989). Occasionally, radiological abnormalities resembling those in MS are found in individuals who undergo neurological investigations for unrelated reasons. Most of these patients eventually develop MS symptoms, or new lesions. Some of these patients, however, never develop clinical symptoms, and may be diagnosed with 'benign MS' (Okuda et al. 2009, Goodin 2014).

1.1.3.1 Relapse-remitting MS

The first clinical signs or attack of MS patients are usually referred to as clinically isolated syndrome (CIS). Further criteria need to be met before a diagnosis of 'clinically definite MS' (CDMS) can be made (further discussed in 1.1.6). Approximately 85% of these individuals will exhibit RRMS and will have 'clearly defined relapses with full recovery or with sequelae and residual deficit upon recovery; periods between disease relapses are characterised by a lack of disease progression' (Lublin and Reingold 1996). These relapses develop over days or weeks, and may persist for days to months.

1.1.3.2 Secondary progressive MS

Almost 50% of RRMS patients will develop SPMS 10-15 years after disease onset, irrespective of treatment regime (Ransohoff, Hafler, and Lucchinetti 2015, Hauser and Oksenberg 2006). In SPMS, relapses become less frequent and remissions are only minor if they take place at all. Remissions present merely as plateaus of disease progression, as patients become gradually, but constantly more disabled (Lublin and Reingold 1996). During progression, acute inflammation becomes less prominent, while neurodegeneration becomes more obvious, which makes it harder to treat (Goodin 2014). This is exemplified by an increase in brain atrophy on MRI scans, and the lack of drug effectiveness, even by the newest treatments available. There are two major barriers for the development of more effective therapies for progressive MS: (1) that disease progress is manifested only when any compensation mechanisms are exhausted, and (2) because no reproducible models of clinically relevant progressive disease currently exist.

1.1.3.3 Primary progressive MS

Less than 15% of MS patients begin with a purely progressive form of MS. These patients tend to have a later age of onset, a more severe disease course, and the prevalence is equal in men and women (Hauser and Oksenberg 2006). PPMS can be described as 'disease progression from onset with occasional plateaus and temporary minor improvements allowed' (Lublin and Reingold 1996). There are similarities between PPMS and SPMS, for example, the rate of disability worsening, and the histopathology (Confavreux et al. 2000, Confavreux, Vukusic, and Adeleine 2003). Though PPMS has a distinct nature compared to other MS subtypes, and it would not be surprising that PPMS may eventually be shown to be a distinct neurological entity.

1.1.3.4 Progressive-relapsing MS

Finally, PRMS, a rare form of MS that can be characterised by its progressive nature from onset, like PPMS. It affects less than 5% of patients with MS. As the disease progresses, patients present with clear relapses, with or without recovery, and an always continuing disease progression between relapses (Lublin and Reingold 1996).

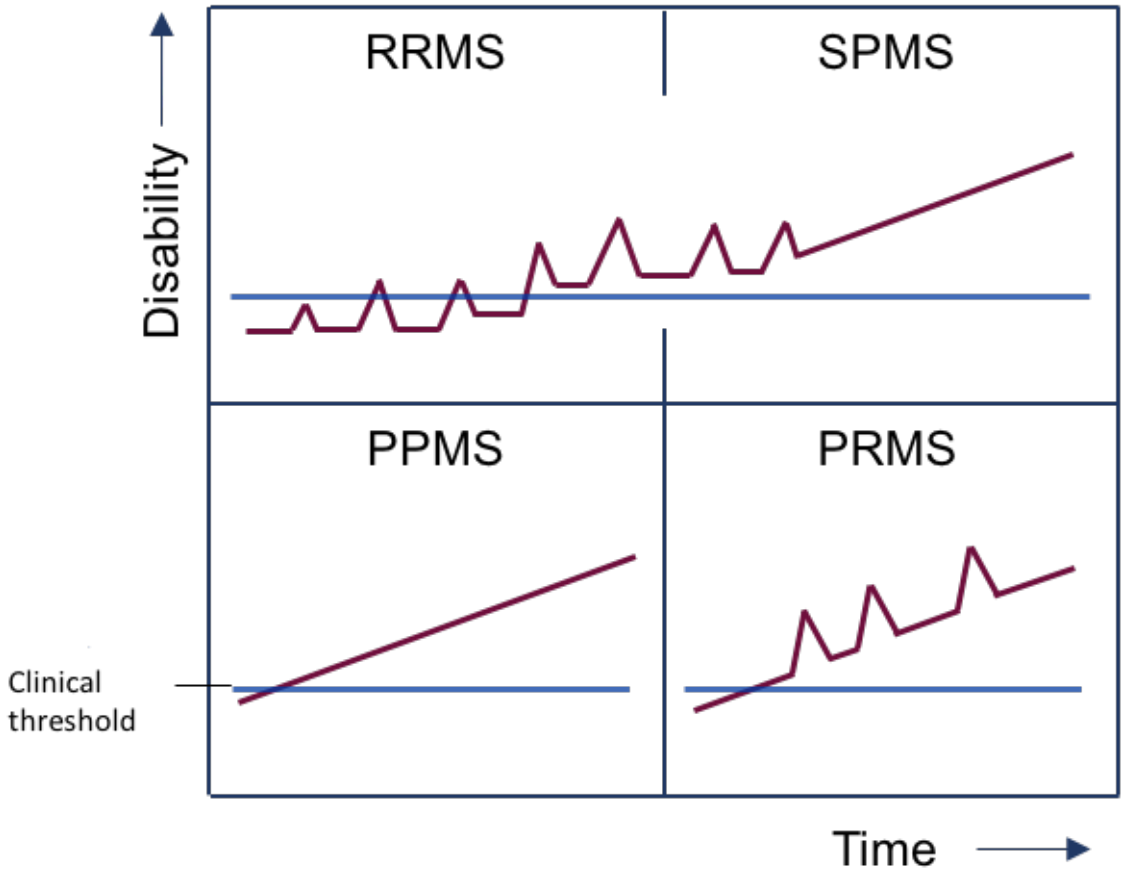


Figure 1.1 Forms of multiple sclerosis. RRMS is characterised by clearly defined relapses with full or partial recovery. RRMS patients can convert into SPMS, where relapses become sparser and disease progression more prominent. PPMS is characterised by disability worsening from disease onset, without any remissions. Patients with PRMS have progressive disease from onset and can experience clinical attacks followed by incomplete remission. RRMS, relapse-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; PPMS, primary progressive multiple sclerosis; PRMS, progressive-relapsing multiple sclerosis.

1.1.4 Other relevant diseases that may mimic MS

1.1.4.1 Neuromyelitis optica and other diseases in the spectrum

Like MS, neuromyelitis optica (NMO), or Devic's disease, and NMO spectrum disorders (NMOSD) are chronic autoimmune demyelinating diseases of the CNS. Their clinical symptoms can be similar, and are also characterised by relapses and remission. In NMO, aquaporin-4 (AQP4) water channels on astrocytes are targeted by pathogenic autoantibodies, leading to inflammatory lesions that mainly affect the optic nerves and the spinal cord. Unlike MS, NMO disability is entirely dependent on 'not full recovery from relapses', while MS progression can be independent of relapses. Correct diagnosis is important as many MS therapies often do not work for NMO patients and may even worsen NMO (Lennon et al. 2005, Kawachi and Lassmann 2017, Kleiter et al. 2012). A subset of patients with NMOSD phenotype that lacks autoantibodies against AQP4, have been shown to produce pathogenic autoantibodies against myelin oligodendrocyte glycoprotein (MOG) (Kitley et al. 2012). These MOG-antibody disease patients share clinical features with both MS and NMO, however, the underlying pathogenic mechanisms and metabolic profiles differ, suggesting it is again a different condition (Waters et al. 2015, Juryńczyk et al. 2017, Jurynczyk et al. 2017).

1.1.4.2 Tuberculosis meningitis and other meningoencephalitis diseases

Some inflammatory brain diseases, such as tuberculosis meningitis or luetic meningoencephalitis can resemble MS histologically, with meningeal inflammation and associated demyelinating cortical lesions. Brain lesions in tuberculosis meningitis can also be characterised by widespread microglial activation, macrophage infiltration, and meningeal inflammation. Subpial cortical lesions and primary demyelination, however, are largely absent in other inflammatory brain diseases that affect the cortex, and seem to be MS specific (Fischer

et al. 2013, Moll et al. 2008). Moreover, no demyelination is detected in B cell-lymphoma patients with meningeal infiltration of B cells.

1.1.5 Aetiology

Like other complex disorders, the pathogenesis of MS is the result of a combination of genetic and environmental factors. We now have a slightly better understanding of susceptibility to the disease. But how each of these factors contribute to the disease course, remains unclear (Ransohoff, Hafler, and Lucchinetti 2015).

In the mid nineteenth century, it had been suggested by Ernst Lyden that MS may have a genetic element, mainly implied by the high incidence in specific ethnic groups, and by the increased occurrence of MS in certain families. Individuals have a 20-30-fold increased risk of developing MS if a sibling has MS, or a 200-300-fold increase if their monozygotic twin does (Ebers, Sadovnick, and Risch 1995, Robertson et al. 1996, Goodin 2014).

Over 200 gene variants appear to increase the risk of developing MS, most involve immune mechanisms, such as T cell immunology (IL2 AND IL7R), or TNF (De Jager et al. 2009). Yet most studies point to the major histocompatibility complex as the most important one (Hauser and Oksenberg 2006, Dendrou, Fugger, and Friese 2015, Reich, Lucchinetti, and Calabresi 2018). The HLA-DRB1 gene is the strongest genetic connection so far. The *DRB*1501* allele, specifically, with each allele contributing independently to an individual's susceptibility for developing MS (Hauser and Oksenberg 2006, Goodin 2012). Many of these genes that are associated with an increased risk for MS are also associated with other autoimmune diseases, including rheumatoid arthritis, and psoriasis (Beecham et al. 2013).

There are more than just genetic factors that come to play in MS pathogenesis: if one monozygotic twin has MS, the chance of the other one developing MS is only 30.8% (Sadovnick et al. 1993). Furthermore, adoptees have been found to exhibit a comparable risk

to the general population, which indicates that living with an affected individual has little or no impact on susceptibility in the absence of biological relatedness (Goodin 2012). However, there are many environmental factors, including smoking, diet, chemicals, vitamin D, infections, and toxic exposures that have been associated with MS pathogenesis (Goodin 2014). Exposure to sunlight seems to be an important factor involved in MS and related studies are quite consistent (Ascherio, Munger, and Simon 2010, Simpson et al. 2010). As previously mentioned, the prevalence of MS is greater in the higher latitudes, where sunlight is scarcer and vitamin D deficiencies are more common (Rosati 2001, Group 2017). UVB light catalyses the body's production of vitamin D, and vitamin D is required for various mechanisms, including bone health and immune system maturation. Moreover, vitamin D deficiency has been associated with autoimmunity in general, and with cancer (Holick 2007, Goodin 2014). Besides this, vitamin D is involved in regulating *HLA-DRB1*1501* (Ramagopalan et al. 2009).

Other ways in which environmental factors may trigger autoimmunity is by directly, or indirectly, stimulating autoreactive T or B cells through molecular mimicry, or by simply promoting a proinflammatory state. For example, infectious agents have been shown to play a role in relapses (Dendrou, Fugger, and Friese 2015). Several viral and bacterial agents have been linked to MS. Not only are infections associated with exacerbations, they were also hypothesised to play a causative role in MS pathogenesis, though this view has lost popularity (Buljevac et al. 2002, Correale, Fiol, and Gilmore 2006). However, interest remains in the role of Epstein-Barr virus (EBV). The occurrence of other virus induced demyelinated diseases, such as progressive multifocal leukoencephalopathy, may explain this popularity. EBV infection is very common in humans, with an occurrence of over 90% in the total population, and nearly 100% in MS patients (Goodin 2014). The infection is often asymptomatic, but can express itself as mononucleosis. EBV infection has been implicated to play a role in the pathogenesis of diseases other than MS, such as Burkitt's lymphoma, rheumatoid arthritis, and Sjögren's syndrome (Goodin 2014, Vaughan et al. 1995). Although other infectious agents,

such as the herpes simplex virus and measles, have been linked to MS, none other than EBV have compelling evidence to play a role in MS pathogenesis (Goodin 2014, Ascherio and Munger 2007).

1.1.6 Diagnosis

In 1965, the Schumacher et al. criteria were introduced to make the definition for diagnosing MS more uniform internationally - patients had to present with dissemination in space (DIS) and with dissemination in time (DIT). These criteria (table 1) were used to diagnose clinically definite MS (Poser and Brinar 2004, Schumacher et al. 1965). More generalised diagnostic meters and new diagnostic tools evolved from this, which improved MS diagnosis.

	Clinically definite multiple sclerosis
1	Objective signs of dysfunction of the CNS
2	Dissemination in space, shown by clinical evidence of CNS damage in two or more areas
3	Evidence of damage to the white matter
4	Dissemination of time, shown by either two or more relapses, or disability progression
5	Age of onset should be between 10 and 50 years
6	Clinical signs cannot be explained by another disease

Table 1-1 The Schumacher criteria. The Schumacher criteria were established as a way of diagnosing clinically definite MS more uniform (Schumacher et al. 1965).

The use of neuroimaging and cerebrospinal fluid (CSF) analysis became part of diagnosing MS as a component of the Poser criteria in 1983, an update to the Schumacher criteria (Poser and Brinar 2004). The Poser criteria based diagnosis on a combination of clinical signs and the presence of oligoclonal bands in the CSF (Poser et al. 1983). The McDonald criteria were introduced in 2001, they form an updated set of diagnostic guidelines that combines clinical criteria with magnetic resonance imaging (MRI) visual evoked potentials (VEP), and CSF analysis, and have been updated thrice since (Poser and Brinar 2004, McNicholas et al. 2018). Testing VEP can be helpful in finding disturbances in functional pathways, and CSF analysis can assist diagnosis as well (Goodin 2014). The primary criteria to diagnose CDMS (to verify whether a person actually has MS) include two or more neurologic attacks (DIT), and two or more lesions as visualised by MRI (DIS). DIS now involves ≥1 lesion in ≥2 characteristic locations (periventricular, juxtacortical, infratentorial, or spinal cord), and DIT requires a new T2 lesion on a follow-up MRI at any point after the baseline scan. Additional clinical and/or laboratory evidence is necessary if at least one of these criteria score one or less. These criteria allow for progressive MS diagnosis as well, with the straightforward rule showing that there is clinical evidence of at least one year of disease progression (McNicholas et al. 2018).

MRI has become increasingly more important in diagnosing MS. It can be used to examine the criteria for both DIT and DIS, and can also reveal potential atypical features of a lesion (McNicholas et al. 2018). The use of MRI will be discussed further in section 1.2.5.2. The latest revisions to the McDonald criteria emphasised the importance of MRI and suggested that oligoclonal bands may now substitute imaging evidence in certain circumstances (McNicholas et al. 2018). The new revisions focussed on earlier diagnosis, allowing the start of treatment sooner. The use of oligoclonal band analysis will be discussed further in section 1.2.5.4.

1.1.6.1 Clinical signs

MS can present in many ways, depending on which area of the CNS is affected. Initial symptoms often include limb weakness, visual loss (optic neuritis), double vision (diplopia), sensory disturbance, ataxia, and clumsiness. Such clinical attacks build up over days or weeks and tend to diminish gradually, after a peak severity within 2 to 3 weeks. Eventually, as the disease progresses, other symptoms may occur, such as bladder dysfunction, fatigue, depression, and progressive sensory loss (Hauser and Oksenberg 2006, Noseworthy et al. 2000).

One way to score clinical disability in MS patients, in a way that is held to be objective and reproducible, is by using the expanded disability status scale (EDSS). In 1955 John F. Kurtzke introduced the disability status scale, which later evolved into the more sensitive EDSS (Kurtzke 1955). This scale takes into account 9 functional groups: pyramidal, cerebellar, brain stem, sensory, bowel & bladder, visual, cerebral and other. Each are scored from 0 to 10 for disability, 0 represents normal functionality and 10 represents death due to MS (Kurtzke 1983, 2013).

1.1.6.2 MRI

The use of MRI as a non-invasive way of visualising MS lesions in clinical practice, and then in clinical trials and research was introduced by Ian Young in 1981 (Young et al. 1981). MRI offers the possibility of studying the brain and the spinal cord. It also allows abnormalities in the brain or spinal cord to be studied. It allows for the number and size of lesions in the CNS to be examined, as well as calculating degeneration by measuring the size of the brain and spinal cord. The McDonald criteria rely heavily on MRI. Not only has MRI become essential in MS diagnosis, it is also crucial in the development of new therapies as it allows for an estimate of what the nature of the lesions are like (acute, chronic, active chronic) (Reich,

Lucchinetti, and Calabresi 2018). MS can be diagnosed by MRI by identifying focal lesions in the white matter in T2-weighted MRI scans, or blood-brain barrier (BBB) breakdown on contrast enhanced T1-weighted scans (Kutzelnigg and Lassmann 2014, Reich, Lucchinetti, and Calabresi 2018). T2-weighted images are used to determine the number and size of WM lesions, and to determine dissemination in space (DIT) and dissemination in time (DIS). T1-weighted images can also be used in chronic disease to get a crude picture of axonal loss. Together, the number, location, and total volume of CNS lesions are used as a surrogate marker for disease severity (Lund et al. 2013). By using a contrast agent, most commonly gadolinium, one can detect BBB breakdown on T1-weigted images (Saade et al. 2018). Gadolinium is a chelated paramagnetic contrast agent that is not able to cross the BBB, but can enter the CNS parenchyma where the BBB is disrupted. The contrast agent increases the signal intensity on T1-weighted images by shortening the relaxation time of adjacent protons, providing a picture of ongoing inflammation (Grossman et al. 1986). BBB breakdown can be found in all stages of MS, but in variable degrees (Lucchinetti et al. 1996).

MRI can provide information about the number and size of active inflammatory CNS lesions, and is one of the few tools to look 'into' the CNS in a living person. As with any other tool, MRI is not perfect. Recently, there has been a shift in research interest from white matter (WM) damage to grey matter (GM) damage and cortical involvement in MS pathology. Although MRI can show alterations in WM quite well, it is not particularly sensitive when it comes to providing insight into the structure and composition of a GM lesion (Filippi et al. 2012, De Groot et al. 2001). Furthermore, contrast enhancement in MRI is not a marker for the presence or absence of inflammation, but merely for BBB breakdown, as inflammation can take place behind an intact BBB (Keaney and Campbell 2015, Kutzelnigg et al. 2005). This is the case in SPMS, where there is less gadolinium enhancement in the MRI scans, yet post-mortem histopathological studies have shown presence of infiltrated immune cells in actively demyelinating lesions throughout the entire disease course (Frischer et al. 2009). In summary,

MRI reveals acute inflammation reflected by gadolinium enhanced lesions in T1-weighted images. Although chronic lesions do not show gadolinium enhancement, demyelination can still be visualised in the WM, or as generalised atrophy (Goodin 2014).

1.1.6.3 Oligoclonal bands

The latest McDonald criteria emphasize the importance of the presence of oligoclonal bands (OCBs) in the CSF. The presence of OCBs in the CSF is analysed by running the CSF on a gel, which separates immunoglobulins (Igs) present in the CSF. Although this does not tell us much about the OCB function, their presence alone represents CNS-restricted inflammation. OCB patterns vary greatly between patients, but remain relatively unchanged throughout a patient's disease course over time (Owens et al. 2006). The role of OCBs in MS pathogenesis is not certain: OCBs are not MS-specific – they can be present in other inflammatory CNS diseases as well (Chu et al. 1983, Greenfield and Hauser 2018). It has been shown that in some of these diseases, Immunoglobulin G (IgG) in OCBs are directed against the cause of the disease, e.g. the measles virus in subacute sclerosing panencephalitis (Vandvik et al. 1976, Owens et al. 2007).

1.2 Pathophysiology of multiple sclerosis

The regions of the CNS that are affected by pathology are not the only determinant of the clinical symptoms observed in MS as the pathophysiology is also stage dependent. Typical MS pathology is characterised by inflammatory demyelinating lesions, containing infiltrated lymphocytes and plasma cells, widespread microglial and astrocyte activation, loss of axons and oligodendrocytes, and a transient breakdown of the BBB (Hauser and Oksenberg 2006, Dendrou, Fugger, and Friese 2015). Although focal demyelinating lesions are a classic hallmark of MS, normal appearing white matter (NAWM) represents diffuse pathology that is not visible macroscopically. RRMS patients may have some NAWM abnormalities, but changes become more severe in the progressive phases of disease, and can occur independently of the major focal lesions (Kutzelnigg and Lassmann 2005). Furthermore, MS used to be thought to only affect the WM, however, the demyelinating lesions can also occur in the GM, especially within the cortex (Kutzelnigg and Lassmann 2014). GM lesions differ from WM lesions in that they tend to exhibit limited BBB breakdown, and are often associated with meningeal inflammation and neuronal loss (Magliozzi et al. 2007, Howell et al. 2011, Kutzelnigg et al. 2005). GM lesions can be classified into different types: type I, leukocortical lesions affect both the grey and the white matter without reaching the surface of the brain. Type II lesions are often located around blood vessels and only affect the grey matter, but do not extend to the surface of the brain. Type III lesions, or subpial lesions, are the most common type of lesions in MS, especially in progressive MS. They are located in the most outer layers of the cortex, where they can spread out over a large surface area, but they never extend into the WM. Type IV lesions on the other hand do extend through all layers of the cortex, but, again, do not penetrate the subcortical white matter (Figure 1.2) (Kidd et al. 1999, Peterson et al. 2001, Geurts et al. 2005, Bø et al. 2003a, Calabrese, Filippi, and Gallo 2010). Cortical demyelination, and merely diffuse WM injury are characteristic of progressive MS, although GM demyelination occurs in early phases

of disease as well (Kutzelnigg et al. 2005). GM lesions tend to have less BBB breakdown, oedema, and fewer activated microglia and infiltrated lymphocytes compared to white matter lesions (Peterson et al. 2001). Lesions in progressive disease also tend to be less inflammatory active, yet the number of lymphocytes in the brain of progressive MS patients is similar to those in inactive lesions from RRMS patients, and 10-100 times higher than age-matched patients with a different neurodegenerative disease (Frischer et al. 2009).

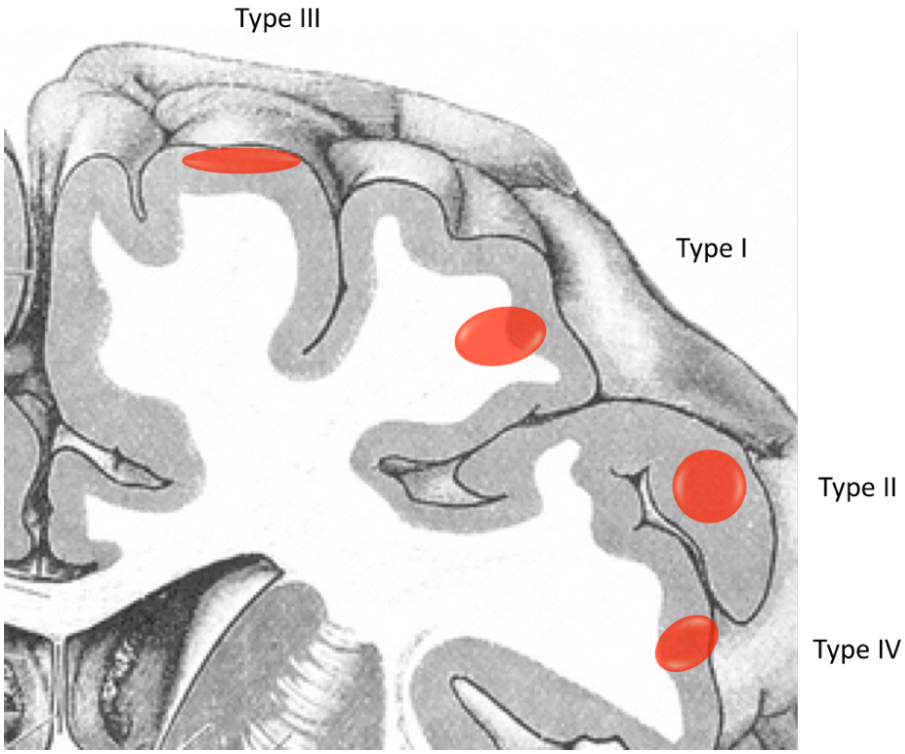


Figure 1.2 Types of cortical lesions. Type I lesions affect both the grey and the white matter. Type II lesions are located within the cortex, without extending to the white matter, or the brain surface. Type III lesions affect the outer layers of the cortex and can extend over a large area. Type IV lesions extend through all layers of the cortex, without affecting the white matter. Adapted from Kawachi and Lassmann 2017.

1.2.1 Neuroinflammation

Rudolf Virchow introduced the concept of neuroglia in 1856, describing them as a mechanical support cells with repair functions in the CNS. After that, more cells of the CNS were identified and so was the nature and composition of a MS plaque. MS has been described as a neuroinflammatory disease, which converts to being neurodegenerative in later stages of disease. MS lesions often seem to initiate around inflamed blood vessels, where inflammatory cells can first enter and will often accumulate in cuffs. The cellular composition of these infiltrates is dependent on the type and stage of the lesion (Lassmann 2018, Dendrou, Fugger, and Friese 2015). The major inflammatory components of a MS lesion include microglia and macrophages, astrocytes, T cells, B cells and plasma cells, and the BBB.

1.2.1.1 The blood-brain barrier and endothelial cells

The BBB is a multilayer interface that separates the CNS from the periphery. It tightly regulates the movement of nutrients, ions, cells, toxins, and other molecules to and from the CNS, and protects the CNS form toxins and pathogens. It allows for tight regulation of homeostasis, allowing for normal neuronal function. The BBB is composed of endothelial cells, the basement membrane, pericytes, and astrocyte endfeet (Figure 1.3) (Daneman and Prat 2015).

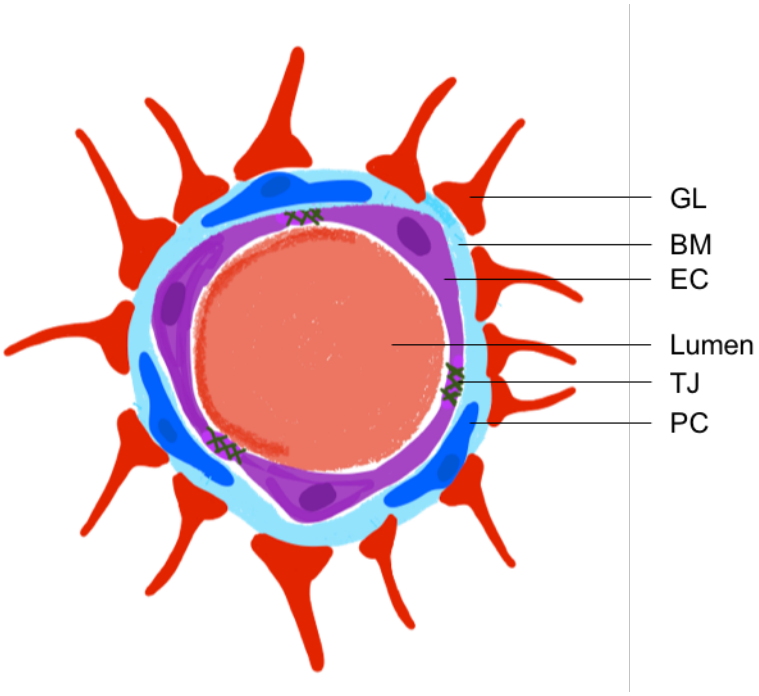


Figure 1.3 Schematic overview of the blood-brain barrier. The BBB is composed of endothelial cells, the basement membrane, pericytes, and astrocyte endfeet. BBB, blood-brain barrier; GL, glia limitans; BM, basement membrane; EC, endothelial cell; TJ, tight junction; PC, pericyte.

Endothelial cells are part of the walls of all blood vessels, including those in the CNS. CNS endothelial cells are unique for their special transport systems, reduced pinocytic activity, and an increased number of mitochondria (Ballabh, Braun, and Nedergaard 2004). Moreover, they express tight junction complexes on their cell surface that bind to adjacent endothelial cells, forming a tight barrier that allows regulation of the flow of ions and other molecules more tightly (Figure 1.3). These complexes include tight and adherens junctions, that block penetration of macromolecules and prevent diffusion of smaller substances from the blood to the brain (Daneman and Prat 2015). BBB endothelial cells (BBB-ECs) express highly specific nutrient transporters that allows them to transport nutrients into the CNS and waste products from the CNS into the blood (Mittapalli et al. 2010). Furthermore, BBB-ECs express a very low level of leukocyte adhesion molecules (LAMs) compared to endothelial cells elsewhere in the body, which gives a stronger control over the number of immune cells entering the CNS. All these properties together make BBB-ECs central regulators of CNS homeostasis (Daneman and Prat 2015).

Pericytes are located on the abluminal surface of the endothelium, and are key in regulating blood vessel diameter, transcytosis, and leukocyte transmigration across vessels (Figure 1.3) (Daneman and Prat 2015). Pericytes are also involved in angiogenesis, and produce extracellular matrix components and growth factors, including TGFβ, angiopoietins, PDGF, and S1P (Shepro and Morel 1993, Alvarez, Cayrol, and Prat 2011). Furthermore, loss of pericytes results in increased BBB permeability, and upregulation of associated genes, including ICAM-1 and angiopoietin (Daneman et al. 2010).

Pericytes and BBB-ECs are separated by the basement membrane (Figure 1.3). The basement membrane is an extracellular matrix structure made up out of collagens, laminin, heparin sulphate proteoglycans, as well as other glycoproteins. It forms another physical barrier to prevent molecules from migrating into the CNS, but it can be disrupted by the action of by matrix metalloproteinases (Daneman and Prat 2015). The basement membrane may facilitate BBB-component crosstalk by serving as a base for signalling molecules (Keaney and Campbell 2015).

Astrocytic endfeet, or glia limitans, form the last and outer major component of the BBB (Figure 1.3). They line CNS blood vessels almost entirely, and serve as a link between the neuronal circuitry and the peripheral blood flow, regulating CNS metabolite levels and water content (Attwell et al. 2010, Keaney and Campbell 2015). The end feet are highly abundant in AQP4 water channels and Kir4.1 potassium channels. Astrocytes release other molecules that regulate BBB integrity, including Hedgehog family proteins regulating BBB-EC function, and VEGF for promoting angiogenesis (Keaney and Campbell 2015). Furthermore, astrocytic endfeet produce tight junction proteins, which are upregulated in inflammatory conditions. These tight junction complexes reinforce the BBB, controlling leukocyte entry into the CNS (Horng et al. 2017).

Despite the complex structure of the BBB and the great capacity of regulating its permeability, the BBB is not entirely non-permeable. Up to 0.1% of large molecules, such as Igs can cross the BBB from the blood through either non-specific vesicle trafficking, lack of tight junction continuation, or other mechanisms, such as receptor mediated transcystosis (Freskgård and Urich 2016). Hence, systemic factors can constantly influence the CNS (Yu et al. 2011, Daneman and Engelhardt 2017).

The BBB can be affected in several ways in MS and other neuroinflammatory conditions. Under pathological circumstances in different neurological disorders, including MS, the BBB can be disrupted (Daneman and Prat 2015). In most neurological disorders, BBB breakdown is caused by inflammation, injury or degenerative mechanisms. The excessive immune cell infiltration in early MS is paired with transient BBB breakdown, which can last for weeks, months, or even years. Neuroinflammation, causes both BBB disruption and activation of its cellular components (Alvarez, Cayrol, and Prat 2011). Tight junction abnormalities can be detected in early stages of lesion formation and may result in a leaky BBB. Inflammatory cytokines can decrease the expression of tight junction proteins, such as occludin and VE-cadherin, causing the barrier to become leakier (Oshima et al. 2001). Whether BBB breakdown occurs before or after initiation of a MS lesion remains a topic of debate, but in EAE, leukocyte recruitment precedes BBB breakdown (Hickey, Hsu, and Kimura 1991, Hickey 1999, Reboldi et al. 2009).

Activated BBB-ECs contribute to further inflammation by producing chemokines such as CCL2, CCL5, and CXCL10, but also adhesion molecules, including ICAM1, VCAM1, PECAM, E-selectin, and ALCAM (Kebir et al. 2007, Cayrol et al. 2008, Daneman and Prat 2015). Expression of these molecules leads to subsequent mobilisation recruitment of immune cells that can cross the BBB more effectively under inflammatory circumstances. Infiltration of lymphocytes, monocytes, and macrophages is in turn correlated with the production of

further proinflammatory molecules, such as TNFα, IFNγ, IL-17, IL-22, and GM-CSF (Daneman and Prat 2015, Alvarez, Cayrol, and Prat 2011). All these events lead to more inflammation, accompanied by oedema, glial activation, axonal loss, and further instability of the BBB (Frohman, Racke, and Raine 2006, Alvarez, Cayrol, and Prat 2011).

In progressive MS, the BBB is often patent, and inflammatory aggregates around vessels and in the meninges develop behind an intact BBB (Hochmeister et al. 2006). MRI often does not show gadolinium enhanced lesions in progressive MS, even though we know from post-mortem analysis that inflammatory lesions are present (Hochmeister et al. 2006, Lassmann 2018). Even tertiary lymphoid structures (TLS) can be detected in post-mortem tissues in absence of any damage to the BBB, indicating compartmentalised inflammation (Serafini et al. 2004, Magliozzi et al. 2010, Howell et al. 2011).

1.2.1.2 Microglia and Macrophages

Microglia were named by Pío del Río-Hortega, who divided the term 'neuroglia' or Santiago y Cajal's 'third element' of the CNS into microglia and oligodendrocytes (Tremblay et al. 2015). Microglia are yolk-sac-derived residential macrophages of the CNS (Ginhoux et al. 2010). They constantly monitor the brain environment in a 'hexagonal-close packing' array, using their ramified processes (Lawson et al. 1990, Nimmerjahn, Kirchhoff, and Helmchen 2005). Microglia play many roles in normal brain functioning and maintenance, where they interact with neurons, astrocytes and oligodendrocytes, and are quick to respond to changes by modulating their environment in different activation states. They play roles in synapse modulation and pruning, phagocytosis, tissue repair, debris removal and neurogenesis. Microglial activation and/or dysfunction can be observed in almost all CNS diseases and injuries (Aguzzi, Barres, and Bennett 2013). They can become activated by inflammatory challenges or damage. When they do so, they retract their processes and turn into an 'amoeboid' morphology, characterised by a larger, rounder cell body. Depending on the type

of challenge, microglia will become more phagocytic, express different genes, and secrete context-dependent cytokines, chemokines, and trophic factors (Li and Barres 2018).

Together with infiltrating macrophages, activated microglia are the most common cell type in MS lesions, especially around actively demyelinating plaques (Lassmann 2014b). Activated microglia are present in all stages of MS and they are associated with tissue damage (Ferguson et al. 1997). The number of activated microglia and macrophages is highest in actively demyelinating MS lesions, and, as the disease progresses, microglial distribution becomes less focal and more diffuse. Clusters or nodules of microglia can be present at any stage of MS, and are not restricted to lesions (De Groot et al. 2001). In MS, activated microglia are known to secrete proinflammatory cytokines such as IL-1β, TNFα, IL-6, and IFNγ, which may damage neurons and oligodendrocytes (Correale 2014, Correale et al. 2017). Moreover, MS microglia upregulate the expression of proteins related to phagocytic activity, such as CD68, and antigen presentation, such as MHC class I and II (Zrzavy et al. 2017). Besides this, microglia can interact with the adaptive immune system, and can also contribute to tissue damage directly. They are a major source of reactive oxygen and nitrogen species, leading to oxidative damage and mitochondrial dysfunction in MS (O'Loughlin et al. 2018, Mahad, Trapp, and Lassmann 2015). At the same time, microglia can contribute to repair by clearing myelin debris, or by trimming dysfunctional synapses (Aguzzi, Barres, and Bennett 2013, Reich, Lucchinetti, and Calabresi 2018).

In CNS homeostasis, three other types of macrophages can be found: meningeal, perivascular, and choroid plexus macrophages. All of them are located at the interface between the circulation and the brain parenchyma (Prinz, Erny, and Hagemeyer 2017). Their unique position allows them to influence migration, sprouting, and refinement of the CNS vasculature, but also to be an efficient part of immune surveillance. In healthy brains, very few peripheral immune cells can enter the specialised compartments of the CNS: the perivascular,

leptomeningeal and ventricular space (Prinz, Erny, and Hagemeyer 2017). The exact contribution of each cell type remains unclear, because only recently has progress been made to distinguish microglia from different macrophages (Bennett et al. 2016).

1.2.1.3 Astrocytes

As for microglia, astrocytes are another type of glial cells found in the CNS. Traditionally they were thought to only play a supporting role for surrounding neurons. However, more recently astrocytes emerged as key players in the inflamed CNS and in MS. In normal, resting, physiological circumstances, the star-shaped cells each survey a non-overlapping territory. Astrocytes play many roles such as regulating blood flow, providing trophic support to neurons, engaging in synaptic plasticity, and regulating ion and fluid balances. Moreover, they play an essential role in maintaining BBB integrity. The astrocytic end feet, glia limitans, form the outer membrane separating the CNS parenchyma with non-CNS tissue (section 1.3.3). Similar to microglia, astrocytes can become reactive, and are involved in both acute injuries to the brain and chronic neurological diseases (Anderson et al. 2016, Nedergaard, Ransom, and Goldman 2003, Sofroniew 2014, Liddelow and Barres 2017). Astrocytes get activated through astrogliosis, a complex process during which an astrocyte can undergo a wide range of changes, accompanied by relevant cell-signalling specific to the local microenvironment (Sofroniew 2014). In active MS lesions, astrocytes become reactive - they proliferate, become hypertrophied, upregulate GFAP (glial fibrillary acidic protein), retract their cell processes that formed the glia limitans, and start overlapping their territories, until it is virtually impossible to delineate individual cells (Brosnan and Raine 2013, Sofroniew 2009, Misu et al. 2013, Sharma et al. 2010). Astrocyte activation can spread to the NAWM, and hypertrophied astrocytes around the demyelinating lesion can contain myelin debris or even (parts of) oligodendrocytes (Ponath et al. 2017, Brosnan and Raine 2013, Wu and Raine 1992). In chronic lesions,

astrocytes can form glial scars, consisting of overlapping, proliferating astrocytes with an elongated shape (Sofroniew 2014). Glial scars border lesion cores in MS, and can cause permanent rearrangement of tissue structure. These 'scars' are, seemingly, positioned here to form a barrier between the highly-inflamed tissue compartment and normal functioning tissue, and to limit 'inflammation' from spreading from the perivascular space and the meninges into the CNS parenchyma (Sofroniew 2015, Khakh and Sofroniew 2015, Brosnan and Raine 2013). Chronic lesions can contain astrocytes in different activation stages, though often with thickened processes (Ludwin et al. 2016). Activated astrocytes in MS lesions secrete cytokines, such as TNFα, IL-1β, and IL-6, as well as chemokines, such as CCL2, CCL20, and CXCL10, leading to further activation of glia cells and the recruitment of more leukocytes (Tanuma et al. 2006, Rothhammer and Quintana 2015, Farina, Aloisi, and Meinl 2007). Finally, astrocytes can produce BAFF, a B cell survival factor that is highly upregulated in the brains of MS patients (Krumbholz et al. 2005).

1.2.1.4 T cells

T cells are lymphocytes that are key players in cell-mediated immunity. They use their T cell receptor (TCR) to recognise peptides presented by MHC molecules. T cells are present in the MS brain from the early stages of disease, and they are mostly located around perivascular cuffs, but they are also present around the lesion core in a more scattered pattern in actively demyelinating lesions. In progressive MS, these cuffs can spread throughout the NAWM, distributed throughout the entire CNS (Seewann et al. 2009). Animal models that are employed in MS research, especially experimental autoimmune encephalomyelitis (EAE), have fostered the idea that MS is an autoreactive $CD4^+$ T-cell mediated disease, but thorough investigation using immunohistochemistry showed that the number of $CD4^+$ T cells is much lower in MS lesions than previously thought (Machado-Santos et al. 2018, Dendrou, Fugger, and Friese 2015). Circulating $CD4^+$ T cells from MS patients have been shown to recognise several myelin

proteins, although similar results have been shown for $CD4^+$ T cells from healthy individuals (Hellings et al. 2001). Together with unsuccessful clinical trials targeting $CD4^+$ T cells specifically, such as cM-T412 (anti-CD4 antibody) and ustekinumab (anti-IL12 and IL23 antibody), the data now suggests a lessor role for $CD4^+$ T cells in MS than originally thought (van Oosten et al. 1997, Segal et al. 2008). Approximately 76% of T cells present in MS lesions are MHC-class-I-restricted $CD8^+$, and their number seem to correlate with the extent of axonal damage (Machado-Santos et al. 2018, Frischer et al. 2009).

$CD8^+$ cells are thought to contribute to demyelination and axonal/neuronal damage in MS directly. However, the exact molecular mechanism remains unclear. It is likely that these cells cause damage by the release of proinflammatory cytokines, such as TNFα and IFNγ, or by interaction of their Fas ligand with the Fas antigen on neurons (Mizuno et al. 2008, Meuth et al. 2009, Giuliani et al. 2003, Huse, Quann, and Davis 2008).

Studies of both patients with MS and animal models for MS have indicated an imbalance between regulatory T cells (T_{reg}) and effector T cells that are reactive against CNS components (Kaskow and Baecher-Allan 2018). MS patients have fewer T_{reg} cells circulating in their blood compared to healthy controls, but with a more proinflammatory phenotype (Dhaeze et al. 2015). T_{reg} cells have many functions, including the regulation of some B cell functions (Gotot et al. 2012, Jang et al. 2011, Nakamura, Kitani, and Strober 2001).

1.2.1.5 B cells and plasma cells

It has now become clear that pathogenic T cells are not the only contributors to MS pathogenesis. B cells also play a large role in many ways. The number of brain B cells is variable between patients, and throughout disease progression. While disease progresses, the proportion of B cells has been demonstrated to increase (Frischer et al. 2009). As for T cells, B cells and plasma cells are mostly present in perivascular cuffs around blood vessels, and only

relatively few cells are found to be scattered around the focal MS lesion (Machado-Santos et al. 2018). Although $CD8^+$ T cells can be dominant in the brains of many other neuroinflammatory diseases, the presence of B cells in these situations is limited, making the presence of brain B cells more specific for MS (Machado-Santos et al. 2018). Moreover, tertiary lymphoid structures (TLS) have been found in the meningeal compartment of MS patients, they are rich in B cells and associated with a more severe pathology (Serafini et al. 2004, Magliozzi et al. 2007).

B cells are thought to play many roles in MS, including antibody production, antibody presentation, and cytokine secretion (Greenfield and Hauser 2018). Unlike in other neurological diseases, antibody producing plasmablasts can remain in the CSF of MS patients for a long time. They contribute to IgG production, and their numbers are associated with disease severity (Obermeier et al. 2011, Cepok et al. 2005). Studies investigating CSF B cells have revealed that plasmablasts present in the CSF are derived from a small number of CNS B cells that have been activated and have undergone somatic mutation (Eggers et al. 2017, Greenfield and Hauser 2018). Moreover, in a single patient, the same B cell clones can exist in different compartments within the CNS, such as the meninges, and the CSF (Lovato et al. 2011). Interestingly, some of these clones are not CNS restricted, and can be found in the periphery (von Büdingen et al. 2012, Palanichamy et al. 2014, Stern et al. 2014). These studies suggest that cells are able to traffic in and out the CNS, crossing the BBB, which is known not to be a tight barrier for leukocytes.

Exploring the contribution of antibody-independent roles for B cells in MS has become more popular in recent years after the initial successful trials with the anti-CD20 antibody rituximab, which was found to reduce relapsing disease activity (Hauser et al. 2008, Bar-Or et al. 2008). CD20 is a B-cell specific non-glycosylated transmembrane protein (Singh et al. 2014). CD20 is present on the B-cell lineage from pre-B cells through plasmablasts, and is involved in B cell

proliferation and differentiation. Plasmablasts and plasma cells do not express CD20 on their surface membrane, and OCBs are not affected by this therapy, suggesting that it is $CD20^-$ plasmablasts that produce the intrathecal Ig and not $CD20^+$ B cells (section 1.4.1.3) (Cross et al. 2006, Monson et al. 2005).

B cells are antigen-presenting cells (APCs) that can present antigenic peptides to T helper cells. B cells recognise specific peptides through the B-cell receptor (BCR), a transmembrane complex consisting of a pair of heavy and light-chain IgGs with a unique antigen-binding site (Seda and Mraz 2015). Binding of an antigen to the BCR initiates B-cell activation, which triggers B-cell proliferation and differentiation, but also causes the B cell to internalise and process the antigen. Subsequently, the B cell loads the antigenic peptide on a MHCII molecule to present it on the outer cell membrane to T cells. T cells then get activated by binding to the antigen-MHCII complex with their TCR and to costimulatory molecules simultaneously (Rodríguez-Pinto 2005, Batista and Harwood 2009, Lanzavecchia 1985).

In MS, genomic studies have pointed out that MHCII and other genes related to antigen presentation, including CD40 and CD80, are risk factors for MS (Sawcer et al. 2011). MHCII and other costimulatory molecules are more upregulated in B cells from MS patients than from healthy controls after in vitro stimulation (Mathias et al. 2017). As discussed earlier, the gene with the strongest link to increased susceptibility for developing MS is the DR beta chain of the MHCII molecule. In a mouse model of MS, knocking out MHCII on B cells specifically reduces the disease severity considerably, supporting a pathogenic role of B cells as APCs (Molnarfi et al. 2013).

Another difference between B cells isolated from MS versus those from healthy controls is their cytokine expression profile. B cells, especially memory B cells, can produce high amounts of the proinflammatory cytokines TNFα, LTα, IL-6, and GM-CSF (Duddy et al. 2007, Barr et al. 2012, Bar-Or et al. 2010). B cells can regulate different immune mechanisms by changing

their profile of cytokine production/secretion. B cells also produce anti-inflammatory cytokines, such as IL-10 and IL-35, and this production is often downregulated in MS patients' B cells (Shen and Fillatreau 2015, Duddy et al. 2007). Thus, B cells from MS patients exhibit increased expression of proinflammatory cytokines and decreased expression of anti-inflammatory cytokines. Depleting B cells in MS patients with anti-CD20 antibodies results in a decreased inflammatory response by both T cells and myeloid cells (Bar-Or et al. 2010, Li, Rezk, Healy, et al. 2015). After B cells reconstitution in these patients, their cytokine secretion profile becomes less proinflammatory, and they start producing more IL-10 (Duddy et al. 2007, Bar-Or et al. 2010, Li, Rezk, Miyazaki, et al. 2015).

Finally, B cell tolerance is affected in MS. In healthy individuals, two major checkpoints regulate autoreactive B cells during development – central tolerance occurs at early stages of B cell development in the bone marrow and peripheral tolerance in secondary lymphoid organs at later stages of B-cell development. Mechanisms of peripheral tolerance regulate the activation and survival of B cells, and include antigen-receptor desensitization and the induction of anergy, a process during which B or T cells become functionally impaired, usually as a result of self-recognition (Nemazee 2017). The number of B cells expressing autoreactive and polyreactive antibodies was found to be increased in MS patients, due to defects in peripheral tolerance (Hervé et al. 2007, Kinnunen, Chamberlain, Morbach, Cantaert, et al. 2013). The defects in peripheral tolerance are thought to be dependent on B-T cell interactions. In MS, the decrease in number and regulatory functions of T_{reg} cells is thought to lead to an increase of autoreactive B cells (Kinnunen, Chamberlain, Morbach, Choi, et al. 2013, Kinnunen, Chamberlain, Morbach, Cantaert, et al. 2013, Dhaeze et al. 2015, Hervé et al. 2007).

1.2.2 Meningeal tertiary lymphoid structures in MS

In MS, patients can exhibit meningeal inflammation, which is often associated with cortical demyelination. Depending on the disease stage, meningeal cell infiltrates can consist of a

combination of T cells, B cells, and macrophages. Meningeal inflammation can occur in all stages of MS, while B cell aggregates structured in a lymphoid follicle-like manner seem to be more typical for SPMS (in up to 70% of patients (Figure 1.4) (Choi et al. 2012, Serafini et al. 2004, Magliozzi et al. 2007, Howell et al. 2011, Lucchinetti et al. 2011, Magliozzi et al. 2010, Howell et al. 2015, Bevan et al. 2018). The exact structure of a TLS can vary from lymphocytic aggregates to highly organised B and T cell compartments. Traditionally, TLS consist of B cells, T cells, and specialised stromal cells: follicular dendritic cells (FDCs), CXCL12-expressing reticular cells (CRCs), and fibroblastic reticular cells (FRCs) in an organised manner (Howell et al. 2011, Pikor, Prat, et al. 2015, Rodda et al. 2015). TLS can be formed at sites of chronic inflammation in non-lymphoid tissues, and consist of a collection of immune cells that are structured in a way similar to secondary lymphoid organs (Tang et al. 2017). TLS can occur in other diseases, including rheumatoid arthritis, Sjögren's syndrome, and Myasthenia gravis (Pikor, Prat, et al. 2015).

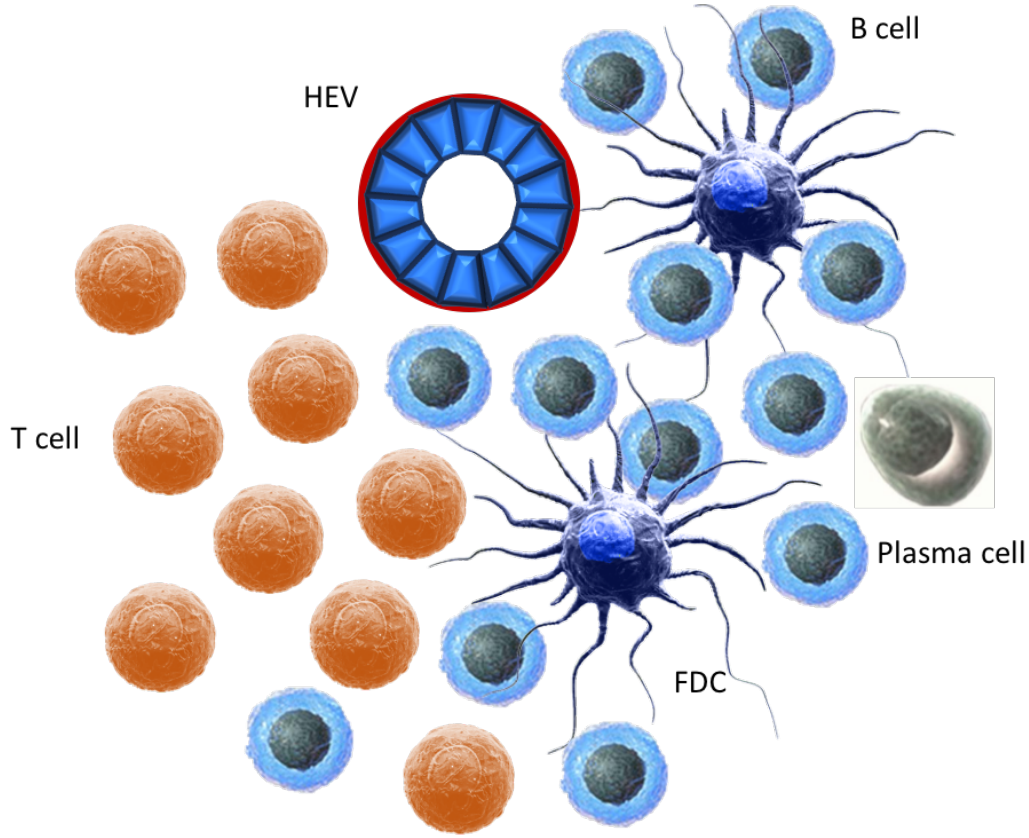


Figure 1.4 Schematic overview of a tertiary lymphoid structure. TLS are characterised by the presence of B cells, T cells, follicular dendritic cells, plasma cells, and high endothelial venules. HEV, high endothelial venule; FDC, follicular dendritic cell.

B cell follicles in lymphoid organs are also rich in FDCs and they express high levels of VCAM1, ICAM1, and complement and Fc receptors (Klaus et al. 1980, Mandel et al. 1980, Cyster et al. 2000, Qin et al. 2000, Batista and Harwood 2009). FDCs are different form classic dendritic cells and in order to develop and function in a germinal centre they require B cells and a variety of chemokines to be present (MacLennan 1994, Fu and Chaplin 1999). At the same time, they express CXCL13, a B cell chemoattractant, as well as other molecules involved in germinal centre responses (Pikor, Prat, et al. 2015). CRCs are the main producers of CXCL12, another B cell chemoattractant. FRCs are more involved in the T cell segment of the TLS, they produce T cell chemoattractant CCL19 and CCL21, as well as B and T cell survival factors IL-7 and BAFF.

Excretion of adhesion molecules and cytokines, such as VCAM1 and lymphotoxin, promotes vascularisation and further release of chemokines by high endothelial venules (HEVs). HEVs are present in most secondary lymphoid organs, where they allow transmigration of B and T lymphocytes through continuous expression of adhesion molecules and chemokines. HEVs can be present in mature TLS as well (Drayton et al. 2006, Ruddle 2014).

Retrospective analysis in post-mortem samples have shown that in MS, TLS are often accompanied by damage to the glial limitans, and neuronal loss, which presents in a gradient-like manner being greater in the outer cortical layers than in the inner ones (Magliozzi et al. 2010). These TLS are associated with a more severe disease course – MS patients with TLS tend to have a younger age of disease onset, become wheelchair dependent earlier, and die at a younger age (Magliozzi et al. 2007). Meningeal TLS are correlated with cortical demyelination, neurodegeneration, and atrophy, which are in turn linked to clinical disability (Magliozzi et al. 2010, Magliozzi et al. 2007, Serafini et al. 2004, Magliozzi et al. 2018, Fisniku et al. 2008, Calabrese et al. 2012). Meningeal inflammation and grey matter demyelination is correlated with an increased expression of proinflammatory molecules in the CSF, with a similar mRNA-

expression profile in the meninges. The upregulation of TNF, LTα, IFNγ, IL2, IL10, IL12p40, IL16, CXCL10, CXLC13, CCL20, CCL17, and CCL22 is also associated with earlier onset of disability and death (Magliozzi et al. 2018). These proinflammatory molecules are likely released by the immune cells making up the TLS-like structures in the meninges of SPMS patients. Many of these markers, including CXL13, CXL12, and TNF, are essential in B cell activation and proliferation, as well as in lymphoid neogenesis (Aloisi and Pujol-Borrell 2006). Furthermore, this particular intrathecal inflammatory expression profile can be identified even in patients in early disease stages, suggesting the proinflammatory meningeal environment persists throughout the disease. On the contrary, patients with a lower degree of meningeal inflammation had a different intrathecal expression profile, one that did not involve such an increase in the expression of inflammatory makers (Magliozzi et al. 2018). A more proinflammatory milieu undoubtedly contributes to further damage and inflammation. Although it has been speculated that meningeal TLS are causal for more severe pathology in SPMS, for which no evidence of this exists at this time. It thus remains unclear what the role of TLS in MS is. They might, however, serve as an extra tool to distinguish those patients who are likely to progress faster and need a more stringent therapy, if we can find a way to reliably image these structures in MS patients.

1.2.3 Demyelination

Primary demyelination remains another important aspect of MS pathology, but it can be very heterogenous, with different degrees of myelin protein preservation, oligodendrocyte loss, and inflammation. (Lucchinetti et al. 1996, Lucchinetti et al. 2000). As described before, demyelination is often associated with aggregates of perivascular lymphocytes and activated macrophages.

Active demyelination in a MS lesion can be recognised by a reduced density of myelinated fibres and irregular myelin sheaths wrapping the axons (Lucchinetti et al. 2000). Histologically, actively demyelinating lesions are defined by colocalisation of myelin debris with macrophages, showing active myelin degradation within the lysosomes (Brück et al. 1995). Small myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG) or myelin associated glycoprotein (MAG) can easily be degraded by macrophages, on average 1-2 days after phagocytosis, whereas larger myelin proteins, including myelin basic protein (MBP) and proteolipid protein (PLP), can persist for up to 10 days inside a macrophage. Over time, the myelin debris will turn into granular lipids, which may persist for several months, reflecting later stages of demyelination (Lucchinetti et al. 1996). This information is used to estimate the age of a lesion. Active demyelination occurs mostly in RRMS, and is often accompanied by BBB breakdown, but can also occur in SPMS patients with active disease (Dulamea 2017). Progressive MS lesions are often demyelinated, while further demyelination does not always occur. Demyelination in chronic lesions can be different from that observed in acute lesions, as it often affects shorter segments of myelin, although it is still the nodules of activated microglia and macrophages doing the damage, as indicated by myelin fragments associated with these nodules (Prineas et al. 2001). As described before, MS is not merely a white matter disease, and especially in progressive disease cortical demyelination is common and extensive, and can be independent from white-matter demyelination (Kidd et al. 1999). Cortical

demyelination occurs preferentially in proximity to enfoldings of the brain, as in the neocortex and the hippocampus, and it was formerly assumed that it occurred in absence of inflammation (Geurts et al. 2007, Kutzelnigg et al. 2005, Bø et al. 2003b). However, it is now evident that cortical demyelination is associated with meningeal TLS, and that there is a correlation between neurodegeneration and meningeal inflammation (Howell et al. 2011, Magliozzi et al. 2007) . A number of mechanisms, in addition to antibody/complement-mediated destruction, that lead to demyelination are known to exist, these include bystander damage caused by the release of proteases by microglia/macrophages, toxic agents produced by lymphocytes, or by oxidative injury (Lassmann 2018).

Demyelination can be spontaneously reversed by remyelination, and the two processes can take place simultaneously (Prineas et al. 1993). Remyelination, recognised by very thin myelin sheaths and shortened internodes, can lead to the repair of small areas within plaques, but also entire plaques, leading to the formation of "shadow plaques" (Périer and Grégoire 1965, Prineas and Connell 1979, Prineas et al. 1993). Remyelinated fibres are not immune to further damage, in fact, they are more prone to be demyelinated again compared to regular, healthy myelin (Prineas et al. 1993, Bramow et al. 2010). Remyelination can take place at any stage of MS, and although longer disease duration seems to be associated with more remyelination, the efficiency of remyelination declines with disease progression (Patrikios et al. 2006). It remains unclear why some patients have remyelinated lesions and others do not. Some genetic components may play a role, but, as yet, no gene candidates have been found. Other factors include age, lesion location, and axonal integrity (Lassmann 2018). The presence and functionality of oligodendrocytes and oligodendrocyte progenitor cells (OPCs) do influence the degree to which a plaque can get remyelinated.

1.2.3.1 Oligodendrocytes

Oligodendrocytes are the myelinating glial cells in the CNS. By ensheathing axons, they allow rapid conduction of electrical signals between neurons. Furthermore, they provide metabolic support, and other factors that help maintain the survival of neurons by producing factors, such as BDNF and NGF (Fünfschilling et al. 2012, Griffiths et al. 1998, Lee et al. 2012, Byravan et al. 1994, Dougherty, Dreyfus, and Black 2000, Gonzalez et al. 1990). Impairment in oligodendrocyte function can lead to impaired signal conduction, and is associated with neurodegeneration (Chamberlain et al. 2016). Oligodendrocytes are affected to a variable degree in MS. In some patients, oligodendrocytes are largely absent from the lesion, whereas in others, oligodendrocytes are mostly preserved or restored by recruited and differentiated OPCs (Lucchinetti et al. 1996). The diverse nature of the oligodendrocyte pathology cannot be explained by the classical view of MS being a primary demyelinating disease, but if we accept that the immunological target may not be restricted to oligodendrocytes, then the pattern of disease becomes easier to accept. NMO, for example, is an antibody-mediated demyelinating disease in which AQP4 on astrocytes are the target. In the CNS, AQP4 is only expressed on astrocytes and thus the demyelination and loss of oligodendrocytes is an example of bystander tissue destruction and demyelination (Tradtrantip et al. 2019). In MS, oligodendrocytes are likely to die because of several reasons, including exposure to proinflammatory cytokines (Buntinx et al. 2004, Lisak et al. 2012). The fate of surviving oligodendrocytes in a demyelinated plaque is unclear. Surviving mature oligodendrocytes seem to no longer myelinate nerve fibres and eventually die by apoptosis. Most of the remyelination, if present, happens by newly differentiated OPCs, that can proliferate and migrate to the site of injury (Young et al. 2013). However, OPCs often fail to mature and differentiate properly under these circumstances (Wolswijk 2000, 2002). There seem to be multiple reasons why this is so. For example, oligodendrocytes have a very high metabolic rate in order to myelinate properly, which leads

to not only toxic by-products, but also great vulnerability to oxidative damage (Juurlink 1997, Bradl and Lassmann 2010). Furthermore, their function can also be impaired by cytotoxic T cells, autoantibodies, activated microglia, and other proinflammatory molecules (Linington et al. 1988, Peferoen et al. 2015, Lassmann 2014b).

1.2.4 Neurodegeneration

Inflammation is only one aspect of MS pathophysiology, another, perhaps more characteristic aspect is the focal demyelination accompanied by axonal and neuronal loss. As the disease progresses, anti-inflammatory treatments are often less effective, which is likely to be a consequence of the neurodegenerative aspects of the MS.

1.2.4.1 Neuronal / axonal loss

Demyelination is not the main cause of increasing, irreversible disability in progressive MS, it rather is the loss of axons and brain atrophy (Prineas et al. 2001, Wegner et al. 2006). Demyelination leads to insufficient signal conduction through axons, which is often associated with loss of motor and cognitive skills in MS (Chamberlain et al. 2016). Although demyelination leaves axons vulnerable and without trophic support, axonal and neuronal loss can occur in areas without demyelination and is not restricted to the cortex (Kutzelnigg et al. 2007, Kutzelnigg and Lassmann 2014, DeLuca et al. 2006). For example, the diffuse inflammation seen in the NAWM in progressive MS is linked to axonal damage without primary demyelination (Kutzelnigg et al. 2005). The extent of axonal loss varies greatly between patients, and between different lesions of the same patient (Bjartmar et al. 2000). Axonal degeneration can start early on in the disease progress, but may not become apparent until much later, when it has slowly but gradually accumulated to significant CNS atrophy (Kawachi and Lassmann 2017). CNS atrophy is the most important marker of neurodegeneration, as it can be visualised in patients using MRI (Popescu et al. 2015). Atrophy happens at a rate of

roughly 0.5% per year and does not only occur in progressive disease, but is present even in the early stages of MS (De Stefano et al. 2010). By the time of clinical worsening, degeneration must have reached a point where compensatory mechanisms cannot be balanced out anymore, and the CNS has exhausted its 'functional reserve' (Friese, Schattling, and Fugger 2014). The concept of functional reserve describes the remaining capacity of an organ to fulfil its physiological function and can be used as a mediator between pathological and clinical outcomes, as the degree of brain loss does not necessarily correlate to clinical decline, and brain function is in this case more relevant than size (Stern 2012, Cader et al. 2006).

Numerous mechanisms have been suggested to play a role in MS neurodegeneration, including microglial/macrophage activation, astrocyte activation, meningeal inflammation, mitochondrial damage and/or failure, hypoxia, and iron accumulation (Kawachi and Lassmann 2017). Activated microglia and macrophages can contribute to neurodegeneration by secreting a range of inflammatory molecules not only in MS plaques, but also in the NAWM and in the grey matter. Furthermore, they produce reactive oxygen species such as myeloperoxidase, contributing to neuronal injury (Fischer et al. 2012). Astrocytes seem to regulate microglial activation and migration, but they also support neurons by producing lactate. In MS, astrocytes produce less lactate, due to a decreased formation of cAMP, resulting in impaired mitochondrial metabolism in the neurons (Mayo et al. 2014, De Keyser et al. 1999). Due to their high metabolic profile, neurons rely strongly on their mitochondria for ATP production. Mitochondrial dysfunction leads to energy deficiency, oxidative injury, and dysregulated calcium homeostasis, all in turn contributing to axonal and neuronal loss. Mitochondrial dysfunction, Na^+ influx, excessive release of Ca^{2+}, and oxidative injury are aspects of hypoxia, which can lead to neurodegeneration via Ca^{2+}-dependent pathways (Kawachi and Lassmann 2017, Mahad et al. 2009, Trapp and Stys 2009).

As described earlier (section 1.3.2), meningeal TLS can contribute to neurodegeneration (Howell et al. 2011). The exact mechanism is unclear, but chronic inflammation comes with chronic secretion of inflammatory products that are neurotoxic, including reactive oxygen species, cytokines, and glutamate (Friese, Schattling, and Fugger 2014). Lastly, iron accumulation occurs in the aging brain. Free Fe_{2+} ions are known to amplify oxidative injury. Degeneration in MS brains is known to cause release of iron, causing further inflammatory neurodegeneration. In progressive disease, this may be worsened by age-related iron accumulation (Mahad, Trapp, and Lassmann 2015, Kawachi and Lassmann 2017).

1.2.5 The acute phase response

Although the contribution of the immune system to tissue destruction in the brain is often considered to be in the absence of any impact on the periphery, it is clear that disease processes within the brain do affect the behaviour of peripheral organs. The acute phase response (APR) is a cascade of systemic inflammatory reactions that is the body's response to injury or infection, which have the primary purpose of restoring homeostasis. Multiple peripheral organs, including the lung and the spleen are involved, but the APR is primarily coordinated by the liver (Campbell, Zahid, et al. 2008, Campbell et al. 2003, Wilcockson et al. 2002). It is now clear that the APR is not just evoked to limit systemic inflammation following injury or disease. For instance, it involves the production of a range of cytokines, chemokines, and traditional acute phase proteins (APPs), which initiate a range of responses, including the mobilisation of leukocytes, fever, secretion of glucocorticoids, complement, and clotting agents (Gabay and Kushner 1999, Moshage 1997, Wilcockson et al. 2002). An APP can be any protein whose plasma concentrations changes by at least 25% in inflammatory disorders. The magnitude of these changes varies and can increase up to 1000-fold in case of C-reactive protein (CRP) and serum amyloid A (SAA), whereas the concentration of other proteins, such as albumin, goes down (Gabay and Kushner 1999).

At the site of injury, or infection, proinflammatory cytokines and other molecules are produced locally. These mediators enter the circulation and switch on the APR by binding to their specific receptors on hepatocytes. IL-1β, TNFα, and IL-6 are key players in this activation process, with IL-6 being responsible for the upregulation of most APPs. They are produced by several different cell types, but macrophages and monocytes at the site of injury are the main source (Kushner 1993, Gabay and Kushner 1999). APPs are very diverse, they include complement proteins, clotting factors, cytokines, chemokines, iron-binding proteins, lectins, pentraxins, and other proteins, and have many different functions (Robinson, Harmon, and O'Farrelly 2016). In addition, IL-1β, TNFα, IFNγ, and IL-6 also induce the expression of adhesion molecules, such as ICAM-1 by endothelial cells in the liver. This contributes to the recruitment of leukocytes to the liver, which in turn exacerbates the local production of cytokines, chemokines and further recruitment of leukocytes (Ando et al. 2007, Ohira et al. 2003, Ohira et al. 1994, Sakamoto et al. 1997). The different pro- and anti-inflammatory mechanisms induced by the APR will affect the site of initial injury to eventually encourage a return to homeostasis. The APR is a complex, tightly regulated mechanism that seems to be highly variable and is dependent on the nature of the disease or injury.

1.2.5.1 The acute phase response after CNS injury

As for any other organ, injury or infection in the CNS leads to activation of the APR. Unlike other organs, the APR following CNS injury is distinct, even differences between brain and spinal cord exist. Equivalent injuries to the brain or the periphery will result in a much stronger inflammatory response in the periphery (Anthony and Couch 2014b). After acute injury or infection, local cells start producing proinflammatory cytokines, such as IL-1β and TNFα. These are secreted in similar degrees in the CNS, mainly by microglia, and in the periphery, mainly by macrophages, yet the response in these different environments is different. This is associated with the induction of downstream chemokines – a focal injection of IL-1β in the

periphery induces more downstream CC-chemokines, while a similar injection in the CNS induces more CXC-chemokines, which results in lower leukocyte recruitment to the CNS (Blond et al. 2002, Schnell, Fearn, Klassen, et al. 1999, Anthony and Couch 2014b).

My laboratory has studied the APR after a focal CNS injection of IL-1β over the last decade (Wilcockson et al. 2002, Campbell, Jiang, et al. 2007, Sá-Pereira et al. 2018) . Briefly, acute injury to the CNS induces local production of proinflammatory cytokines, and the release of extracellular vesicles (EVs). EVs are membrane-enclosed vesicles produced by nearly all cell types under physiological and pathological circumstances (Raposo and Stoorvogel 2013). EVs and other injury signals travel to the liver and the spleen where they get taken up by macrophages and initiate an APR. The secretion of cytokines, chemokines, and APPs by the liver as part of the APR results in an array of changes, including the mobilisation of leukocytes from the bone marrow. Mobilised leukocytes will then transmigrate to the site of injury, as well as to the liver, due to chemotaxis and other stimuli (Figure 1.5) (Couch et al. 2017, Campbell, Zahid, et al. 2008, Dickens et al. 2017).

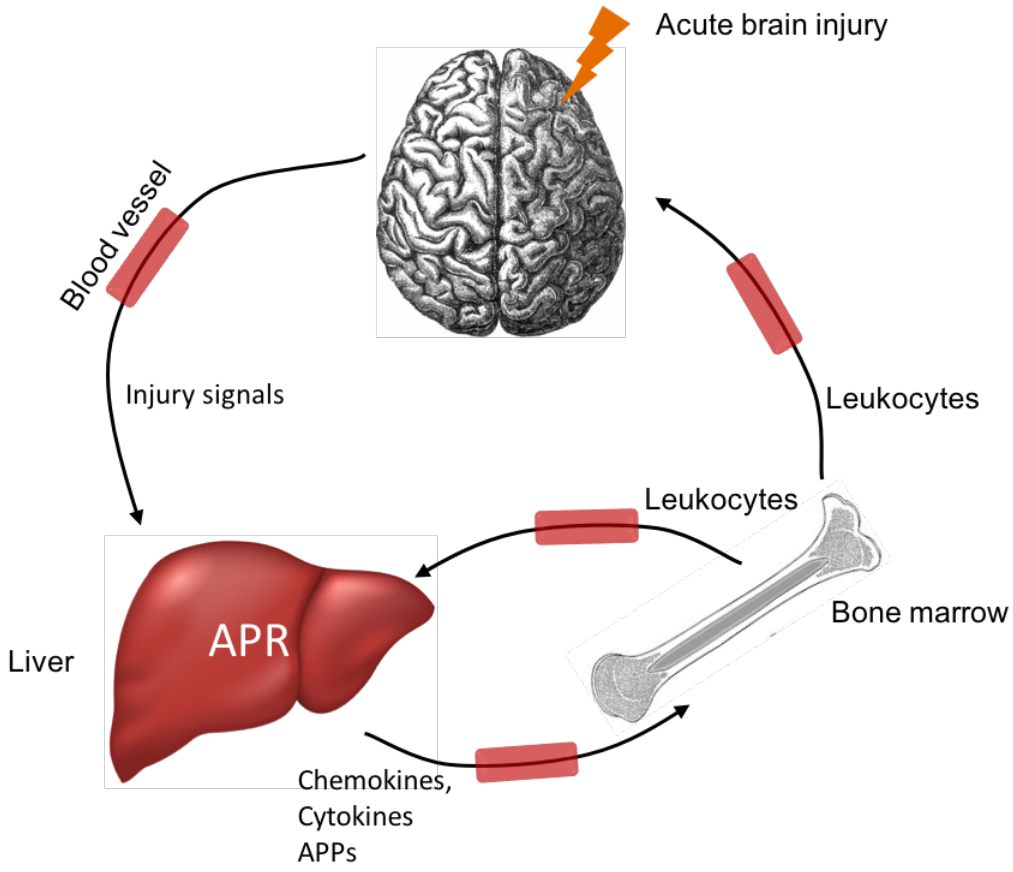


Figure 1.5 The acute phase response after brain injury. Injury to the brain triggers a local immune response, that, if big enough, subsequently induces an APR orchestrated by the liver. The secretion of hepatic APPs, cytokines, and chemokines results in leukocyte mobilisation from the bone marrow. Mobilised leukocytes then travel to the site of injury, as well as to the liver. APR, acute phase response; APP, acute phase protein. Adapted from Anthony and Couch 2014.

IL-1β is also present in the brains and CSF of MS patients and is associated with MS pathology (Brosnan et al. 1995, Hauser et al. 1990, Rovaris et al. 1996). Injecting adenovirus overexpressing human IL-1β in the rat cortex can even develop lesions that model some histological aspects of MS (Silva et al. 2018, Ferrari et al. 2004). Moreover, in a different study, the injection of the same virus into the lateral ventricle resulted in sustained brain inflammation, and sustained elevated cytokine production by the liver (Campbell, Deacon, et al. 2007). It remains unclear whether cytokine production in MS lesions can induce an APR, or whether the APR is affected in MS. We do know, however, that there is an increased recruitment of neutrophils to the liver of MS patients and mice with EAE compared to controls (Campbell et al. 2010). Furthermore, relapses or disease exacerbations in MS are often associated with systemic infections, such as upper urinary tract infections (Buljevac et al. 2002, Correale, Fiol, and Gilmore 2006). We have shown that acute CNS lesions, but also MS-like lesions can be exacerbated by augmentation of the APR (Campbell et al. 2005, Campbell, Anthony, et al. 2008, Mardiguian et al. 2017).

1.3 Treatment of MS

In the last decades, our understanding of the pathological mechanisms behind MS has changed considerably, which had led to the development of better disease-modifying therapies (DMTs) for RRMS. DMTs target the pathogenic mechanisms underlying MS, thereby altering a patient's disease outcomes (Ransohoff, Hafler, and Lucchinetti 2015). RRMS now has several licenced DMTs that suppress or alter the behaviour of the immune system. Some are now so effective at treating relapses that a proportion of patients can expect to live a relapse-free life. However, for SPMS, only siponimod has been shown to have any effect on progression, and only in patients with 'active' disease (Kappos et al. 2018). No single drug works best for all patients – an ideal treatment would result in the elimination of all clinical and radiological disease activity without significant adverse effects. Escalation therapy is currently the most common treatment strategy, which involves patients starting on a first-line therapy that has relatively few adverse effects, while a proportion of MS patients responds well to these drugs. In case of evidence of disease activity, patients will be changed to a more effective second-line treatment (Torkildsen, Myhr, and Bø 2016). The availability of more effective second-line treatments has popularised another treatment strategy: induction therapy. This strategy conceptualises treating patients earlier with more efficacious treatment, thereby preventing accumulative inflammatory damage and related neurodegeneration and disability (Comi, Radaelli, and Soelberg Sørensen 2017).

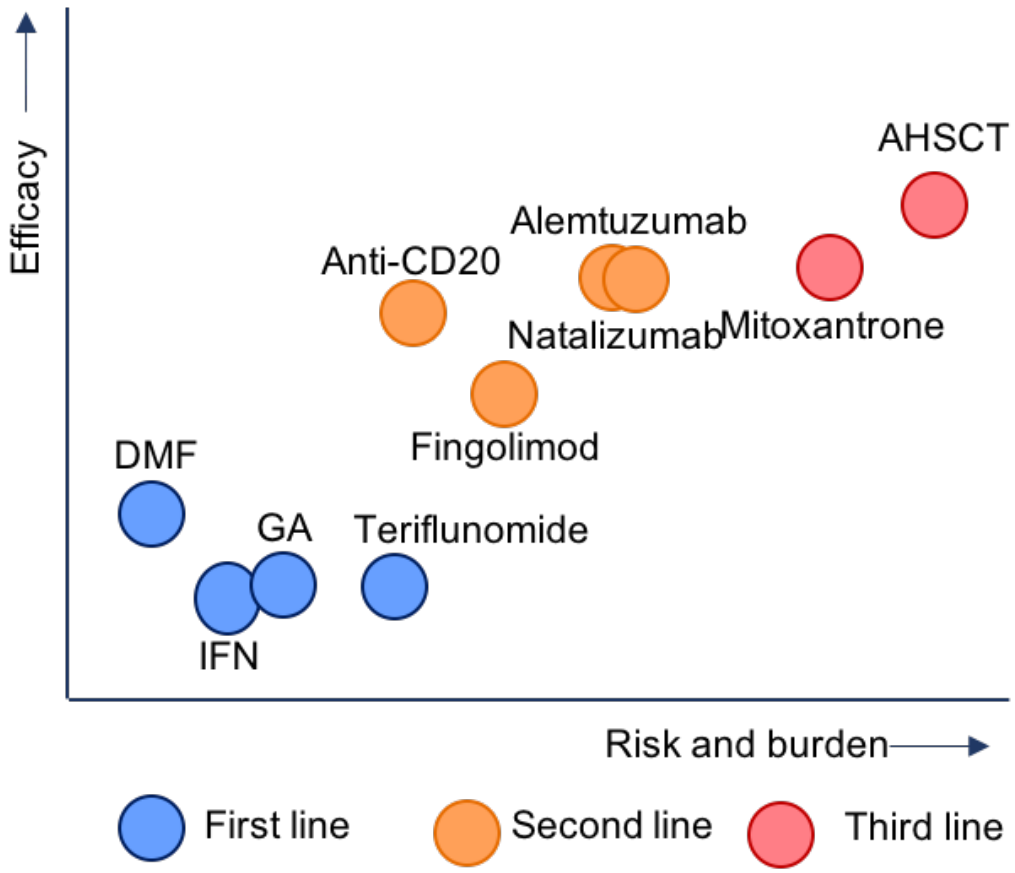


Figure 1.6 The relative efficacy and risk of different immunomodulatory MS therapies. A schematic overview of the relative risk versus efficacy for different first, second and third line therapies for MS. DMF, dimethyl fumarate; IFN, interferon; GA, glatiramer acetate; AHSCT, autologous haemopoietic stem-cell transplantation.

High dose corticosteroids, such as methylprednisolone or dexamethasone, are the first line treatment in MS during relapses. They were the first approved class of drugs to treat MS, but they do not affect disease progression and can only relieve some acute symptoms temporarily (Ransohoff, Hafler, and Lucchinetti 2015). Corticosteroids are usually given for 3-5 days. Other first line therapies include Interferon β (IFNβ) and glatiramer acetate (GA), two of the first DMTs for MS.

1.3.1 First-line therapies

IFNβ was the first DMT for RRMS approved by the food and drug administration (FDA) in 1993(Hauser et al. 1983, Paty and Li 1993, Ransohoff, Hafler, and Lucchinetti 2015). Recombinant IFNβ is the most frequently prescribed therapy to relieve exacerbations in RRMS, although it has also been shown to reduce the annual relapse rate (ARR), slow down disease progression, and lower disease activity (Torkildsen, Myhr, and Bø 2016). IFNβ has immunoregulatory effects via various mechanism, including antagonising IFNγ-mediated MHC upregulation on APCs, altering cytokine expression profiles, and blocking leukocyte transmigration (Hauser and Oksenberg 2006). As of this year, five formulations of IFNβ have been FDA-approved for use in MS. (Reich, Lucchinetti, and Calabresi 2018). Depending on the construct, intramuscular (i.m.) or subcutaneous (s.c.) injections are given between once daily and once weekly. Common side-effects include flu-like symptoms and inflammatory skin reactions at the injection-site, leaving necrotic skin patches in approximately 5% of patients. More serious side effects, such as cardiotoxicity and acute leukaemia are slightly more uncommon (Goldenberg 2012). IFNβ has not been shown to be effective in SPMS (Leary et al. 2003).

GA is a synthetic polymer that was designed to mimic sequences of MBP. The exact mechanism of action is still unclear, but it was designed to manipulate the T helper response by competing with myelin epitopes for APC sites to encourage a less encephalitogenic state,

and by reducing general inflammatory activity (Racke, Lovett-Racke, and Karandikar 2010). GA is injected s.c., daily. GA has been shown to reduce relapse rate and gadolinium-enhanced lesions in RRMS patients (Goldenberg 2012). The most common side effects of GA treatment are injection-site reactions, flu-like symptoms, tight chest, and anxiety (Torkildsen, Myhr, and Bø 2016).

Teriflunomide is another immunomodulatory agent, but how it works in MS is largely unknown. Teriflunomide inhibits de novo synthesis of pyrimidine, and inhibits rapidly proliferating cells. Teriflunomide comes in the form of tablets and is taken orally daily (Torkildsen, Myhr, and Bø 2016). Several trials have shown that teriflunomide reduced the ARR, slowed down disease progression, and reduced the number of gadolinium-enhanced lesions on MRI. Common side effects include respiratory or urinary tract infections, diarrhoea, and leukopenia (Torkildsen, Myhr, and Bø 2016, Miller et al. 2014).

Dimethyl fumarate (DMF) is yet another anti-inflammatory immunomodulatory agent that was introduced in 2013, whose mechanism is not fully understood. The drug is thought to work by driving leukocytes towards a more anti-inflammatory state (Bomprezzi 2015). Two phase III trials showed that DMF reduced the ARR, the disease progression rate, and the number of gadolinium enhancing lesions compared to placebo. DMF is taken orally and dosed twice daily. Gastrointestinal events, including diarrhoea and abdominal pain are amongst the most common adverse effects (Gold et al. 2012, Torkildsen, Myhr, and Bø 2016, Fox et al. 2012).

1.3.2 Second-line therapies

Fingolimod (FTY720) is a sphingosine-1-phosphate (S1P) receptor modulator – it downregulates the expression of the S1P receptor 1 on leukocytes and the endothelium, preventing lymphocytes from leaving the lymph node and subsequently migrating to the CNS (Kappos et al. 2006). The drug was FDA approved for treating RRMS in 2009, after it had

shown efficacy in reducing disease progression, the number of relapses, and improving the loss of brain volume compared to placebo or IFNβ-1a (Kappos et al. 2010, Cohen et al. 2010). Fingolimod is given orally daily (Pelletier and Hafler 2012). Although fingolimod was approved as a first-line treatment, it is often only used as a second-line drug, due to safety issues. Fingolimod is not highly specific and targets four out of five known S1P receptors expressed in various tissues, which can lead to a wide range of potential side effects. The most common serious side effects are cardiovascular problems, and thus patients need to be carefully pre-screened for evidence of cardiovascular disease (Pelletier and Hafler 2012).

Siponimod is another S1P receptor modulator, targeting $S1P_1$ and $S1P_5$. Siponimod, like fingolimod, prevents lymphocytes from leaving lymphoid tissue, thereby preventing infiltration into the CNS. Animal studies have shown that the drug can cross the BBB and is thought to protect against synaptic degeneration (Kappos et al. 2018, Gentile et al. 2016). Siponimod was approved by the FDA earlier this year for the treatment of relapsing forms of MS, after a successful phase III trial (Kappos et al. 2018). The trial showed that treatment with siponimod reduced the risk of disability progression, slowed down the rate the brain volume loss, and lowered the increase lesion volume compared to placebo in MS patients. The observed adverse effects were similar to those of fingolimod and included headache, infections, and hypertension (Kappos et al. 2018).

Natalizumab is a humanised monoclonal antibody (mAb) that binds to integrin-4α on lymphocytes and monocytes. By binding to integrin-4α, natalizumab inhibits the binding of leukocytes to VCAM1 on the inflamed endothelium, thereby preventing leukocyte transmigration into inflamed tissue (Brown 2009). Natalizumab is injected intravenously every 4 weeks (Brown 2009). It was FDA approved as a DMT for RRMS in 2004 on basis of two clinical trials. The clinical trials showed that natalizumab reduced disease progression, the number of relapses, and the number of new gadolinium-enhanced lesions compared to placebo

and as a co-therapy with IFNβ versus IFNβ alone. The last trial had to end early, however, due to the incidence of several patients developing multifocal leukoencephalopathy (PML), a life-threatening disease (Ransohoff 2007). Multiple cases of PML have occurred in patients treated with Natalizumab (Bloomgren et al. 2012). Other side-effects include fatigue, infusion reactions, infections (Polman et al. 2006).

Alemtuzumab is a humanised mAb that binds to CD52, a surface marker expressed by mature lymphocytes, macrophages, and eosinophils. The drug induces cell death of these cell types through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Following binding, the mAb induces antibody-dependent cellular cytolysis and complement-mediated lysis. Alemtuzumab is given intravenously for two treatment courses – during the initial 5-day course, patients receive the drug daily, and 12 months later, during the second course, patients receive alemtuzumab for three days. Additional courses may be given if necessary, 12 months after the last course. In clinical trials, alemtuzumab reduced the annual relapse rate, as well as disease activity compared to IFNβ-1a (Cohen et al. 2012). Despite these good results, treatment with alemtuzumab causes an increased risk for secondary autoimmunity, including thyroid disorders. Other common side effects include infusion reactions, and increased incidence of infection (Goldenberg 2012).

A relatively new, popular type of treatment for MS is the use of mAbs targeting B cells. The recent success of clinical trials proving anti-B cell mAbs to be effective in MS has highlighted the pivotal role of B cells in MS. These B-cell-directed antibodies target CD20. CD20 is a relatively safe target because it is not expressed on B cell precursors and plasma cells, leaving pre-existing humoral immunity, and the capability of B cell reconstitution intact (Greenfield and Hauser 2018). The next section will focus on anti-CD20 therapy in detail.

1.3.3 Anti-CD20 antibodies in MS

Anti-CD20 therapy has proven to be effective for RRMS, but has also shown promising results in PPMS, which has led to ocrelizumab being the only licenced drug for use in PPMS. As described before, B cells may have many potential roles in MS pathogenesis, including antigen presentation and cytokine production (Greenfield and Hauser 2018). How exactly anti-CD20 therapy impacts different B cell populations and how it improves MS patients remains unclear.

Several anti-CD20 antibodies exist and can all be distinguished by their structure, mechanism of action of inducing cell death, and the location on and mode of binding the CD20 molecule (Figure 1.7 and 1.8). Currently there are four different anti-CD20 antibodies in clinical trials for MS: rituximab (RTX), ocrelizumab (OCR), ofatumumab (OFA), and Ublituximab (UBX) (Greenfield and Hauser 2018).

RTX is a chimeric mouse-human mAb and was the first anti-CD20 antibody to be tested in MS (Hauser et al. 2008). RTX has a human IgG part (constant region) with a murine variable region that recognises CD20 (Morrison et al. 1984). The Fc part of RTX plays an essential role in inducing cell death in B cells. The Fc part can interact with and complement protein C1q and Fcγ receptors (FcγR) to switch-on a cascade of events that lead to CDC, and ADCC respectively (Figure 1.7) (Mössner et al. 2010). RTX works mostly through CDC; binding of C1q to the Fc part of CD20-bound RTX triggers the formation of the membrane attack complex via C3 and C5. Several clinical trials have been conducted treating MS patients with RTX or placebo. The dose of RTX was 1000mg intravenously given on days 1 and 15 for RRMS patients in a 48-week trial, or every 24 weeks, through 96 weeks in a PPMS trial. In the RRMS trial, RTX reduced the number of relapses compared to placebo, and the number of gadolinium-enhanced lesions up to 24 weeks after treatment, but not at 4 weeks after treatment. This was paired with a rapid >95% reduction of $CD19^+$B cells in the blood from 2 weeks until 24 weeks after treatment, while by 48 weeks the number of $CD19^+$ cells had

increased to 30.7% of the baseline number (Hauser et al. 2008). The most common side-effects were infusion reactions, such as fever, nausea, and hypotension. Although patients treated with RTX had a smaller increase in lesion volume than those treated with placebo in the PPMS trial, the primary endpoint of the trial, a reduction in confirmed disease progression by RTX, was not reached (Hawker et al. 2009).

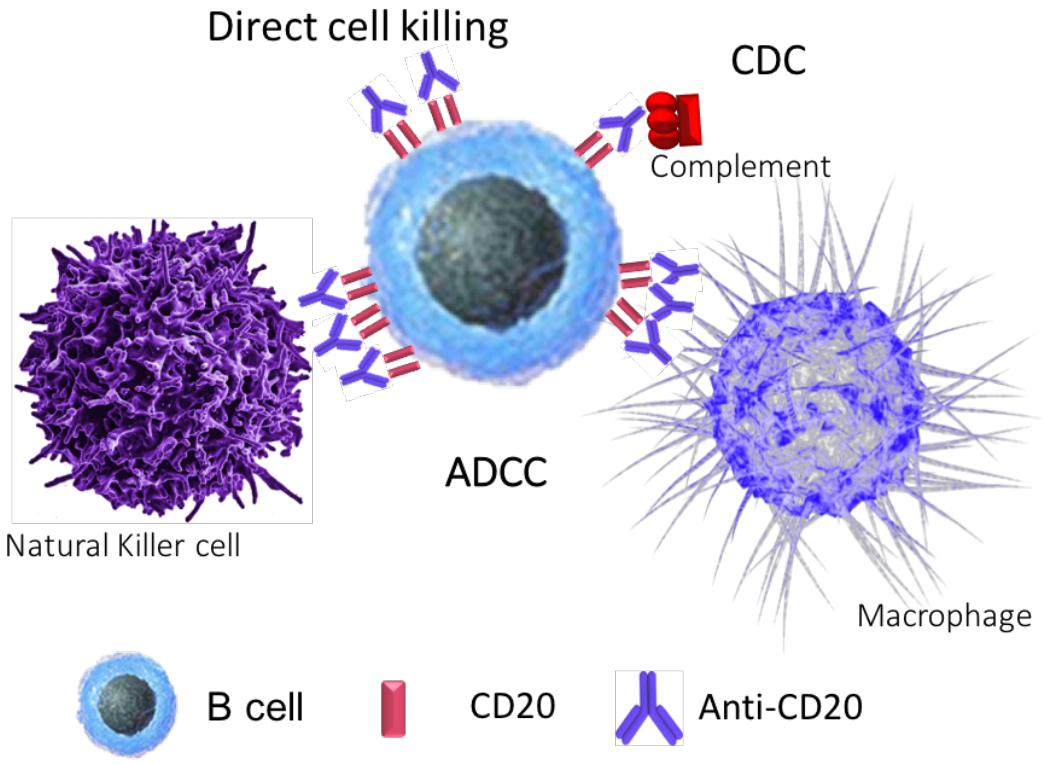


Figure 1.7 Mechanisms of action of anti-CD20 antibodies. Anti-CD20 antibodies can induce cell death in B cells through direct cell killing, ADCC, and CDC. ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity. Adapted from Tobinai et al. (Tobinai et al. 2017).

RTX is currently used in the clinic to treat a variety of B cell malignancies, including off-label treatment for RRMS and PPMS (Salzer et al. 2016). In Sweden, RTX is one of the most frequently used DMTs (Juto et al. 2019). Not uncommonly, patients show a lack of response to RTX treatment, but the exact reason for this remains unclear. RTX resistance can be caused

by a variety of mechanisms, leading to reduced expression of CD20 on the cell surface. Reduced CD20 density can be caused by downregulation of gene expression and translation, significant internalisation of CD20 molecules, or "CD20 shaving", a phenomenon described as trogocytosis. During trogocytosis, $Fc\gamma R^{+}$ cells, such as NK cells, bind to the anti-CD20 antibody attached to a CD20 molecule on a B cell, and instead of phagocytising the B cell only the antibody-antigen complex and surrounding membrane is removed, and internalised by the phagocyte, leaving the B cell intact (Skopelja-Gardner et al. 2017, Beum et al. 2006, Pedersen, Jungersen, and Pedersen 2011). This mechanism leads to fewer CD20 molecules on the surface of B cells, which results in a reduction of antibody binding, and a reduction of effector cell binding. Together, this can lead to a lower drug efficacy, or even drug resistance (Tobinai et al. 2017).

Ocrelizumab (OCR) is a humanised, glycosylated mAb that targets CD20 and is the first FDA approved anti-CD20 mAb for both RRMS and PPMS. Humanised indicates that the parent antibody was not human, however, modifications of the protein sequence of the antibody are applied to make the antibody more like one naturally occurring in humans. Humanised and human antibodies were developed to create a less immunogenic profile of the antibodies, causing fewer infusion-related reactions. Fewer infusion-related problems in turn can benefit patients by the possibility of a more convenient route of administration and a lesser need for glucocorticoids. Compared to RTX OCR has stronger ADCC compared to CDC. OCR and RTX share the same core epitope of the CD20 molecule, but their exact binding sites differ slightly (Figure 1.8) (Klein et al. 2013, McGinley, Moss, and Cohen 2017). In two phase III studies, OPERA I and OPERA II, OCR was compared to IFNβ in RRMS. Patients received either 600mg OCR intravenously every 24 week, or 44μg IFNβ-1a three times a week for 96 weeks. Both trials showed that OCR was better at reducing the annual relapse rate, progression in disability, and the number of gadolinium-enhanced lesions than IFNβ over a period of 96 weeks. Adverse effects occurred in a similar proportion of patients in each treatment group,

and included infusion-related reactions and headaches for OCR (Hauser et al. 2016). In the OTORIO phase III trial PPMS patients were treated with either OCR (600mg intravenously) or placebo every 24 weeks for more than 120 weeks. In this trial patients treated with OCR had reduced disability progression, reduced lesion volume, and a smaller reduction in brain volume compared to patients treated with placebo. The most common side effects were again infusion related. Other adverse effects included urinary tract infection and influenza (Montalban et al. 2017).

Ofatumumab (OFA) is a fully human anti-CD20 mAb that is currently in clinical trials for treating MS. OFA was generated in human-CD20-immunised, transgenic mice expressing human IgG. OFA binds to a different loop of the CD20 molecule from RTX and has more efficient CDC than ADCC activity (Figure 1.8) (Greenfield and Hauser 2018, Zhang 2009). In a phase II trial, OFA was given twice intravenously (300mg) to RRMS patients two weeks apart. No serious safety concerns were observed, but suppression of brain lesion activity was observed. The most common side effects were infusion related, and MRI showed a significant reduction of the total number and of new gadolinium-enhanced lesions compared to placebo (Sorensen et al. 2014). Consequently, in search for a better efficacy/safety profile for anti-CD20 treatment, another trial took place with a different mode of administration (Bar-Or et al. 2018). In the MIRROR study RRMS patients were treated with different doses of subcutaneous (s.c.) OFA or placebo every 4 weeks, or every 12 weeks. A range of doses was given to explore the optimal dose for treatment - 3, 30, or 60mg of OFA was given every 12 weeks, or alternatively 60mg was given every 4 weeks. In this trial, s.c. dose-dependent B cell depletion was observed, although no full depletion was observed (Bar-Or et al. 2018).

Interestingly, quick B cell repletion was observed in the treatment groups with less frequent OFA administration, which was much faster than treatment with intravenous OFA, or other anti-CD20 antibodies (Bar-Or et al. 2018, Sorensen et al. 2014, Hauser et al. 2016). This also

was a dose-dependent effect, with faster B cell repletion in the lower-dose groups. The clinical implications of full B cell depletion or fast repletion remain unclear. OFA did reduce the number of new gadolinium-enhanced lesions compared to placebo, and had no significant effect on the number of relapses, or on EDSS score. The most common adverse effects were injection related, which was independent of the dose of OFA (Bar-Or et al. 2018).

Ublituximab is a chimeric monoclonal antibody that binds to yet another epitope of the CD20 molecule. Due to glycoengineering it has enhanced affinity for FcγRIIIa receptors, leading to enhanced ADCC activity compared to RTX and OFA (Babiker et al. 2018). UBX is currently in a Phase II trial to assess its safety and effectiveness in RRMS (Fox 2018). A previous trial focussing on dose, safety, and tolerability showed efficient B cell depletion, and a reduction in gadolinium-enhanced lesions (Fox 2018).

Although OCR and other anti-CD20 antibodies have proven to improve the prognosis for MS patients by reducing disease activity and slowing down disease progression, patients still deteriorate over time. The trials suggest, however, that B cells must play a role in the disease pathogenesis (Montalban et al. 2017, Hauser et al. 2016). This complicated matter is underlined by failed studies with other B cell targeting therapies, B cell growth factor inhibitor atacicept, for example, increased disease activity in a phase II trial (Kappos et al. 2014).

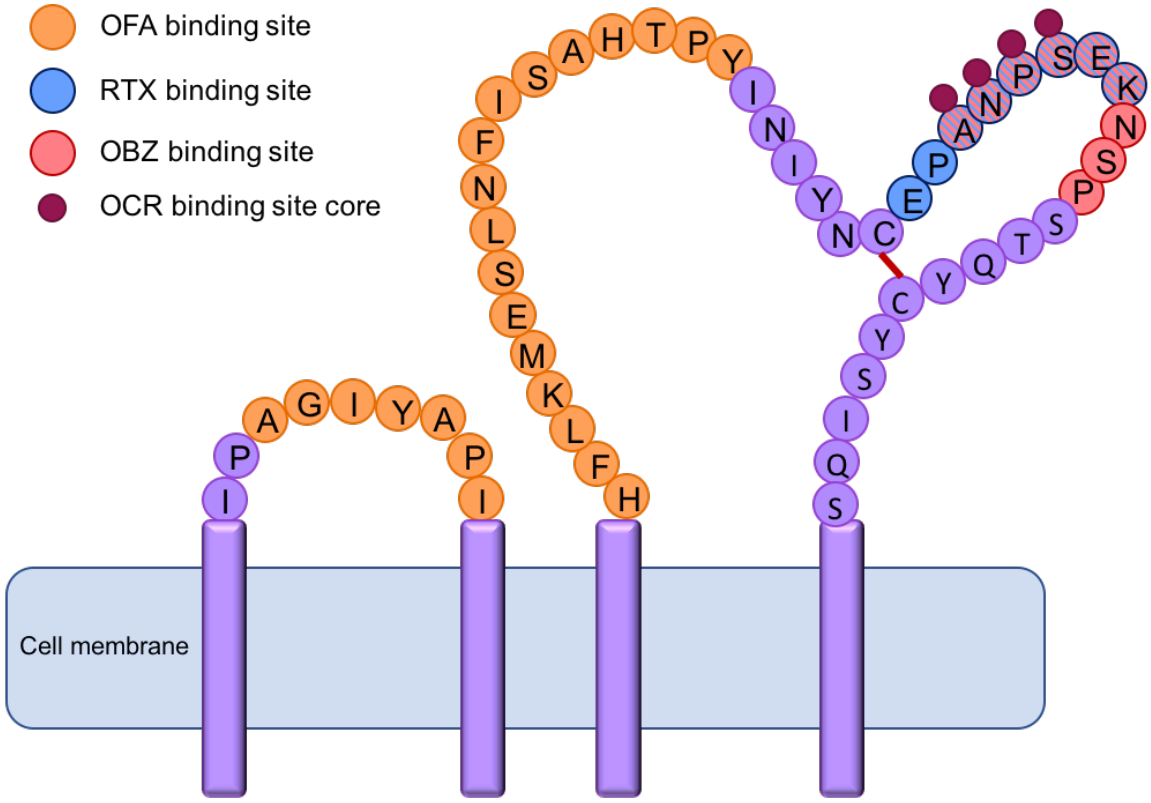


Figure 1.8 CD20 binding sites. The structure of CD20 and the binding sites of rituximab, ocrelizumab, ofatumumab, and Obinutuzumab. Adapted from Klein et al. (Klein et al. 2013). OFA, ofatumumab; RTX, rituximab; OBZ, obinutuzumab; OCR, ocrelizumab.

1.3.3.1 Other Anti-CD20 antibodies – Obinutuzumab/GA101

Most existing anti-CD20 antibodies, including RTX, OCR, and OFA, are type I, which means that upon binding to CD20 on the cell surface they induce redistribution of CD20 into lipid rafts, leading to stronger C1q binding, and subsequently to more efficient complement activation or CDC (Bologna et al. 2013). Obinutuzumab (OBZ) and its parent antibody GA101, are novel, fully humanised mAbs. They both are type II mAbs, which means that although the antibody binds the same area of the CD20 molecule as RTX, it binds at a different angle (Figure 1.8). The binding does not redistribute CD20 into lipid rafts, hence these antibodies have lower CDC activity (Herter et al. 2013). Another feature of type II antibodies is that they are able induce direct cell death in a caspase-independent manner (Mössner et al. 2010). One advantage of the direct cell killing properties is that treatment is not dependent on host's immune system for effector mechanisms. It remains unclear how exactly type II mAbs induce direct cell death, but lysosome-mediated mechanisms have been suggested (Honeychurch et al. 2012). Furthermore, the number of type II antibodies bound to a target cell have been shown to be half the number of type I antibodies. This may have to do with the fact that CD20 molecules often cluster together as complexes consisting of at least three CD20 molecules on the cell membrane of B cells, allowing (type II) antibodies to bind multiple CD20 molecules at once (Tobinai et al. 2017).

OBZ is glycoengineerd and therefore has enhanced ADCC activity. Post-translational glycoengineering of OBZ resulted in a nonfucosylated sugar in the Fc region, causing increased affinity to FcγRIIIa, a Fc receptor present on NK cells, monocytes and macrophages. This increased affinity translates to enhanced ADCC activity (Ferrara et al. 2011, Mössner et al. 2010). FcγRIIIa crosslinking upon binding to the antibody attached to the target cell, triggers the release of enzymes by the effector cell, resulting in killing or phagocytosis of the target cell

(Glennie et al. 2007). GA101 is not glycoengineerd, and is often used for research purposes. GA101 has similar ADCC activity as OFA and RTX (Herter et al. 2013).

Several studies have shown better efficacy of OBZ compared to RTX and OFA in whole-blood B cell depletion and anti-tumour activity in animals (Herter et al. 2013, Mössner et al. 2010, Awasthi et al. 2015). OBZ is in several clinical trials for various B cell malignancies, including non-Hodgkin lymphoma, B cell lymphoma, and chronic lymphocytic leukaemia (Salles et al. 2013, Tobinai et al. 2017). Similar to the other anti-CD20 antibodies, the most common side effects include infusion reactions (Tobinai et al. 2017, Salles et al. 2013). OBZ has only been studied in a cancer-context, which requires high doses of the antibody.

Under certain circumstances it is desired to not recruit any more immune cells via either complement or effector cell activation. Hence, variants of OBZ have been developed that have diminished binding to complement or FcγRs, or do not bind complement or FcγRs at all. The enhanced ADCC activity of OBZ might increase the production of proinflammatory cytokines, which contributes to infusion-related side effects (Herter et al. 2018). The aglycosylated variant of GA101 (Aglyco), contains a mutation in the Fc part of the antibody that prevents glycosylation, which leads to a strongly reduced ADCC activity.

The "PGLALA" variant of OBZ has been generated by the introduction of several Fc mutations: P329G/Leu234Ala/Leu235Ala, the P329GLALA mutations (Schlothauer et al. 2016). PGLALA has undetectable binding to FcγRII and FcγRIII, and merely residual binding to FcγRI, while leaving the direct cell killing properties of OBZ intact (Schlothauer et al. 2016). Herter et al. compared PGLALA's efficacy with both OBZ and RTX in whole blood from healthy donors, and in a mouse cancer model. They found that both OBZ and RTX induced elevated cytokine release upon binding to B cells in peripheral blood, but that PGLALA did not. Furthermore, in an *in vivo* cancer model they showed that PGLALA had a similar efficacy as RTX at slowing down tumour progression (Herter et al. 2018). Although PGLALA was not

as efficacious as OBZ in this study, as treatment with OBZ resulted in full tumour remission, PGLALA's efficacy vs. safety profile fills a spot in clinical needs (Herter et al. 2018). High efficacy and rapid depletion of cancerous B cells is important in aggressive B cell cancers, in other less aggressive diseases, like MS or other autoimmune diseases, a safer treatment may be more desirable, despite its slightly lower efficacy.

1.3.4 Third-line therapies

Mitoxantrone inhibits topoisomerase II, an enzyme involved in DNA repair. The drug inhibits B cell, T cell, and macrophage proliferation, impairing antigen presentation, and cytokine production. Mitoxantrone is given at a 5 to 12 mg/m^2 dose intravenously once every 3 months (Goldenberg 2012). In RRMS, mitoxantrone has shown to reduce relapse rate, disease activity, and to slow down disease progression. Also, it appears to extend the time between relapses in RRMS. In SPMS Mitoxantrone does not improve disability, but rather slows down progression. The use of mitoxantrone has decreased due to long-term safety issues regarding liver injury. Other adverse effects include nausea, hair loss, and infections (Torkildsen, Myhr, and Bø 2016).

Total immune system reset with autologous haemopoietic stem-cell transplantation (AHSCT) is a relatively new, drastic therapy for MS. In trials for this treatment, patients with aggressive MS received a so-called BEAM chemotherapy regimen (carmustine, which prevents DNA replication and transcription; etoposide, a topoisomerase inhibitor; cytarabine, a DNA polymerase inhibitor; and melphalan, which inhibits DNA synthesis), to destroy their bone marrow. After a few days, they received AHSCT. AHSCT has shown to have high efficacy in suppressing clinical activity caused by inflammation in RRMS patients, which later translated into improvement of disability score in a majority of patients (Mancardi et al. 2001, Saiz and Graus 2004, Burt et al. 2009). As all treatments, AHSCT has risks. Especially the early studies showed high risks of death in initial studies (up to 7.3%), but this percentage has gone down

in more recent studies (to ±0.3%). The treatment is still very intense, though intensity depends on chemotherapy, age, and health of the patient. The treatment resets the immune system entirely, by wiping it out first, and allowing it to reconstitute entirely. As with most treatments, the best responders are young otherwise relatively healthy patients with aggressive (RR)MS. No direct comparison has been made to prove it is better than RTX or natalizumab in the ideals subgroup of patients (Burt et al. 2009).

1.3.5 Neuroprotective and neurorestorative agents

Beyond modulating the immune system, effort has been directed towards restoring damaged parts of the CNS. Efforts to promote endogenous remyelination, OPC proliferation, or even to transplant neuronal precursors have been made (Reich, Lucchinetti, and Calabresi 2018). This is especially true in progressive MS, where is a huge unmet need for treating the worsening disability. This has led to a renewed interest in remyelination and neuroprotective therapies.

High-dose biotin, or vitamin B7, has shown to improve vision and EDSS score in some patients with progressive MS. Biotin is thought to promote myelin repair by augmenting fatty acid synthesis, while protecting against axonal loss simultaneously, thereby restoring disability in progressive MS patients (Tourbah et al. 2016). Oral treatment with 100mg biotin thrice daily led to reversal of disability after 12 months in 12.6% of patients in a randomised, double-blind, placebo-controlled trial. During a 12-month extension phase, the percentage of patients with reversed disability rose to 15.4% (Tourbah et al. 2016). Moreover, biotin treatment also reduced the percentage of patients with confirmed disease progression. Furthermore, the treatment was well-tolerated and few adverse effects were observed. Interestingly, in this trial, there were more enlarging or new CNS lesions detected by MRI in the biotin treated group compared to the placebo group, which could indicate that biotin has proinflammatory properties. Taken together, biotin seems to have therapeutic potential, but caution should be taken. Treatment was not effective in every patient, but it may benefit a proportion of patients.

Further trials and research need to be done to explore long-term effects of biotin on neuroprotection in MS (Tourbah et al. 2016, Ciotti and Cross 2018).

Another molecule with neuroprotective features is lipoic acid (LA). LA is an antioxidant that plays a key role in mitochondrial oxidative respiration (Spain et al. 2017). In a double-blind, randomized, placebo-controlled trial, a daily oral dose of 1200mg LA for two years reduced the rate of brain atrophy in SPMS patients. LA was well tolerated, and the few adverse effects were mostly related to an upset gastrointestinal tract. LA had no effect on clinical outcomes in this study. However, as for biotin, LA treatment caused an increase in the total volume of T2-weighted lesions ($p=0.058$), indicating disease activity (Spain et al. 2017, Ciotti and Cross 2018). This makes LA less of an attractive treatment option, especially as the positive changes in preserving brain volume are, as for so many other treatments, a reduction in disability progression, rather than halting or restoring it entirely.

Several trials with other agents, such as ibudilast, a macrophage-migration-inhibitor-factor inhibitor, and simvastatin, a statin have shown some interesting results regarding slowing down brain atrophy (Fox et al. 2018). Many other therapies have failed to meet their primary outcome measures in progressive MS trials. Among these therapies were lamotrigine, a sodium channel blocker, MBP8298, a synthetic amino acid sequence of MBP to stimulate tolerance, and dronabinol, a cannabinoid (Ciotti and Cross 2018, Freedman et al. 2011).

Another strategy for neuroprotection is protecting axons from (further) damage (Altmann et al. 2009, Nandoskar et al. 2017). One way to obtain this is by remyelinating the CNS, which can be beneficial for all types of MS, as well as for other neurodegenerative diseases. A number of therapies with remyelinating properties have been identified via high-throughput screening methods. These include benztropine and clemastine, an anticholinergic drug and an antihistamine that both can cross the BBB (Mei et al. 2014, Ciotti and Cross 2018, Green et al. 2017). Preclinical data has shown that both drugs promote oligodendrocyte differentiation and

remyelination (Deshmukh et al. 2013, Mei et al. 2016, Li, He, et al. 2015). A phase 2 clinical trial showed an improvement visual examination in patients treated with clemastine. On the downside, the drug worsened fatigue in patients, and there is no proof of stability and sustainability of the 'new' myelin. It should be remembered that new myelin is likely to be targeted by the same process that destroyed the original myelin sheaths. Furthermore, no biological effects could be determined with MRI (Green et al. 2017). More research needs to be done to optimise the timing and duration of treatment as the drug has shown benefits in both preclinical work and in MS patients (Mei et al. 2016, Li, He, et al. 2015).

One of the challenges in the remyelination/protection trials is how to determine whether a therapy works or not. In most animal studies, at least one of the outcome measures involves examining a piece of CNS histologically or performing molecular studies on it, which is not possible when treating MS patients. Hence, a range of clinical studies are often performed to evaluate cognitive or physical function, as biomarkers for myelination are lacking. These matters affect the plausibility of clinical trials as the design and the primary endpoints often differ, even though they are crucial to determine the treatment effect.

1.3.5.1 Anti-LINGO-1 antibodies

LINGO-1 (LRR and Ig domain-containing Nogo Receptor-interacting protein) is a transmembrane protein, exclusively found in neuronal tissue, and is a negative regulator of myelination, oligodendrocyte differentiation, and axonal and neuronal regeneration (Mi et al. 2005, Mi et al. 2004). The protein interacts with both NgR1(Nogo-66 receptor) and p75 or TROY to form a receptor complex that, upon activation, can modulate RhoA signalling. RhoA signalling is best known for inhibiting neurite outgrowth, but is involved in a number of processes, including apoptosis (Theotokis and Grigoriadis 2018, Palandri et al. 2015). LINGO-1 knockout mice display increased oligodendrocyte differentiation and myelination compared to WT mice, and the inhibitory properties of LINGO-1 can be (partially) counteracted by anti-

LINGO-1 therapy (Mi et al. 2005, Mi et al. 2004). In EAE, anti-LINGO-1 has shown to reduce demyelination, memory loss, and axonal degeneration, and to promote regeneration ox axons (Gresle et al. 2016, Sun et al. 2015).

BIIB033, or opicinumab, is a fully human monoclonal antibody that targets LINGO-1, which has had some promising results in preclinical EAE studies, activating remyelination and reducing axonal loss (Tran et al. 2014, Mi et al. 2007). By binding to LINGO-1, BIIB033 prevents the protein to form its active form as a tetramer. It is one of the first remyelination therapies tested in humans. Although only a small percentage of treating antibodies are able to cross the BBB of patients, the target is highly CNS specific, which does allow for higher treatment concentrations (Mi et al. 2005). A phase 1 trial showed that opicinumab is very well tolerated, even in doses up to 100mg/kg, which are very high for an antibody-based therapy (Cadavid et al. 2017). In the SYNERGY trial, MS and optic neuritis patients were treated with different doses of opicinumab or placebo, while they were cotreated with IFNβ. The trial did not show great clinical benefits on visual performance, or MRI measurements (Calabresi 2018). Although opicinumab did not reach its primary goal, a new trial has recently been started. Post-hoc analysis of the SYNERGY trial has given a clearer idea of the ideal patient population and dose (Petrillo et al. 2018, Evans et al. 2017). The current trial, AFFINITY, will investigate the effect of opicinumab as add-on therapy to anti-inflammatory DMTs (Biogen 2018).

1.3.6 Limitations and new directions of therapies

Many other therapies are currently in the pipeline. A range of immunomodulators, including ozanimod and other S1P modulators, as well as cladribine, a chemotherapy that targets lymphoctyes; laquinimod, a carboximide derivative; and BTK inhibitors, a group of drugs that target B development, are moving towards approval.

The wide range of currently approved treatments allows for decent disease control in many MS patients, often through anti-inflammatory mechanisms, reducing and managing relapses. Especially if patients get treated early after initiation of the first symptoms, their outcomes appear to be better in the long run. Once patients start progressing, however, these treatments become less effective, as the damage to the brain has been done. And at the moment, there are no treatments available to reverse neurodegeneration. Whether current treatments are able to significantly suppress disease progression all together remains an unanswered question. One of the issues remains the popularity of the escalation treatment strategy. Only after signs of (increased) disease activity, patients will be put on a more efficacious treatment, due to safety reasons. Many patients do respond to first-line treatments, yet the idea that multiple first-line treatments should fail before a patient can move on to a second-line treatment may negatively impact a patient. The better and sooner a patient can be treated, the better the outcome in terms of disease activity, and progression. The time a patient spends being undertreated, exacerbates degenerative mechanisms. Furthermore, individual therapy is essential – there is no 'one treatment fits all' idea in treating MS. In many clinical trials, only patients are recruited who have not received DMT before, or have no existing comorbidities. In the clinic, these factors play a role in deciding how to treat a patient (Piehl 2014). Patients should be monitored frequently to follow treatment effectivity and possible disease progression.

One major shortcoming is the unavailability of a biomarker to see whether a treatment is beneficial, or to predict whether a treatment will be beneficial or not. MRI is still the only tool that can give some quantifiable information on disease status. Although MRI techniques are improving, other biomarkers are also needed to validate treatment effects. Some novel biomarkers under investigation are neurofilaments, optical coherence tomography, and metabolomics on blood and CSF samples.

It seems clear that current anti-inflammatory therapies are not very effective in progressive MS, although some treatments reduce the rate of disease worsening in some patients, the overall progression is not halted or reduced to an impressive degree. More insight needs to be gained in the underlying mechanisms that drive progressive disease in order to treat it more strategically and more accurately.

1.4 Animal models of MS

A large range of different models for MS are available, and not one model represents the entire spectrum of the clinical, pathological, and immunological features of the disease (Lassmann and Bradl 2017). Here, some of the most common and relevant rodent models are described.

1.4.1 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the most common animal model used to study MS. The model was first described in macaque monkeys in the early 1930s, using rabbit brain emulsions and extracts (Rivers, Sprunt, and Berry 1933, Rivers and Schwentker 1935). Complete Freund's adjuvant (CFA), is one of the most commonly used adjuvants for experimental antibody production. After its original formulation, no adjuvant has been developed with stronger immunostimulatory capabilities. CFA is composed of a light mineral oil, a surfactant agent (mannide monoolate), and heat-killed mycobacterium particles (FREUND 1956, Opie and Freund 1937, Stills 2005). Many variations of EAE have been

described, and all methods utilise some form of CNS antigen in combination with an adjuvant to immunise the experimental animal. Current research uses predominantly mice, but EAE can be induced in any vertebrate animal (Lassmann and Bradl 2017).

Reports of using whole spinal cord homogenate, or myelin proteins MBP and PLP have been mixed with CFA to induce EAE (Brown and McFarlin 1981, Sobel, van der Veen, and Lees 1986, Fritz, Chou, and McFarlin 1983a, b). A popular way of inducing EAE in C57Bl/6 mice is to use MOG-peptide emulsified in CFA. This method is inexpensive and relatively reproducible. This way of establishing EAE requires pertussis toxin (PTx) to induce disease, how PTx helps inducing EAE is still unknown (Lassmann and Bradl 2017). Inducing EAE this way is usually referred to as 'active EAE'. 'Passive EAE' can be induced by transferring CNS antigen-specific immune cells, usually MHC-class-II-restricted T cells, from an immunised donor into naive animals (Cross and Raine 1990). The clinical and histopathological features of active and passive EAE are the same. Both result in a quite severe monophasic disease after 10-15 days. The clinical disease progression can be characterised by initial loss of tail tonus, followed by weight loss, paralysis of the hind legs and subsequently the forelegs, and can eventually lead to death. The model causes the animals to suffer and the clinical features are accompanied by severe weight loss. The clinical features are due to acute, $CD4^+$ T cell mediated, inflammatory lesions in the spinal cord. It displays monocyte/macrophage infiltration, activation of microglia and astrocytes, axonal damage, and large areas of secondary demyelination (Lassmann and Bradl 2017, Nikić et al. 2011, Kipp et al. 2016).

The exact disease course and the nature of the lesions are dependent on the strain of animal used and on the method of disease induction. Some genetically modified mice expressing brain antigen-recognising T cell receptors can develop EAE spontaneously, other strains are more susceptible (SJL/J), and some mouse strains, including BALB/c, are relatively resistant to developing EAE (Bettelli et al. 2003, Bernard 1976, Constantinescu et al. 2001). A variant of

EAE that models more progressive MS is chronic relapsing-remitting EAE (CrEAE). In this model, Biozzi AB/H mice are immunised with an emulsion composed of spinal cord homogenate and CFA, and injected intraperitoneally with PTx on multiple days after immunisation. The model displays a relapse-remitting disease course, but also shows an increasing neurological deficit over time. The multifocal lesions are characterised pathologically by astrocytic gliosis, activated microglia, demyelination, and axonal and neuronal loss (Al-Izki et al. 2012, Hampton et al. 2008). Magliozzi and colleagues showed that B cells and small lymphoid-like structures can be present in the meninges lining the brain stems of CrEAE mice (Magliozzi et al. 2004).

Whether or not B cells play a role in EAE remains a topic of debate. EAE can be induced in B cell-deficient mice, although some researchers argue that B cells can only present large proteins as antigens, such as MOG-protein, but not peptides, like MOG_{35-55} peptide in the context of EAE induction (Lyons et al. 1999, Fillatreau et al. 2002). For example, studies have induced EAE in B cell-deficient mice with MOG peptide (Hjelmström et al. 1998, Wolf et al. 1996). Finally, anti-CD20 therapy does not improve EAE in C57BL/6 mice, whereas anti-CD4 therapy does (Sefia et al. 2017, Biasi et al. 1997).

Although multiple variants of EAE can model some aspects of MS, the disease affects almost exclusively the spinal cord and only establishes as a more chronic disease in certain genetically modified animals, and there is a huge intra-experimental variability. The $CD4^+$ T cell mediated inflammatory response usually lacks B cells, and leads to extensive axonal injury and loss, and displays secondary demyelination. This is different in MS pathology, which is characterized by primary demyelination, in both the brain and the spinal cord and presence of B cells (O'Loughlin et al. 2018, Lassmann 2018). Furthermore, with the lesions manifesting randomly throughout the CNS, it is hard to study the effect of treatments on EAE lesions in a systematic, quantitative method.

1.4.2 Delayed type hypersensitivity model

In 1995, Matyszak and Perry published an article describing a demyelinating brain lesion by an immune reaction against a non-CNS antigen (Matyszak and Perry 1995). The demyelinating lesions are induced by first injecting heat–killed bacillus Calmette-Guérin (BCG) into the brain. This results in an inflammatory response driven by neutrophils and round mononuclear phagocytes, accompanied by transient microglial activation and BBB breakdown. This acute inflammatory response subsides after approximately 3-4 weeks, when there is no evidence of BBB leakage. The injected BCG particles are too large to be digested by microglia, and will stay in either the cytoplasm of these phagocytes or in the white matter. To induce a delayed-type hypersensitivity (DTH) lesion, a peripheral challenge can be given anywhere between 2 weeks up to a year after the initial intracranial injection. The challenge is a subcutaneous injection of the same heat-killed BCG, emulsified with CFA, which leads to the development of an extensive inflammatory lesion at the initial BCG deposit in the brain. The granuloma-like lesion core is composed out of round mononuclear cells, and T cells, and is surrounded by activated microglia. Large inflamed blood vessels, surrounded by T cell filled perivascular cuffs, are another hallmark of this lesion. The lesions' cores can be necrotic and are associated with loss of myelin. Primary demyelination with relative sparing of axons can extend along the corpus callosum. These DTH lesions can remain in the brain up to at least 5 months, at which point the brain is mostly remyelinated again (Matyszak and Perry 1995). DTH lesions can also be reactivated using heat-killed mycobacterium tuberculosis (TB) in CFA (Serres et al. 2009).

This model has been used in rats and made great contributions to preclinical trials for potential MS drugs (Anthony, Dickens, et al. 2014, Anthony, Sibson, et al. 2014). The T cell mediated pathology in this model represents a type I pattern MS lesion with primary demyelination. The model tends to not work very well in mice, however, and the humoral components of progressive MS lesions are not well characterised (Serres et al. 2009).

Injecting live BCG or TB into the CNS results in the formation of brain granulomas similar to those in tuberculosis meningitis. The granulomas are accompanied by severe microglial activation, and sometimes perivascular and meningeal inflammation (Zucchi et al. 2012, van Well et al. 2007, Tsenova et al. 2007).

1.4.3 Focal MOG model

EAE lesions can form anywhere along the neuraxis, which makes studying treatments difficult. To overcome this problem, Kerschensteiner and colleagues developed a focal EAE model in rats, that targets a specific area in the spinal cord (Kerschensteiner et al. 2004). The mode of induction involves immunisation with a MOG protein and incomplete Freund's adjuvant emulsion, followed by a stereotaxic injection of cytokines in the spinal cord 2 weeks later. The cytokine solution contained IFNγ and TNFα, which produced a lesion infiltrated by T cells and foamy macrophages, demyelination, axonal loss, and glial scar formation (Kerschensteiner et al. 2004). This model was later adapted to induce cortical brain lesions. The brain lesions involve widespread cortical demyelination, and transient T cell and macrophage infiltration, that peak 7 days after cytokine injection. The model involves merely limited axonal damage, and the demyelination is followed by extensive remyelination, which is paired with oligodendrocyte proliferation (Merkler et al. 2006).

Gardner et al. explored this model further, moving the cortical injection site closer to the sagittal midline of the brain and using dark agouti rats, to study meningeal inflammation. They show that the combination of both IFNγ and TNFα is required to establish subpial demyelination and inflammation, and that using either cytokine alone does not achieve this. Microglial activation and meningeal infiltration of both T and B cells peaks at 7 days after the cytokine injection, which is associated with complement deposition and oligodendrocyte loss. After approximately 21 days, the brain returns back to normal, with hardly any lymphocytes

present in the meninges, and no activated microglia detected in the brain parenchyma, making it a poor model to study chronic disease (Gardner et al. 2013).

1.5 Virus-induced models of MS

Viral infections have been linked to MS and disease progression, however, no MS-specific MS virus has been discovered. Yet, some viral infections can induce inflammatory demyelinating disease, and scientist have developed models for these diseases to study specific disease mechanisms. The two most popular models for chronic demyelinating encephalomyelitis are induced by the Theiler's virus (TMEV), and the mouse hepatitis (corona) virus (MHV). Both viruses are usually injected intracranially. TMEV first induces a transient acute encephalomyelitis, which in some mouse strains can be followed by chronic demyelination with autoimmune aspects that mainly affects the spinal cord (Lassmann and Bradl 2017, Denic et al. 2011). Inflammatory demyelinating lesions contain T cells, B cells, and plasma cells (Pachner, Li, and Lagunoff 2011, Njenga et al. 2000). The severity of the model varies, depending on which strain of virus and mouse are used. One of the crucial differences with MS is that B cell depletion worsens the disease in this model (Gilli et al. 2015).

MHV-induced demyelinating encephalomyelitis follows a similar two-phase pattern, with the second phase resulting in demyelinated, chronic inflammatory lesions. Again, depending on the viral- and mouse strain, the nature of the lesions varies, though all are mediated by virus-reactive $CD8^+$ T cells (Bender and Weiss 2010). As for the TMEV model, this model is severe, and induces extensive microglial activation all over the CNS. The exact mechanism of tissue injury and demyelination remains unclear, but has likely to do with remaining viral particles switching on an immune response (Cross et al. 1987).

1.5.1.1 Toxin-induced models of demyelination

For a long time, MS disability was considered to be due to demyelination only, which lead to the popularity of studying this phenomenon to investigate its mechanisms, and potential therapeutic interventions. The most common models for toxic demyelination uses either exposure of cuprizone or an intracranial injection of lysolecithin. The advantage of these models is that they are quite reproducible, and allow studying demyelination largely absent of inflammation.

Cuprizone (bis-cyclohexnone-oxalyldihydrazone) is typically fed to mice as a 0.2% food supplement for 4-6 weeks, resulting in demyelinating lesions that spontaneously recover after withdrawal of the cuprizone (Hiremath et al. 1998, Praet et al. 2014). Feeding the mice cuprizone for 12 weeks continuously, however, leads to chronically demyelinated lesions that fail to remyelinate properly (Matsushima and Morell 2001). Although the demyelination is not immune mediated, it is paired with microglial and astrocyte activation, due to the toxic effect cuprizone has on oligodendrocytes (Bénardais et al. 2014, Praet et al. 2014).

Focal injections of lysolecithin result in a rapid demyelination within 1-2 weeks, followed by remyelination (Zendedel, Beyer, and Kipp 2013). The toxin damages the lipids in the myelin sheath directly, thereby transiently recruiting different types of immune cells, including macrophages, B cells, and T cells, which all play a role in repair (Bieber, Kerr, and Rodriguez 2003).

In summary, toxin-induced models do not reflect MS histologically or mechanistically. They have, however, provided insight in not only myelination mechanisms, but in remyelinating strategies as well.

1.6 Summary

This chapter provides an overview of MS pathophysiology, to describe the histological features, and how it has been modelled in animals. It also describes how MS is currently diagnosed and treated, and how all of these topics need improvement, especially for progressive MS. One crucial reason for the lack of effective DMT's for progressive MS is the lack of a suitable animal model. Existing MS animal models better resemble RRMS, making them a poor model for studying progressive MS. There is a need for an animal model that represents progressive MS in order to study its mechanisms and to study new potential therapies, both immunomodulatory and neuroprotective.

1.7 Aims of this thesis

The principal aim of this study is therefore to model progressive MS in mice, to provide a platform to study histological and molecular aspects of the disease, and to explore the efficacy and effectiveness of new treatment strategies. Treatment options for MS are emerging, but remain unsatisfactory, especially for progressive disease. And although we understand the disease pathogenesis better, a lot of processes and mechanisms remain unknown. Recent clinical studies have shown that B cells and the humoral immune system play an important role in MS pathology and that depleting B cells with anti-CD20 is an effective therapy for a large group of MS patients. However, it remains unclear how exactly B cells contribute to MS pathogenesis, and the mechanism behind anti-CD20 therapies is not fully understood and needs further exploration, which can be achieved with the help of suitable animal models. Other than immunomodulatory therapies, a clinical unmet need exists for restorative therapies, especially for progressive MS. A candidate restorative drug for MS is the remyelinating antibody, anti-LINGO-1, which is currently in clinical trials. Studies in animal models of progressive MS can give more insight into whether and how anti-LINGO-1 can benefit MS patients. The work in this thesis was conducted to investigate the following hypotheses:

- That anti-CD20 and anti-LINGO-1 therapy are effective in a new focal, chronic, B cell-rich MS-like model in mice.
- That anti-CD20 therapy affects the acute phase response.
- That anti-CD20 therapy affects meningeal TLS-like structures associated with MS-like lesions in mice.

2. Methods

2.1 Animals

Adult humanised CD20 mice with a BALB/c or C57Bl/6 background and their wildtype (WT) littermates were used throughout this thesis. The mice were initially obtained from JANVIER LABS, France, before being bred in house. Mice were bred heterozygous x WT. WT mice for breeding were obtained from Charles River, UK. Mice were kept in pathogen-free facilities under a standard light/dark cycle with food and water ad libitum. All experiments were performed with UK Home Office approval, under licence number 30/3076.

2.2 Reagents

The MOG_{35-55} peptide was purchased from Innovagen, Sweden (MEVGWYRSPFSRVVHLYRNGK; SP-5360-5). MOG protein (1-125) was purchased from Eurogentech, Belgium (AS-55150-1000). The heat-killed mycobacterium tuberculosis (TB) was purchased from BD biosciences (Difco Mycobacterium Tuberculosis H37Ra). Monoclonal treatment antibodies (several anti-CD20 (GA101, GA101-Aglyco, GA101-PGLALA and Rituximab) and anti-LINGO-1) were generously donated by Hoffmann-La Roche, Switzerland. The gadolinium contrast agent (Omniscan) was purchased from GE Healthcare, UK. Rat recombinant interleukin-1 beta (rrIL-1β) was purchased from R&D Systems UK. Mouse recombinant tumour necrosis factor alpha (TNFα), and interferon gamma (IFNγ) were purchased from Peprotech. CFA (F5881) was purchased from Sigma-Aldrich, Dorset, UK as well as all other reagents unless otherwise stated.

2.3 In vivo procedures

All in vivo procedures were carried out as below in all the experiments described throughout this thesis, unless stated otherwise.

2.3.1 Anaesthesia

All surgical procedures were carried out under general anaesthesia using isoflurane (Isoflo). Anaesthesia was maintained at 2-2.5% in oxygen at a flowrate of 1.5 l/min. Excess isoflurane was passively scavenged, using a Fluovac scavenging system (Harvard Apparatus, UK).

2.3.2 Immunisation

The optimised immunisation procedure is described here. An emulsion was made using two luer lock glass syringes, joined with a 21-gauge needle, containing MOG peptide (0.5 mg/ml) and CFA in a 1:1 ratio. 80µl of TB (66mg/ml in saline) was added to every 1000µl of the MOG fraction increase the proportion of TB in the CFA. Animals were briefly anaesthetised (1.5–2 % isoflurane in oxygen), their legs were shaved, and they received an intradermal injection of approximately 20µl of the mixture in each hind leg.

2.3.3 Stereotaxic surgery

Animals were anaesthetised as described above. The head of the anaesthetised animal was shaved and placed in a stereotaxic frame. All surgical procedures were performed under an operating microscope. The animal's head was sterilised and bupivacaine (Marcain) was administered locally. The skull was exposed by making a small sagittal incision, and stereotactic coordinates were used to determine the site of injection. A dental drill was used to make a burr hole in the skull, exposing the dura of the animal. A finely pulled glass microcapillary was used to inject 1µl of substance, which was delivered over a period of approximately 5 minutes. Exact coordinates and substances will be described in the relevant chapters. The wounds were closed with 6-0 vicryl sutures. Animals were allowed to recover in a heated chamber before being returned to their home cage.

2.3.4 Administration of drugs

Monoclonal antibodies or a vehicle control were administered via the tail vein under general anaesthesia (1.5–2 % isoflurane in oxygen). The antibodies were diluted accordingly in 0.9% saline to a 100µl injection volume. The exact dose of the antibodies will be described in the relevant chapter. For LINGO-1 treatment, mice were cotreated with dexamethasone (0.1 mg/kg in 50µl saline intraperitoneal, Sigma UK) to supress the development of the inflammatory lesion. Anti-LINGO-1 therapy, if it is successful in clinical trials, would normally be used in conjunction with a DMT. If it were not, the ongoing demyelinating process in the immune-mediated MS lesion would reverse any benefit gained by the anti-LINGO-1 therapy. Thus to mimic the clinical scenario more closely, we chose to give a steroid to arrest the ongoing immune-mediated demyelination in our new focal mouse DTH-MOG model, with anti-LINGO-1 as adjunct therapy.

2.4 Behavioural testing

Behaviour was tested in animals with the DTH-TLS (tertiary lymphoid structures) model at day 1, day 6, day 12, day 28, and day 60, after initiation of the lesion, to measure motor coordination and strength. Mice were allowed to habituate to the procedure room for 30 minutes before starting behavioural testing. They then underwent testing on the inverted screen and the static rod test as described previously (Deacon 2013). Between tests, mice were returned to their home cage and allowed to recover for 30 minutes.

2.4.1 Inverted screen

For the inverted screen test, mice were placed on a 45cm^2 square covered with a 1 mm diameter wired mesh, surrounded by a 4cm deep wooden frame, preventing mice from climbing on the other side. The screen was then slowly inverted, and the time was recorded for how long it took for the mouse to fall off onto a soft, padded surface. If the mouse did not fall off, it was assigned the maximum experimental time of 60 seconds.

2.4.2 Fixed rods

Two wooden rods of varying diameters (18 and 9mm), both 1m in length, were fixed to a bench on one end, with 60cm sticking out in space. 60cm below the rods was a padded surface. Animals were placed at the far end of the rod, facing away from the bench. The test was carried out on the thicker rod before the thinner one. The time the animal took to orientate towards the bench, as well as the time the time it took to walk back to the bench was recorded. If the mouse fell from the rod, it was assigned the maximum experimental time of 120 seconds. Animals were allowed to rest between tests.

2.5 MRI

MRI experiments were carried by professor Daniel Anthony and Dr. Jack Miller. Animals were anaesthetised with 2% isoflurane in oxygen, a cannula for gadolinium (Gd) administration was inserted into the tail vein, and animals were positioned in a quadrate birdcage coil. During MRI, anaesthesia was maintained with 1.5-1.8% isoflurane, ECG was monitored and body temperature was maintained at roughly 37 ºC. Spin-echo T_1-weighted images (TR = 500 ms; TE = 20 ms; FOV 25 mm x 25 mm; matrix size, 128 x 128) were acquired pre- and 5 min post-Gd-DTPA injection (30µl, i.v.).

2.6 Tissue sample collection and preparation

Animals were deeply anaesthetised with pentobarbital (20% w/v sodium pentobarbital, JM Loveridge Ltd, UK) and blood was collected via cardiac puncture and transferred to EDTA coated tubes. Animals were then transcardially perfused with cold heparinised saline, containing 5000 U/l heparin, until the liver was cleared of blood. Samples for mRNA extraction were immediately taken and snap frozen on dry ice, or in case of brain, frozen down in optimal cutting temperature compound (O.C.T., CellPath, UK) in an isopentane bath on dry ice. Fresh frozen samples were then stored at -80ºC until further use. For flow cytometry, a piece of spleen was stored in RPMI (Roswell Park Memorial Institute medium) containing 1% FBS on ice. For histology, mice were then transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. Organs were dissected, post-fixed in 4% PFA at 4ºC for 24 hours, and dehydrated in a 30% sucrose solution afterwards. Organs were then embedded in OCT, frozen down in an isopentane bath on dry ice, and stored at -20 ºC until further use.

2.7 Blood analysis

Blood analysis was performed reading the blood samples in triplicate on an ABX Pentra 60 haemocytometer (Horiba, Japan). The average of the three readings was calculated for the number of total white blood cells, lymphocytes, monocytes, neutrophils, basophils, and eosinophils.

2.8 Histology

Neuropathology was examined using histochemistry and immunohistochemistry. We evaluated the extent of microglial and astrocyte activation as a marker for the extent of a generalised inflammatory response. Pan B and T cell markers were used to examine lymphocyte infiltration, and demyelination was measured by stains for myelin. Neuronal and oligodendrocyte survival and proliferation was also evaluated. Peripherally, B cell depletion was measured in the spleen and lymph nodes, while the crude extent of peripheral inflammation was determined by calculating the average number of macrophages and neutrophils per mm^2 in the liver.

2.8.1 Gelatinising slides

Slides were put into racks, washed for 30 seconds in 95% ethanol and dried in a 37ºC oven for one hour. A 0.5% gelatine solution was made in 0.05M phosphate buffer and 0.05% of chromium potassium sulphate was added as hardening agent. Slides were incubated in the gelatine solution for 1 minute before being dried overnight at 37ºC.

2.8.2 Sectioning of tissues

Cryosections were cut at 10μm using a cryostat at -19ºC (Leica Microsystems, UK). Serial sections were collected with 0.1M phosphate buffer on gelatinised slides in case of fixed tissue, or collected on positively charged slides directly in case of fresh frozen tissue. Fixed tissue was allowed to dry overnight, while fresh tissue was only dried briefly before being stored at -20ºC until further use.

2.8.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed using the avidin-biotin-peroxidase method. The frozen slides were dried at 37ºC for 15 minutes, rehydrated and quenched for endogenous peroxidase activity in a 0.03% hydrogen peroxide in methanol solution. The slides were then dipped in PBS and put in a Shandon Sequenza® staining system (Thermo Fisher, UK). Next, the slides were blocked for non-specific Fc-dependent binding with 10% serum in PBS-T, and endogenous biotin in peripheral tissues was blocked using an avidin-biotin blocking kit (Vector Laboratories, UK). The sections were incubated with primary antibodies at the appropriate dilution in 1% serum in PBS at 4ºC overnight (Table 2.1). The sections were then incubated for two hours with the appropriate biotinylated secondary antibodies in 1% serum in PBS (Vector Laboratories, UK) and subsequently with the avidin-biotin-peroxidase solution (Vector Laboratories, UK) for one hour at room temperature. The slides were developed in diaminobenzidine hydrochloride (0.5μg/ml) in 0.1M phosphate buffer until the optimal contrast was obtained. Finally, the slides were washed in 0.1M phosphate buffer and in distilled water (dH_2O). If required by the antibody, heat-mediated antigen retrieval in 0.1M citrate buffer was applied (Table 2.1).

Antigen	Target	Dilution	Source
Iba-1	Microglia/ Macrophages	1:2000	Abcam
CD68	Microglia/Macrophages	1:200	Biorad
HLA-DR	MHC class II	1:200	BD
GFAP	Astrocytes	1:700	Dako
CD45R/ B220	B cells	1:900	BD
CD20	B cells, some T cells	1:100 + AG	Dako
CD3	T cells	1:300 + AG	BD
MBP	Myelin	1:1000	Abcam
NeuN	Neurons	1:2000 + AG	Abcam
MBS, HB199	Neutrophils	1:10.000	Made in house
APC/ CC1	Immature oligodendrocytes	1:200	Merck
NG2	OPCs	1:200	Millipore

Table 2.1 Primary antibodies used for immunohistochemistry. AG, antigen retrieval

2.8.4 Counterstaining and mounting

After IHC, brain sections were placed in 400ml of dH_2O containing 200µl of 100% glial acetic acid for 5 minutes. The sections were then counterstained in a 0.5% cresyl violet acetate solution (pH 3.3) for 10 minutes and placed back in dH_2O. Sections from peripheral tissues were counterstained with haematoxylin for 30 seconds, washed in tap water, and developed in Scott's tap water. All sections were then dehydrated by passing through increasing concentrations of alcohol (80, 95, and two times 100%), and cleared in xylene, before being mounted on coverslips with DPX mounting medium.

2.8.5 Quantification and analysis of the histology

All slides were randomised and blinded until analysis was complete. For brain analysis, the two sections with the largest lesions of each animal were analysed using a light microscope (Leitz Dialux 20). The area of activated microglia and astrocytes in the MS-like lesions was carefully traced using a Camera Lucida. Tracings were scanned, scaled, and the area of activated glia calculated using ImageJ. The total number of B and T cells was obtained by counting the total number in the ipsilateral hemisphere. The number of OPCs and immature oligodendrocytes was obtained by counting cells in the striatum that were positive for the respective markers and matched the cell phenotype as well. To determine neuronal survival, a ratio was calculated by dividing the number of neurons in the ipsilateral piriform cortex by the number of neurons in the contralateral piriform cortex. All numbers acquired were averaged to obtain one value per animal per marker. In smaller lesions (IL-1β induced), the total numbers of neutrophils and activated microglia were counted in the ipsilateral hemispheres on two sections per animal. Activated microglia were characterised with Iba-1 as marker and an enlarged cell body with shortened processes.

For peripheral tissues, the number of neutrophils and macrophages was determined by direct cell counting using a light microscope (Leitz Dialux 20) with an eyepiece grid and a 40x ocular lens. Per animal, 3 areas with the highest density of cells of interest in 3 different sections were analysed. The average for each animal was determined from these recordings (cells/mm^2). To determine the level of B cell depletion, three representative lymph node sections or splenic nodules were traced using the Camera Lucida. The tracings were scanned, the surface area of the B cell compartment and the non-B cell compartment within these structures were determined using ImageJ. A ratio of the two compartments was calculated to express the proportion of area occupied by B cells.

2.9 Molecular biology

2.9.1 mRNA extraction

For mRNA extraction, the QIAshredder and the RNEasy kit from Qiagen was used following the manufacturer's guidelines. An appropriate sized piece of tissue was cut on a sterilised plate on dry ice, and subsequently put in lysis buffer containing 1% β-mercaptoethanol on ice. All following steps took place on ice. The tissue was homogenised using a pestle homogeniser (Kontes, UK) and vortexed vigorously. Next, the tissue lysate was transferred into a QIAshredder spin column and centrifuged for 2 min at full speed. The supernatant was carefully mixed with an equal volume of 70% ethanol transferred to a RNeasy spin column and centrifuged for 30s at 10000 rpm. The flow-through was discarded. Then, RW1 buffer, and subsequently RPE buffer were added twice to the spin column, each followed by a 30s centrifugation at 10000 rpm, after which the flow-through was discarded every time. The spin column was then transferred to a clean collection tube, and 25µl of nuclease free water was added to the spin column. The column was allowed to stand for a minute and then centrifuged for 1minute at 10000 rpm to elute the mRNA. The quantity and quality of the mRNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, UK). The mRNA quality was considered good if the ratio of sample absorbance at 260 and 280 was ±2.0 and the ratio of sample absorbance at 260 and 230 was ± 1.8-2.2.

2.9.2 cDNA conversion and quantitative real-time polymerase chain reaction

The nanoScript2™ reverse transcript kit (Primerdesign, UK), or the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, UK) was used to convert mRNA to cDNA. During this process, a thermocycler (Bio-Rad, UK) was used for the temperature controlled incubations. Real-Time polymerase chain reaction (RT-qPCR) was performed on a LightClycler® 480 (Roche Diagnostics, UK) with SYBR green mastermix containing inert blue dye (Primerdesign, UK). Primers were obtained from Primerdesign or Sigma. All experimental samples were compared to Actb and/or Gapdh. ΔΔCT analysis was used for relative quantification of gene expression. The results were presented as fold change compared to naïve or control expression levels.

Gene	Primer sequence forward	Primer sequence reverse
Tnf	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
Saa	CCAGGATGAAGCTACTCACCA	TAGGCTCGCCACATGTCC
Il-6	AATTCCAGAAACCGCTATGAAGT	ATCCTCTGTGAAGTCTCCTCTC
Cxcl1	GCTGGGATTCACCTCAAGAAC	TGTGGCTATGACTTCGGTTTG
Cxcl10	CATCCCGAGCCAACCTTCC	CACTCAGACCCAGCAGGAT
Mbp	TCACAGAAGAGACCCTCACA	GCCGTAGTGGGTAGTTCTTG
Plp	TGCTCGGCTGTACCTGTGTACATT	TACATTCTGGCATCAGCGCAGAGA

Table 2.2 Primers and sequences

2.10 Flow cytometry

2.10.1 Cell isolation procedure

Animals were deeply anaesthetised with pentobarbital and transcardially perfused with heparinised saline. Using sterile instruments, half the spleen was isolated from the animal, and stored in RPMI containing 1% FBS on ice. The animals were then decapitated, the skin and eyes were removed from the skull, and the cervical vertebrae, the cheekbones, and the lower jaw were removed. Then, the premaxilla was cut, and the skull was cut in two halves, horizontally, leaving the brain intact. Using a dissecting microscope, the skull was carefully cut in two halves. First, one half of the skull was separated from the brain, and placed on ice. Then, the brain was removed carefully and placed in a petri dish on ice, containing RPMI and 1% FBS. The meninges were scraped off both halves of the skull, using extra fine forceps, and placed in the petri dish with the brain. Using fine forceps, the meninges were gently peeled off the brain. The skull and brain were finally rinsed with RPMI.

A single cell suspension was made by pushing the cells through a 70µm cell strainer, using the plunger of a sterile 10 ml syringe. The single cell suspension was kept on ice in RPMI containing 1% FBS until further use.

2.10.2 Cell staining procedure

All cell staining procedures took place on ice. After the red blood cells were lysed in the splenocyte samples with red blood cell lysing buffer (R7757, Sigma), cells were washed twice in PBS. $1*10^6$ for splenocytes were divided in the staining tubes. Cells were then blocked with Fc block (BD sciences) for 15 minutes, before being washed and incubated with fluorescently labelled antibodies for 30 minutes (CD19-Alexa Fluor 488) in 100 µl RPMI containing 1% FBS. Cells were fixed in 2% PFA for 20 minutes, washed twice and resuspended in 500 µl PBS.

2.10.3 Gating strategy

Cell data was collected using a FACSCanto II (BD Biosciences). To obtain accurate cell counts, cells were first gated using the forward versus side scatter to remove debris. The cells were then gated for singlets (FSC-H versus FSC-A), then by the appropriate cell marker, and finally for live/dead stain. In total, 10.000 singlet events were collected per sample. All samples were analysed using FlowJo software (v. 10.3.0, Tree Star, Inc).

2.11 Statistical analysis

All data were analysed with the GraphPad Prism software (v.6, GraphPad Software Inc. USA). To identify differences between the groups, unpaired t-test, or one-way ANOVA with Tukey's multiple comparison test was used, depending on the number of groups analysed. All data are presented as mean ± standard error of the mean (SEM) and were considered statistically significant if $p<0.05$.

3. The development of a new focal model of multiple sclerosis in mice as a platform to study new therapy.

3.1 Introduction and aims

The pathophysiology of multiple sclerosis is characterized by inflammatory demyelinating lesions or plaques, but is also disease stage dependent (Popescu and Lucchinetti 2012). RRMS is characterized by active lesions with infiltration of T and B lymphocytes, monocytes, activated microglia, demyelination and oligodendrocyte loss, increased cytokine production, and disruption of the BBB (Kirk et al. 2003, Grossman et al. 1986). This is different from the progressive stages of MS, where the most striking feature is brain atrophy and lesions become less actively demyelinating. In SPMS, gadolinium-enhanced lesions become sparse, and the composition of immune cells changes as the proportion of B cells and plasma cells in the brain increases (Fischer et al. 2013, Machado-Santos et al. 2018).

The differences in the histopathology may explain why a range of therapies exist for RRMS are ineffective for SPMS. The recent success of anti-CD20 in clinical trials for RRMS has raised interest in this class of drug for progressive disease, but the mechanisms through which it benefits MS patients remain unclear. Anti-CD20 modulates the immune system by depleting B cells. B cells are thought to play a number of roles in MS pathology, including antigen presentation and cytokine production. MS B cells are known to have a more proinflammatory expression profile than B cells from healthy people (Duddy et al. 2007, Li, Rezk, Miyazaki, et al. 2015, McWilliam et al. 2018). GA101 is a type II anti-CD20 antibody because it can induce direct-cell killing upon binding CD20 on B cells, as well as through ADCC and CDC (Herter et al. 2018, Mössner et al. 2010). Other variants of GA101 exist, the aglycosylated variant (Aglyco) has strongly reduced ADCC activity, while the PGLALA variant of GA101, does not have any noticeable ADCC or CDC activity at all (Herter et al. 2018, Mössner et al. 2010). The lack of ADCC and CDC activity are expected to make PGLALA a safer drug, which is exemplified by the lack of cytokine release upon binding to B cells, which is seen with other anti-CD20 molecules.

Beyond modulating the immune system, effort has been directed towards repairing damaged parts of the CNS. To overcome the demyelinating and neurodegenerative aspects of MS, studies have explored neuroprotective and remyelinating strategies (Reich, Lucchinetti, and Calabresi 2018). Opicinumab is an antibody directed against LINGO-1, a negative regulator of myelination, oligodendrocyte differentiation, and neuronal regeneration (Mi et al. 2005, Mi et al. 2004). Treatment with anti-LINGO-1 has been successful in mice, improving demyelination and axonal loss in EAE (Gresle et al. 2016, Sun et al. 2015). However, clinical trials with opicinumab have not reached their primary endpoint. A new trial, AFFINITY, will explore the effect of anti-LINGO-1 as additional (adjunct) therapy with anti-inflammatory treatments.

Usually, drug candidates are studied in animals before entering clinical trials. The most common model for studying MS is EAE, which led to MS being traditionally viewed as being mediated primarily by $CD4^+$ T helper cells. EAE is usually induced by immunization with myelin proteins, which generates a T cell driven autoimmune reaction against myelin proteins, causing inflammatory demyelinating lesions in the CNS (Rivers, Sprunt, and Berry 1933, Lehmann et al. 1992). However, classical EAE primarily targets the spinal cord and rarely the brain, usually in a random pattern, which makes it a poor model to study treatments that might target brain lesions. It is of note that MRI of the brain, rather than the spinal cord, is more commonly used by physicians that are seeking to diagnose or stage MS.

In rats, focal models have been established in the brain and spinal cord, resulting in lesions in a particular area of interest. Histologically, these lesions are similar to those of MS patients, involving demyelination and inflammatory cell infiltration. BBB disruption is transient in these models: after initial disruption, the BBB shows no signs of leakage after approximately 30 days (Kerschensteiner et al. 2004, Merkler et al. 2006, Anthony et al. 2011). Hitherto in mice, focal lesions for MS have not been studied extensively (Chaudhary et al. 2015). Anti-CD20 therapy

has previously been shown to be beneficial in a focal model of MS. However, this was a T cell-mediated model in rats (Anthony, Dickens, et al. 2014).

In order to study the effect of human anti-CD20 antibodies in a disease model, huCD20 BALB/c mice, expressing both human and mouse CD20 were used in my experiments. An additional challenge for developing a relevant model for MS is that many mouse strains, including the BALB/c mice, are reported as being resistant to the development of EAE (Hurwitz et al. 2002). Other advantages for developing a relevant mouse model include the availability of transgenic animals, costs, and the possibility to run larger studies.

The principal aims of this chapter, therefore, were (1) to establish a focal model for progressive multiple sclerosis in mice, (2) to study the effect of anti-LINGO-1 therapy in this model, (3) to study the effect of anti-CD20 therapy in this model.

3.2 Methods

3.2.1 Induction of focal EAE in mice

BALB/c mice were used for these studies. Focal EAE lesions were established as described before (Mardiguian et al. 2017, Merkler et al. 2006). On day -21, mice were immunised with a subcutaneous injection in the hind limbs (25 µl in each side) with a 1:1 MOG_{35-55}peptide (MEVGWYRSPFSRVVHLYRNGK; Innovagen SP-5360-5) (0.5 mg/ml in saline) and IFA emulsion. Alternatively, mice were immunized on day -14, and the MOG peptide was substituted by MOG_{1-117} protein. On day 0, mice received an intracranial injection of a cytokine mixture (1µg TNFα and 0.5µg IFNγ in 1 µl saline) in the left striatum (Bregma: AP +0.5, ML +1.5, DV -2.0). Lesions were allowed to develop before the animals were perfused and organs were collected.

3.2.2 Induction of the new focal DTH-MOG model

Focal lesions were established in huCD20 BALB/c and CD1 mice. On day -12, mice were immunised by an intradermal injection in the hind legs (±15µl in each side) with a 1:1 MOG_{35-55}peptide (0.5 mg/ml in saline) and CFA emulsion, topped up with heat-killed TB (5mg/ml in the total volume). On day 0, the animals received a 1µl stereotaxic intracranial injection in the left striatum (Bregma: AP +0.5, ML +1.5, DV -2.0) with heat-killed TB resuspended in saline (30mg/ml for the anti-LINGO-1 experiment, which was brought down to 10 mg/ml for the anti-CD20 experiments after optimisation).

3.2.3 Treatment with anti-Lingo-1

Treatment started at day 40. Animals with a MS-like lesion were given four 100µl intravenous injections *via* the tail vein of either anti-LINGO-1 (10mg/kg), or a vehicle control spread over 10 days (day 0, day 3, day 6, day 9). The chosen dose was advised after by our colleagues at Hoffmann-La Roche. Each intravenous injection was followed by an intraperitoneal injection of dexamethasone (Dex) (0.1mg/kg). The dose chosen was based on previous work that had shown that the rat DHT lesion was sensitive to steroid therapy (Matyszak and Perry 1996).

3.2.4 Treatment with anti-CD20

Treatment started at day 40, at a time the BBB was assumed to not be leaky anymore (Newman et al. 2001). Animals were given 4 intravenous injections of either the PGLALA variant of OBZ (10mg/kg), or a vehicle control (saline) spread over 10 days (day 0, day 3, day 6, day 9). For the Fc comparisons experiment, 40 animals were randomly divided into four different groups (n=10 mice per group). Each group was given three 100µl intravenous injections of the different variants of GA101 (GA101, Aglycosylated GA101, or PGLALA) 10 mg/kg), or a vehicle control spread over 15 days (day 0, day 5, day 10).

3.3 Results

3.3.1 Model development

3.3.1.1 The focal MOG model does not induce MS-like lesions in mice

In order to generate MS-like lesions, focal EAE lesions were established in mice by immunising them with MOG peptide emulsified in IFA (Figure 3.1A). Mice were perfused 14 days after the intracranial injection to examine the lesion development. Immunolabelling for Iba-1 revealed the extent of the inflammatory response of the brain. Small patches of rounded, activated microglia could be found in the striatum and the corpus callosum of the ipsilateral hemisphere (Figure 3.1B). Because the animals were perfused just 14 days after lesion initiation, brains were stained for mouse IgG to reveal BBB breakdown. Little BBB breakdown could be observed in these lesions, as visualised by the presence of endogenous IgG in the brain (Figure 3.1C). The lesions in the EAE-resistant mice were very small and were not felt to be representative of MS lesions, as they lacked any evidence of demyelination, or significant lymphocyte infiltration.

3.3.1.2 MOG protein does not change the focal MS-like lesions.

In order to obtain larger lesions, that would more closely resemble those seen in MS, mice were immunised with MOG protein instead of peptide. They also received an intraperitoneal injection of PTx, a day after the intracranial cytokine injection, to promote an inflammatory response (Figure 3.2A). Lesions were allowed to develop for 28 days before the mice were perfused. This time, lesions were marginally larger than with the peptide immunisation and cytokine injection alone. The clusters of activated microglia were somewhat larger than before, and there was a clear difference between the ipsilateral and contralateral hemisphere (Figure 3.2B). Anti-MBP staining revealed some demyelination in the corpus callosum of the ipsilateral hemisphere (Figure 3.2B). Furthermore, these mice did not display any clinical symptoms

related to classical EAE. Although lesions showed slightly more microglial activation, they still did not resemble MS lesions.

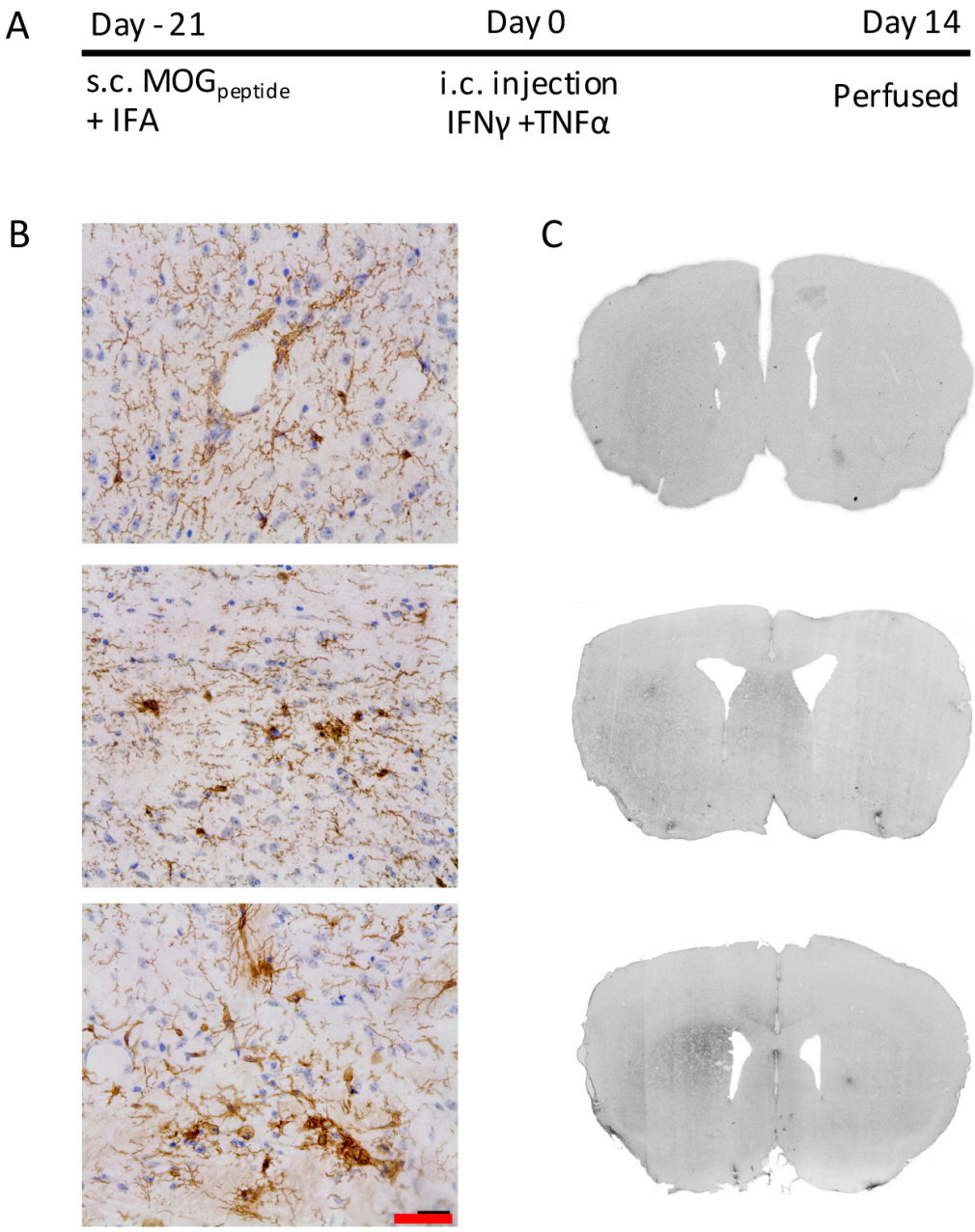


Figure 3.1 The focal EAE model does not induce MS-like lesions in mice. Focal MOG lesions were established in BALB/c mice. (A) Mice were immunized with MOG peptide and IFA three weeks before they received an intracranial cytokine injection. Mice were perfused on day 14. (B&C) Sections were stained for microglia and IgG. (B) Representative photomicrographs of microglial staining show small nodules of activated microglia in brown. (C) IgG stain revealed little BBB breakdown. Red scale bar represents 50µm. MOG, myelin oligodendrocyte glycoprotein; IFA, incomplete Freund's adjuvant; IgG, immunoglobulin G; BBB, blood-brain barrier.

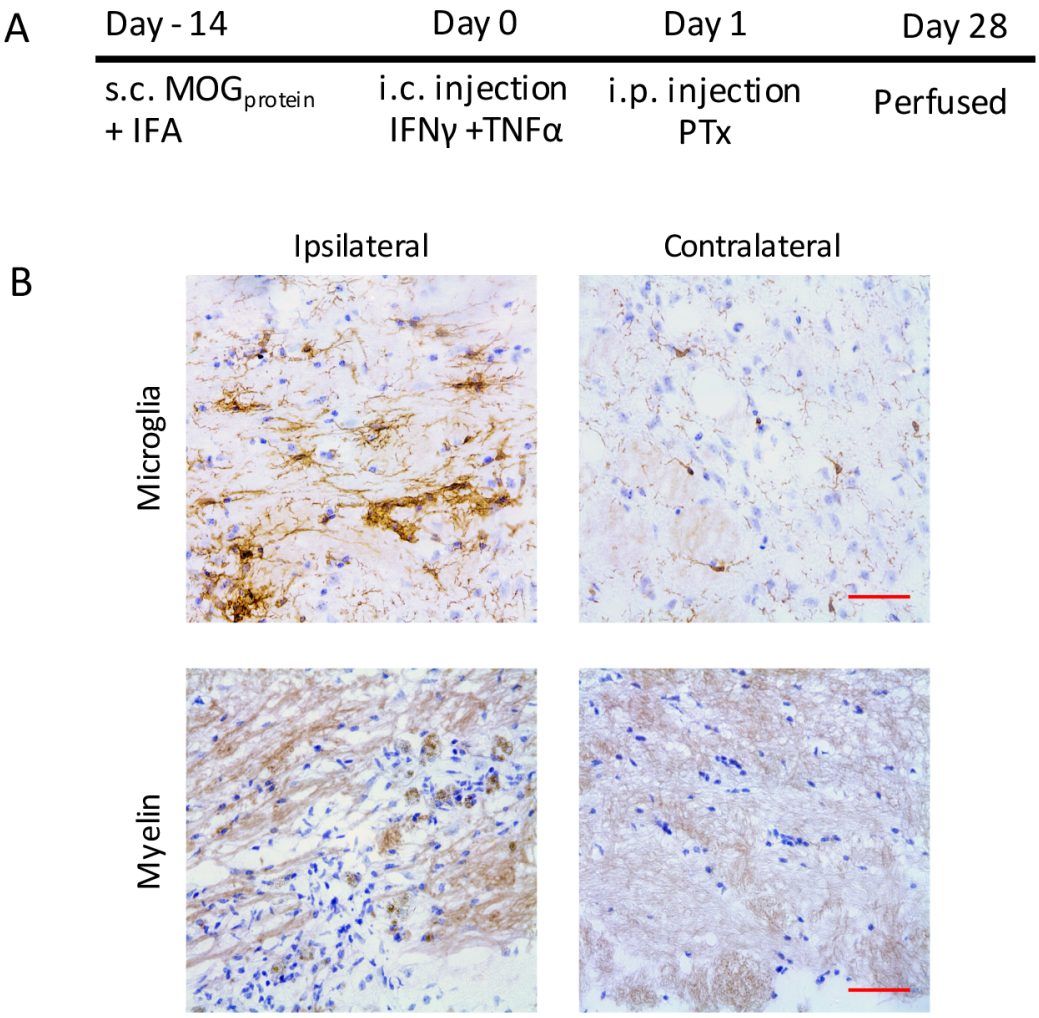


Figure 3.2 The adapted focal EAE model does not induce MS-like lesions in mice. Focal MOG lesions were established in BALB/c mice. (A) Mice were immunized with MOG protein and IFA two weeks before they received an intracranial cytokine injection. Pertussis toxin was given on day 1 to promote lesion formation. Mice were perfused on day 28. (B) Sections were stained for microglia with anti-Iba-1 and myelin with anti-MBP. (B) Representative photomicrographs of microglia and myelin in the ipsilateral and contralateral hemisphere of the brain. Iba-1 staining revealed small nodules of activated microglia. Focal demyelination can be observed in the ipsilateral hemisphere of injected mice. Red scale bars represent 50μm. MOG, myelin oligodendrocyte glycoprotein; IFA, incomplete Freund's adjuvant; PTx, pertussis toxin.

3.3.1.3 Intracranial TB generates MS-like lesion in MOG-immunised mice.

As existing models do not recapitulate progressive MS-like lesions in mice, a new model was developed. A focal DTH-MOG model was established by injecting heat-killed TB intracranially in MOG immunised mice. For the immunisation, CFA was used to emulsify the MOG, instead of IFA, and the injection was given intradermally, rather than subcutaneously, to illicit a response that was hoped to promote more of a B cell response (Figure 3.3A). After allowing the lesion to develop for 40 days, inflammatory demyelinating lesions were found in the brain parenchyma. Immunohistochemistry revealed the presence of large, evolving (expanding) lesions with activated microglia and astrocytes. A granulomatous core similar to the lesions in rats with the DTH model was occasionally observed (Figure 3.3B&C). Lymphocytic infiltrates were present as cuffs around blood vessels, and both B and T cells were found to be present in the brain parenchyma (Figure 3.3D&E). Finally, the mice did not display any clinical features.

3.3.2 Treatment with anti-Lingo1

The lesions induced using the DTH-MOG model are reproducible and have all of the hallmarks of a progressive MS lesion. With this new model in mice I sought to investigate the potential of a remyelinating strategy to promote repair. 40 days after lesion induction, the new DTH-MOG lesions were treated with anti-LINGO-1 antibodies (10mg/kg) with dexamethasone (0.1mg/kg), or with dexamethasone alone. Anti-LINGO-1 has been shown to be effective in models of toxic demyelination in the absence of an immunological assault. Although these models are useful for investigating proof-of-principle remyelinating strategies, they cannot be used to evaluate whether such therapies would work in the more typical environment of an immune-mediated MS lesion.

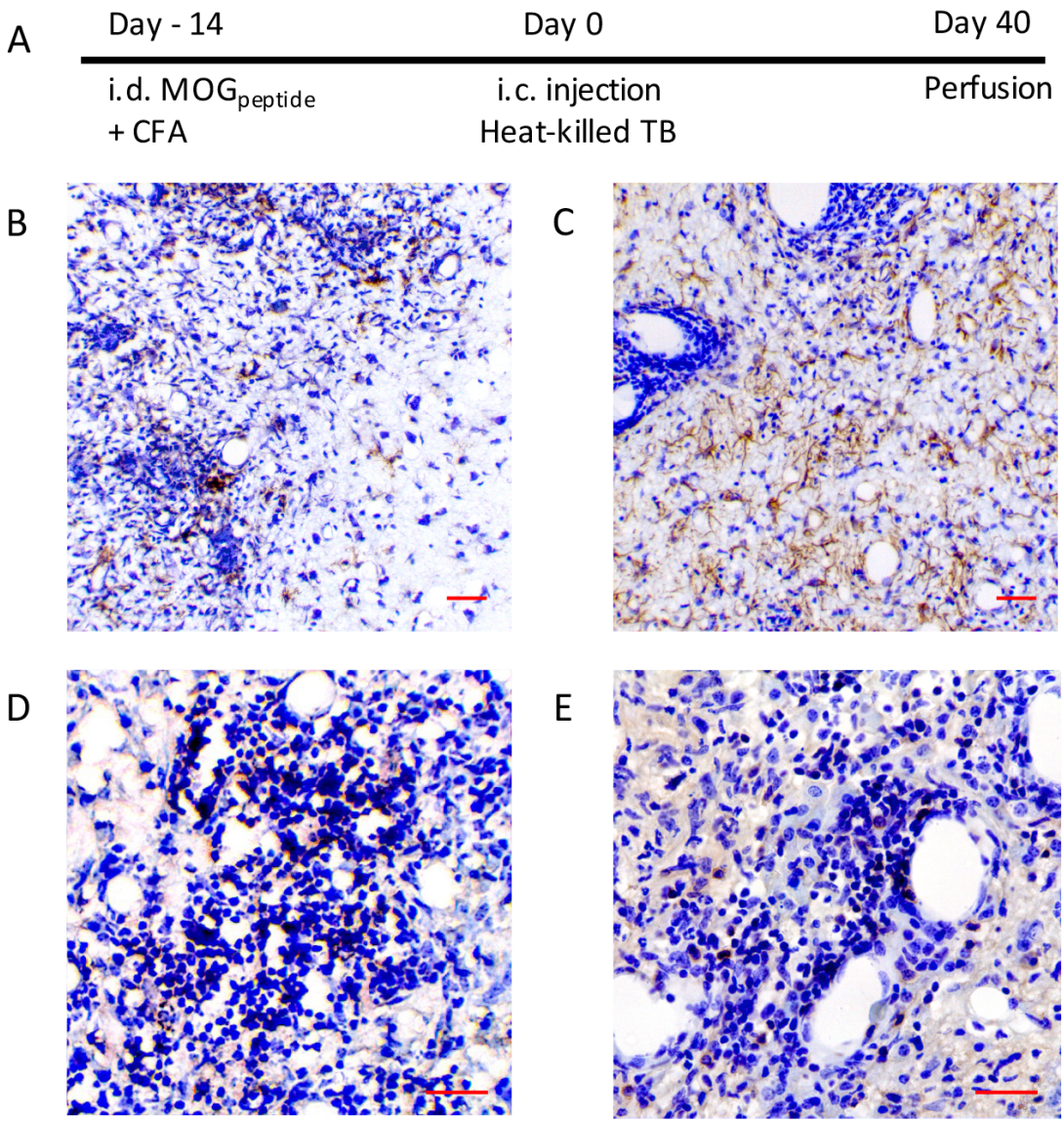


Figure 3.3 A new focal model for MS. (A) Focal MS-like lesions were established in BALB/c mice by immunising the mice with an intradermal MOG peptide and CFA emulsion on day -14, followed by an intrastriatal injection with heath-killed TB on day 0. Lesions were allowed to develop for 40 days. Focal lesions can be characterised by Iba-1$^+$ activated microglia (B), GFAP$^+$ reactive astrocytes (C), infiltrated CD20$^+$B cells (D), and infiltrated CD3$^+$ T cells (E). Red scale bars represent 50μm. MOG, myelin oligodendrocyte glycoprotein; CFA, complete Freund's adjuvant; TB, mycobacterium tuberculosis.

3.3.2.1 Anti-Lingo-1 increased mRNA expression levels of myelin proteins in the DTH-MOG lesion.

LINGO-1 is known to be a negative regulator of myelination and oligodendrocyte differentiation. Here the anti-LINGO-1 therapy increased myelin protein mRNA expression. Expression levels of myelin basic protein (MBP) and proteolipid protein (PLP) transcripts were calculated as relative-fold change in expression from the contralateral hemisphere of control treated animals. Two-way ANOVA revealed a significant difference in MBP mRNA expression between the treatment groups ($F\ 1, 18=10.13$, $p=0.0052$) (1.47 ± 0.170 fold change anti-LINGO-1 vs 1.04 ± 0.053 fold change control). No difference in MBP expression was observed between the ipsilateral and contralateral brain hemispheres in both treatment groups (Figure 3.4A). Anti-LINGO-1 treatment had no significant effect on PLP expression (1.23 ± 0.136 fold change anti-LINGO-1 vs 1.11 ± 0.097 fold change control; $p=0.15$). However, using two-way ANOVA, PLP expression was upregulated in the ipsilateral hemisphere compared to the contralateral hemisphere. Multiple comparisons with a Sidak correction showed that PLP was significantly upregulated in the ipsilateral hemisphere compared to the contralateral hemisphere in anti-LINGO-1 treated animals, and not in controls, suggesting an interaction of the antibody with the lesion (anti-LINGO-1 1.361 ipsilateral vs 1.090 contralateral; control 1.206 ipsilateral vs .012 contralateral) (Figure 3.4A). Immunostaining of MBP showed demyelination in the ipsilateral corpus callosum of animals treated with anti-Lingo-1 and treated with vehicle (Figure 3.4C).

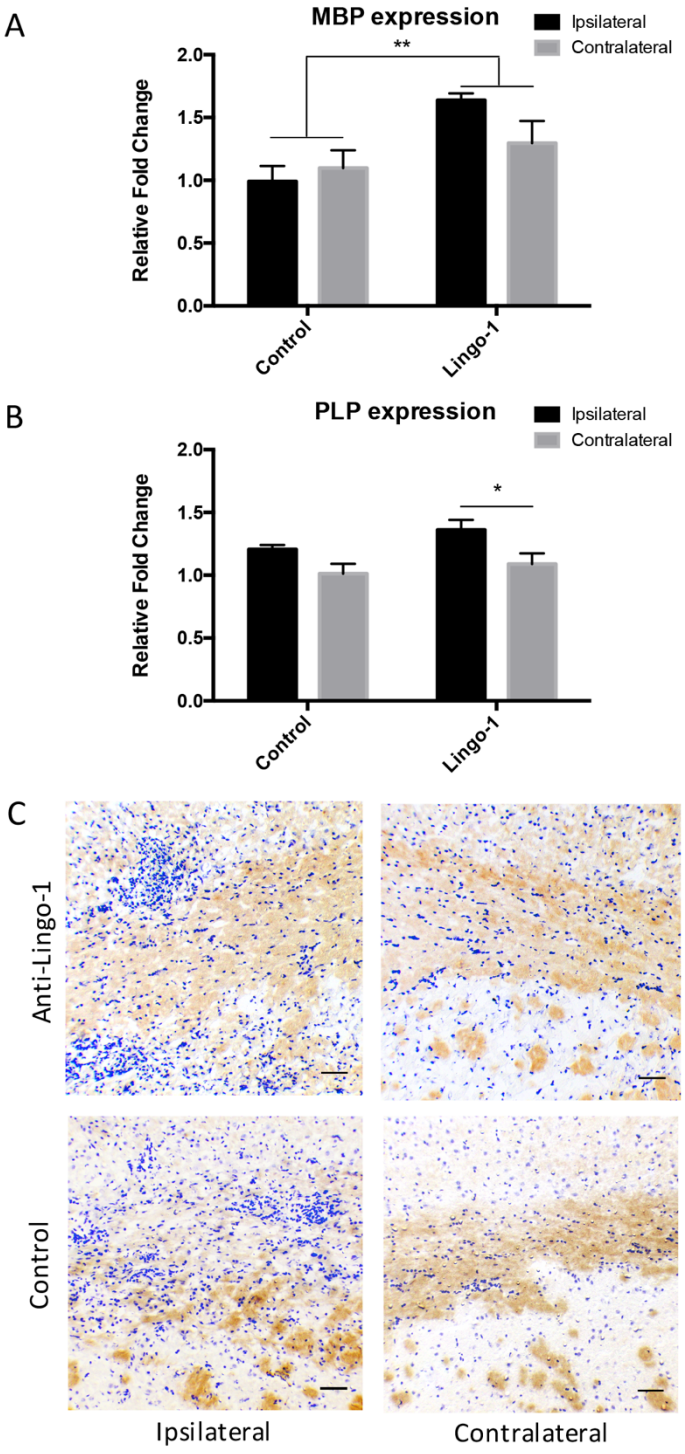


Figure 3.4 Anti-Lingo-1 treatment increases myelin expression in the MOG-DTH model. Focal MS-like lesions were induced in CD1 mice. At day 40, they were treated with either anti-LINGO-1 or a vehicle control. MBP mRNA was measured by RT-qPCR in the ipsilateral and contralateral hemispere. (A) anti-LINGO-1 treatment increased MBP mRNA expression. (B) PLP mRNA expression is increased in the ipsilateral hemisphere in the anti-LINGO-1 groups. (C) Representative photomicrographs of myelin, stained with anti-MBP, in the ipsilaeral and contralateral corpus callosum in anti-LINGO-1 treated animals and controls. Data represent mean ± SEM; **p<0.01. Statistics: two-way ANOVA with Sidak's multiple comparison test, vehicle n=6, lingo-1 n=6. Scale bars represent 50μm. MBP, myelin basic protein; PLP, proteolipid protein.

3.3.2.2 Anti-LINGO-1 increases the number of oligodendrocytes and their precursor cells.

To assess the effect of anti-LINGO-1 on oligodendrocyte proliferation, the number of OPCs and mature oligodendrocytes was obtained from mice with a MS-like lesion treated with anti-LINGO-1 or control (Figure 3.5). OPCs were labelled with anti-Neural/glial antigen 2 (NG2). The number of OPCs was determined by counting anti-NG2 labelled cells with an OPC morphology in the corpus callosum (Figure 3.5C). More OPCs were present in the corpus callosum of the anti-LINGO-1 treated mice. Anti-LINGO-1 significantly increased the number of OPCs to 61.94±2.32/mm^2 compared to 46.07±1.399/mm^2 in the control treatment group ($p=0.0002$) (Figure 3.5A). The number of mature oligodendrocytes was assessed in a similar way – brains were immunolabelled for this cell type with an APC-CC1-antibody (Bin, Harris, and Kennedy 2016). The number of mature oligodendrocytes was doubled in the corpus callosum of the anti-LINGO-1-treated mice compared to vehicle-treated mice (Figure 3.5B&D). The number increased from 30.71±2.285/mm^2 in the vehicle-treated mice to 65.04±4.2464/mm^2 in the anti-LINGO-1-treated mice. An unpaired t-test revealed that the number of premature oligodendrocytes was significantly higher ($p<0.0001$) in the corpus callosum of the anti-LINGO-1-treated mice (Figure 3.5B). This data suggests that anti-LINGO-1 as adjunct therapy significantly promotes remyelinating better than Dex control therapy alone.

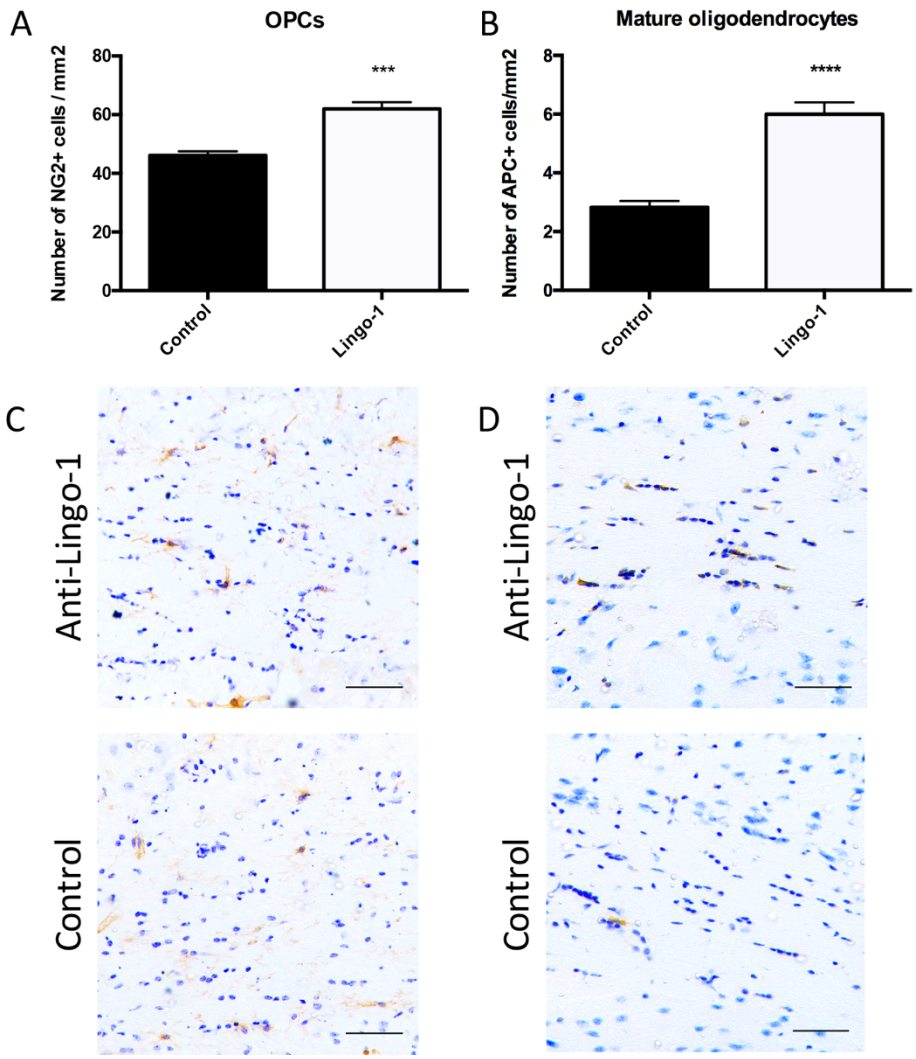


Figure 3.5 Increased proliferation of oligodendrocyte precursor cells. Focal MS-like lesions were induced in CD1 mice. At day 40, they were treated with Dex and either anti-LINGO-1 or a vehicle control. Brain sections were immunostained for oligodendrocyte precursor cells and immature oligodendrocytes, and their numbers were counted in the corpus callosum. Anti-LINGO-1 treatment increased the number of OPCs (A) and mature oligodendrocytes stained with anti-APC-CC1 (B). Representative photomicrographs of striatal OPCs stained with anti-NG2 (C) and corpus callosum mature oligodendrocytes (D) of anti-LINGO-1 treated animals and controls. Data represent mean ± SEM; ***p<0.001, ****p<0.0001. Statistics: unpaired t test, vehicle n=6, lingo-1 n=6. Scale bars represents 50μm. OPC, oligodendrocyte precursor cell; NG2, neural/glial antigen 2.

3.3.2.3 Anti-LINGO-1 treatment reduces neuroinflammation in the DTH-MOG model.

After observing that anti-LINGO-1 treatment had an impact on myelination, we sought to discover what other effects the treatment had on the lesions. To test the effect of the treatment on neuroinflammation and glial activation, cryosections were immunolabelled for astrocytes and microglia. Astrocytes were labelled with anti-GFAP and microglia with anti-CD68. Because these mice were not perfused-fixed, our conventional marker Iba-1, did not work in this tissue. Camera lucida drawings of the area of immunolabelled cells with an activated morphology were generated in a blinded fashion. Analysis showed that the area occupied by activated astrocytes was decreased after treatment (anti-LINGO-1 1.688±0.09226mm^2 vs control 3.359±0.44 mm^2, $p<0.01$) (Figure 3.6A&C). The area occupied by activated microglia followed a similar pattern (anti-LINGO-1 1.440±0.2705 vs control 2.327±0.2806, $p<0.05$) (Figure 3.6B&D). This data shows that the anti-LINGO-1 adjunct therapy had both remyelinating and anti-inflammatory effects compared to the steroid treatment (Dex) alone, and that the DTH-MOG lesions re susceptible to therapy.

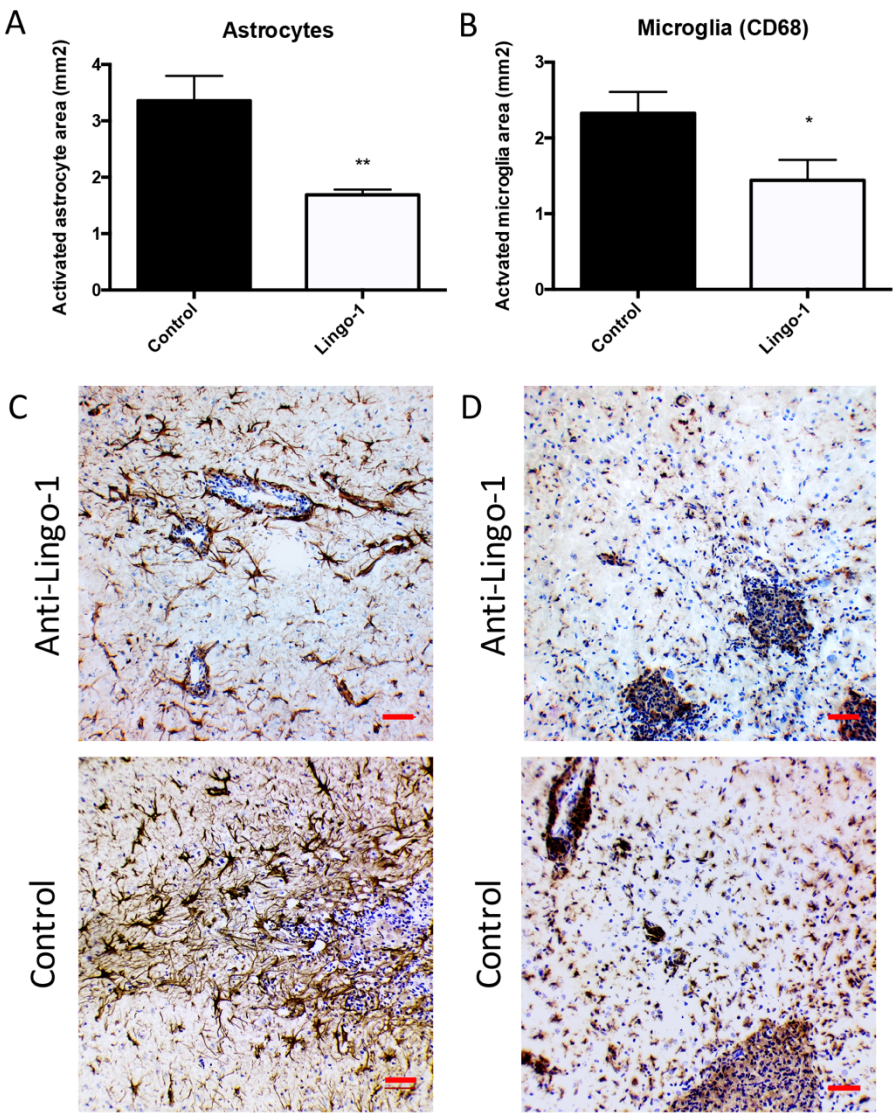


Figure 3.6 Anti-LINGO-1 treatment reduces astrocyte and microglial activation in the DTH-MOG model. Focal MS-like lesions were induced in CD1 mice. At day 40, they were treated with Dex and either anti-LINGO-1 or a vehicle control. Immunohistochemistry showed a reduction in the area of astrocyte activation (A) and of microglial activation (B) after anti-Lingo-1 treatment. (C) Photomicrographs show activated astrocytes stained with GFAP in the brain lesions of control animals and treated animals. (D) Representative photomicrographs show microglial activation stained with CD68 in control animals and treated animals. Data represent mean ± SEM; *$p<0.05$,**$p<0.01$. Statistics: unpaired t test, vehicle n=6, lingo-1 n=6. Scale bars represents 50µm.; GFAP, glial fibrillary acidic protein; CD68, cluster of differentiation 68.

3.3.3 Treatment with anti-CD20

A previous study showed a therapeutic effect of anti-CD20 in a T-cell mediated focal model of MS in rats, but in conventional EAE, results with anti-CD20 therapy have been mixed. However, in EAE, anti-CD20 has been studied in the early induction process. With the new DTH-MOG model, we sought to explore the efficacy of anti-CD20 therapy in a mouse model with a treatment regime in the chronic phase of disease.

3.3.3.1 Different Fc variants of GA101 deplete peripheral B cells equally.

To assess the efficacy of the different Fc variants of the anti-CD20 antibody, GA101, and to explore how different modes of action of GA101 might alter the histological profile of the lesions, animals were treated with either GA101, Aglyco, PGLALA, or a vehicle control. Immunohistochemistry for B cells was performed on spleens and lymph nodes. The area around the B cell follicles was determined using a camera lucida and a ratio was calculated between the B cell proportion in the follicle and the non-B cell proportion. GA101, Aglyco and PGLALA all reduced the proportion of B cells in the spleen significantly compared to control animals (GA101 0.224±0.201, Aglyco 0.504±0.105, PGLALAs 0.519±0.231 vs control 2.276±0.972; $p<0.0001$) (Figure 3.7A). In the cervical lymph nodes, we saw a similar pattern. Compared to vehicle treated animals, the B cell proportion was significantly reduced after Aglyco treatment (0.173±0.052, $p<0.001$) and further reduced by PGLALA or GA101 treatment (0.130±0.050 and 0.0429±0.020 respectively, $p<0.0001$) (Figure 3.7B). GA101 depleted B cells significantly better than Aglyco in cervical lymph nodes ($p<0.05$) (Figure 3.7B).

To understand what systemic changes might accompany the generation of the MS-like lesions, the hepatic response was also examined in this model, and what affect anti-CD20 therapy would have on the number of neutrophils and Kupffer cells in the liver. The level of neutrophil recruitment to the liver has been used as a surrogate marker for the acute phase response. Interestingly, all anti-CD20 Fc variants reduced the number of neutrophils in the liver. GA101 reduced the neutrophil number most ($28.64\pm14.50/mm^2$, $p<0.0001$), followed by Aglyco ($30.65\pm17.88/mm^2$, $p<0.001$), and PGLALA ($44.43\pm13.51/mm^2$, $p<0.05$), compared to control (69.12 ± 13.69) (Figure 3.7C). Anti-CD20 treatment did not affect the number of Kupffer cells present in the liver (GA101: $12.7\pm38.78/mm^2$, Aglyco:$122.3\pm29.39/mm^2$, PGLALA: $147.6\pm31.19/mm^2$ vs control: $102.9\pm36.19/mm^2$) (Figure 3.7D). No differences in glial activation, or lymphocyte infiltration were observed between the different Fc variants (data not shown).

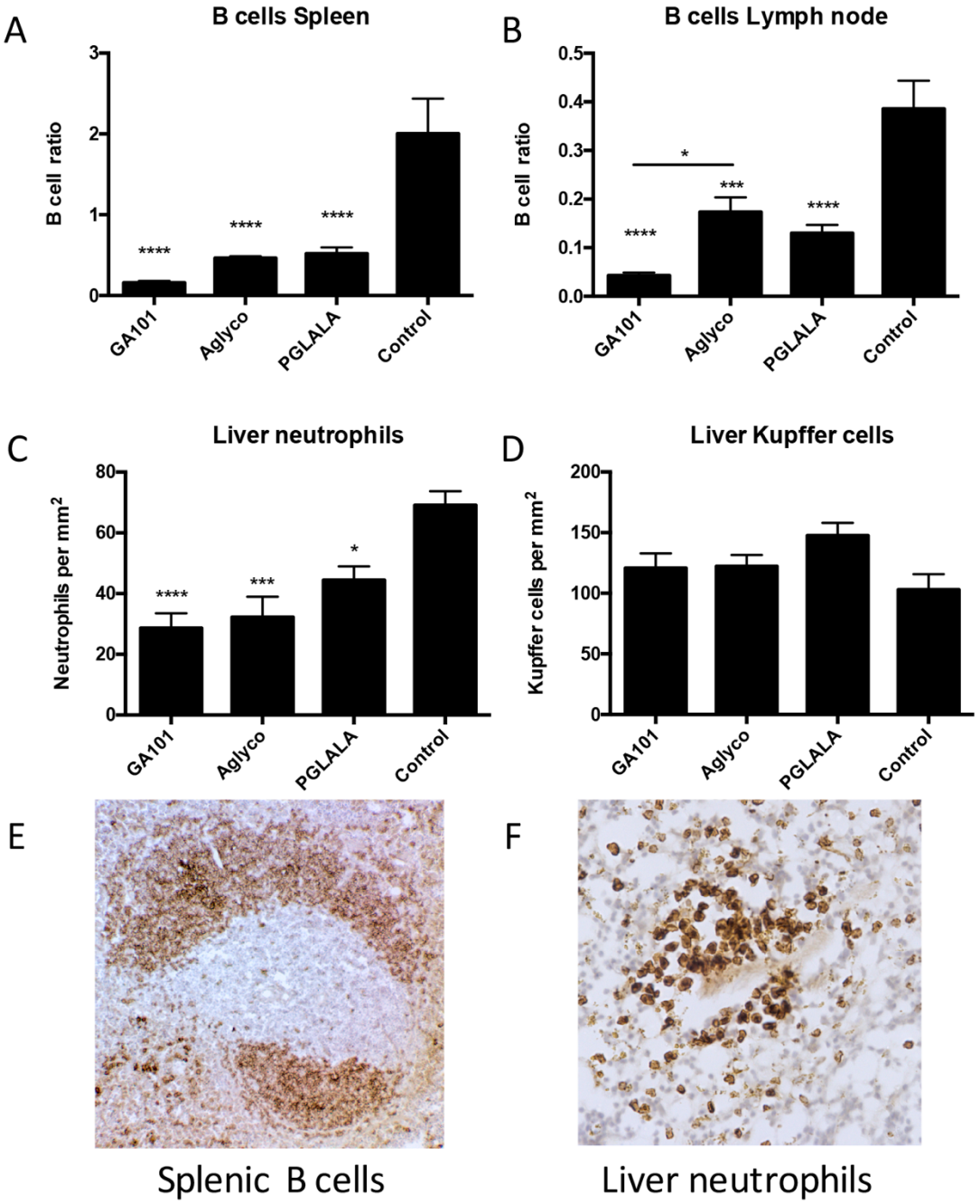


Figure 3.7 Peripheral effects of the different Fc variants of GA101. Focal MS-like lesions were induced in huCD20 BALB/c mice. At day 40, they were treated with either GA101, Aglyco, PGLALA, or a vehicle control. Immunohistochemistry showed the effect of the different Fc variants on peripheral immune compartments. All three Fc variants reduced the ratio of B cells in follicles in the spleen (A) and lymph nodes (B). In the lymph nodes, GA101 depleted B cells more effectively than Aglyco (B). In the liver, all Fc variants reduced the number of neutrophils(C). Treatment had no effect on the number of liver Kupffer cells (D). (E) Representative photomicrograph of splenic B cells in a control treated animal. (F) Representative photomicrograph of liver neutrophils of a control treated animal. Data represent mean ± SEM; *p<0.05,***p<0.001, ****p<0.0001. Statistics: one-way ANOVA with Tukey's multiple comparison test, n= 9 mice per treatment group.

3.3.3.2 PGLALA reduced microglial and astrocyte activation.

To test the effectiveness of the direct cell-killing properties of anti-CD20 in the DTH-MOG model, mice were treated with the PGLALA variant of GA101 (10 mg/kg), or a vehicle control, 40 days after lesion initiation. Immunohistochemistry was performed on perfused fixed brains or microglia and astrocytes. Astrocytes were labelled with GFAP, and microglia with Iba-1. The area of activated glial cells was determined as described before (1.3.2.3). Analysis showed a significant reduction in the area occupied by activated microglia after PGLALA treatment ($0.447\pm0.158mm^2$) compared to control ($1.542\pm0.375mm^2$, $p=0.024$) (Figure 3.8A&C). The area of astrocyte activation followed the pattern of the microglia. Although the area tended to be more spread out, PGLALA treatment still reduced the area of activated astrocytes (Figure 3.8B). The area of activated astrocytes in controls ($2.385\pm0.518mm^2$) was significantly higher than that in PGLALA treated animals ($0.7422\pm0.234mm^2$, $p=0.017$) (Figure 3.8B&D).

3.3.3.3 PGLALA significantly reduces B and T cells in the brain.

The new mouse DTH-MOG model features infiltration of both B and T cells to the brain lesions, and we have shown anti-CD20 to be effective at depleting peripheral B cells. However, it is known that only a small percentage of antibodies is able to cross the BBB. We were thus curious to see the effect of anti-CD20 therapy on brain lymphocyte populations. 10 μm brain sections were stained for B cells (anti-B220) and T cells (CD3) and their total numbers were counted in the ipsilateral hemisphere. Treatment with PGLALA reduced the number of brain B cells compared to control (PGLALA: $7.125\pm2.00/mm^2$ vs control: $58.33\pm14.04/mm^2$, $p=0.012$) (Figure 3.9A&C). Although anti-CD20 targets B cells, PGLALA treated animals had a lower number of brain T cells compared to vehicle treated animals (PGLALA: $18.50\pm4.264/mm^2$ vs control: $61.31\pm15.11/mm^2$, $p=0.016$) (Figure 3.9B&D).

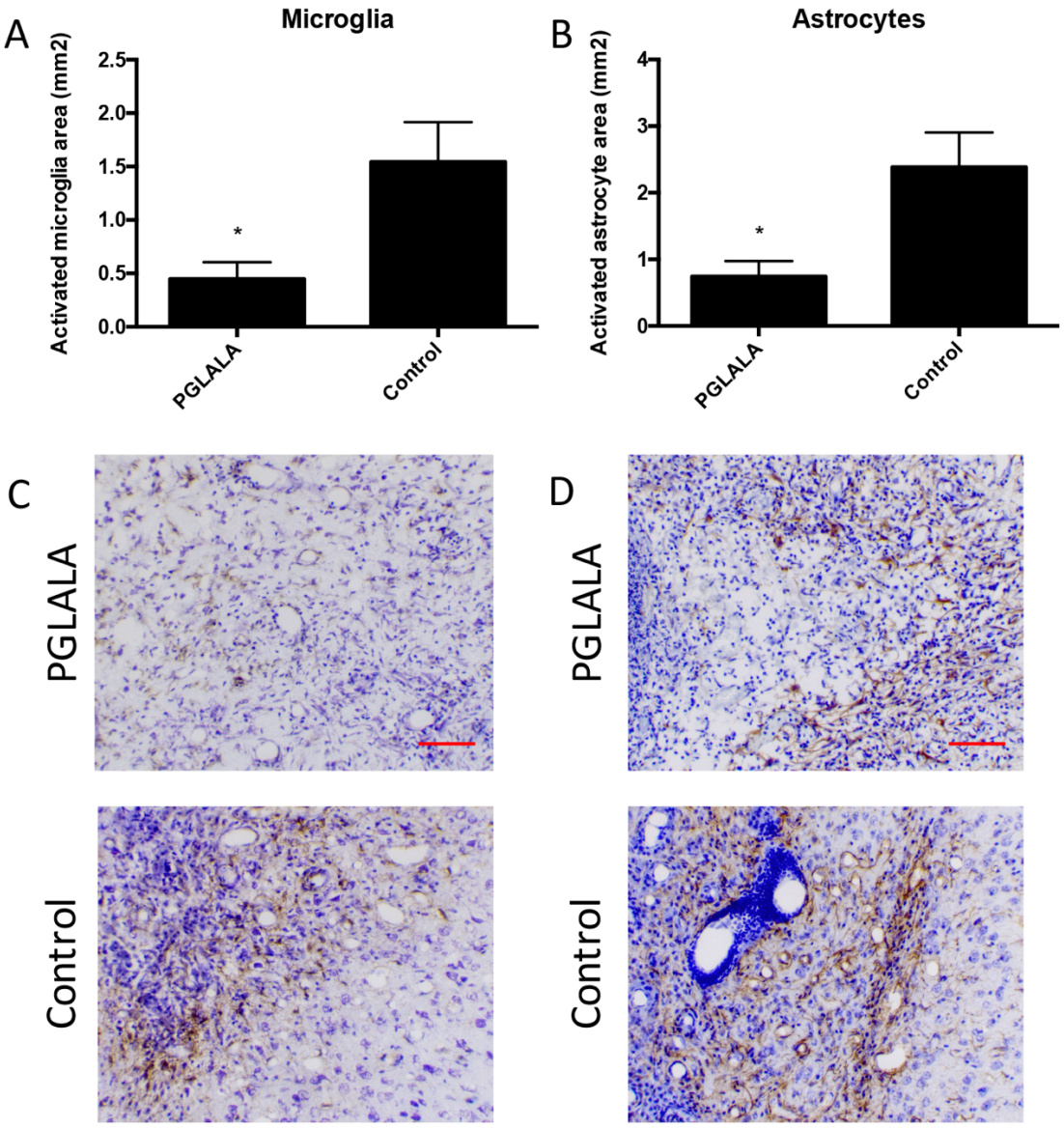


Figure 3.8 Anti-CD20 reduced microglial and astrocyte activation. Focal MS-like lesions were established in MOG-immunised huCD20 BALB/c mice by immunising the mice with MOG peptide and CFA on day -14, followed by an intrastriatal injection with heath-killed TB on day 0. Treatment with either PGLALA or a vehicle control started after 40 days. PGLALA treatment reduced microglial activation (A) and astrocyte activation (B) in focal lesions. (C) Representative photomicrographs of microglia in the lesions of treated (i) and control animals (ii). Representative photomicrographs of activated astrocytes in the lesions of treated (i) and control animals (ii). Note the cuffed hypercellular blood vessels (Di). Red scale bars represent 100µm. Data are mean +/-SEM; *$p<0.05$. Statistics: unpaired t test, n=8 mice. MOG myelin oligodendrocyte glycoprotein, CFA complete Freund's adjuvant, TB tuberculosis.

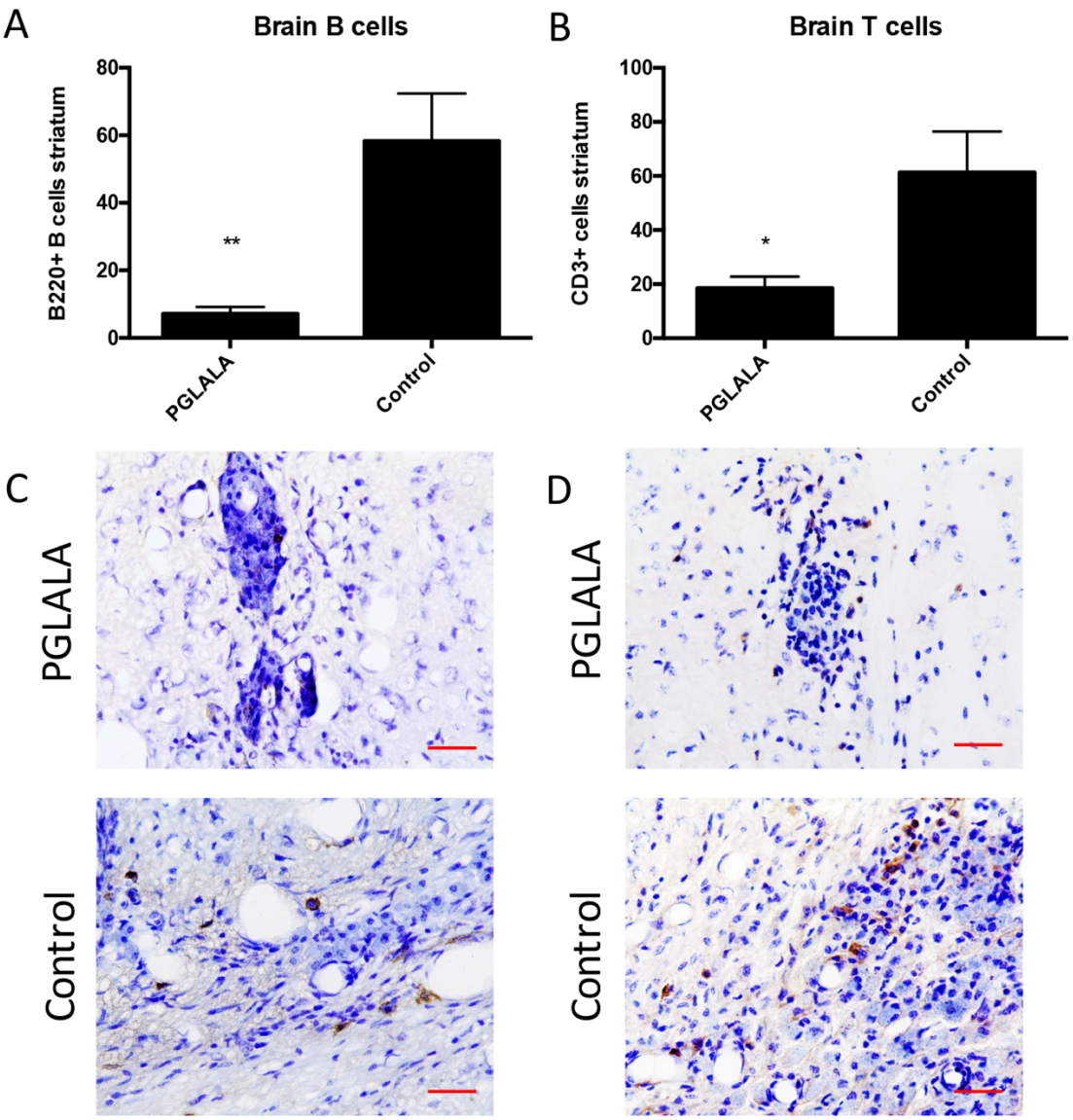


Figure 3.9 PGLALA significantly reduces brain lymphocytes. Focal MS-like lesions were established in MOG-immunised huCD20 BALB/c mice by immunising the mice with MOG peptide and CFA on day -14, followed by an intrastriatal injection with heat-killed TB on day 0. Treatment with either PGLALA or a vehicle control started after 40 days. PGLALA treatment reduced the number of B cells (A) and T cells (B) in focal lesions. (C) Representative photomicrographs of B cells in the lesions of treated (i) and control animals (ii). Representative photomicrographs of T cells in the lesions of treated (i) and control animals (ii). Red scale bars represent 50μm. Data are mean +/-SEM; *$p<0.05$. Statistics: unpaired t test, n=8 mice. MOG myelin oligodendrocyte glycoprotein, CFA complete Freund's adjuvant, TB tuberculosis.

3.3.3.4 PGLALA significantly depletes splenic B cells and liver neutrophils.

To examine peripheral B cell depletion by PGLALA, the proportion of B cells was analysed in the spleen. Peripherally, PGLALA decreased the B cell number in the spleen (Figure 3.10A&B). The proportion of B cells in the splenic follicles decreased to 0.383±0.137 after PGLALA treatment, compared to 2.365±0.334 in control animals ($p<0.0001$) (Figure 3.10A). Liver neutrophil numbers were counted as a surrogate marker for the acute phase response (APR). Interestingly, the number of neutrophils was also decreased in the liver (Figure 3.10C&D). As mentioned above, liver neutrophils can be used as a surrogate marker for magnitude of the hepatic acute phase response to disease. The neutrophil number was increased in this model, compared to controls (data not shown). PGLALA therapy reduced the number of infiltrated neutrophils significantly compared to control treated animals (PGLALA: 42.68±1.996/mm^2 vs control: 84.46±6.688/mm^2, $p<0.0001$) (Figure 3.10C). The decrease was liver specific, as the neutrophil number was the same for PGLALA and control treated animals in the spleen (PGLALA: 1717±199.8/mm^2 vs control: 1960±139.3/mm^2, $p=0.336$) (Figure 3.10E&F).

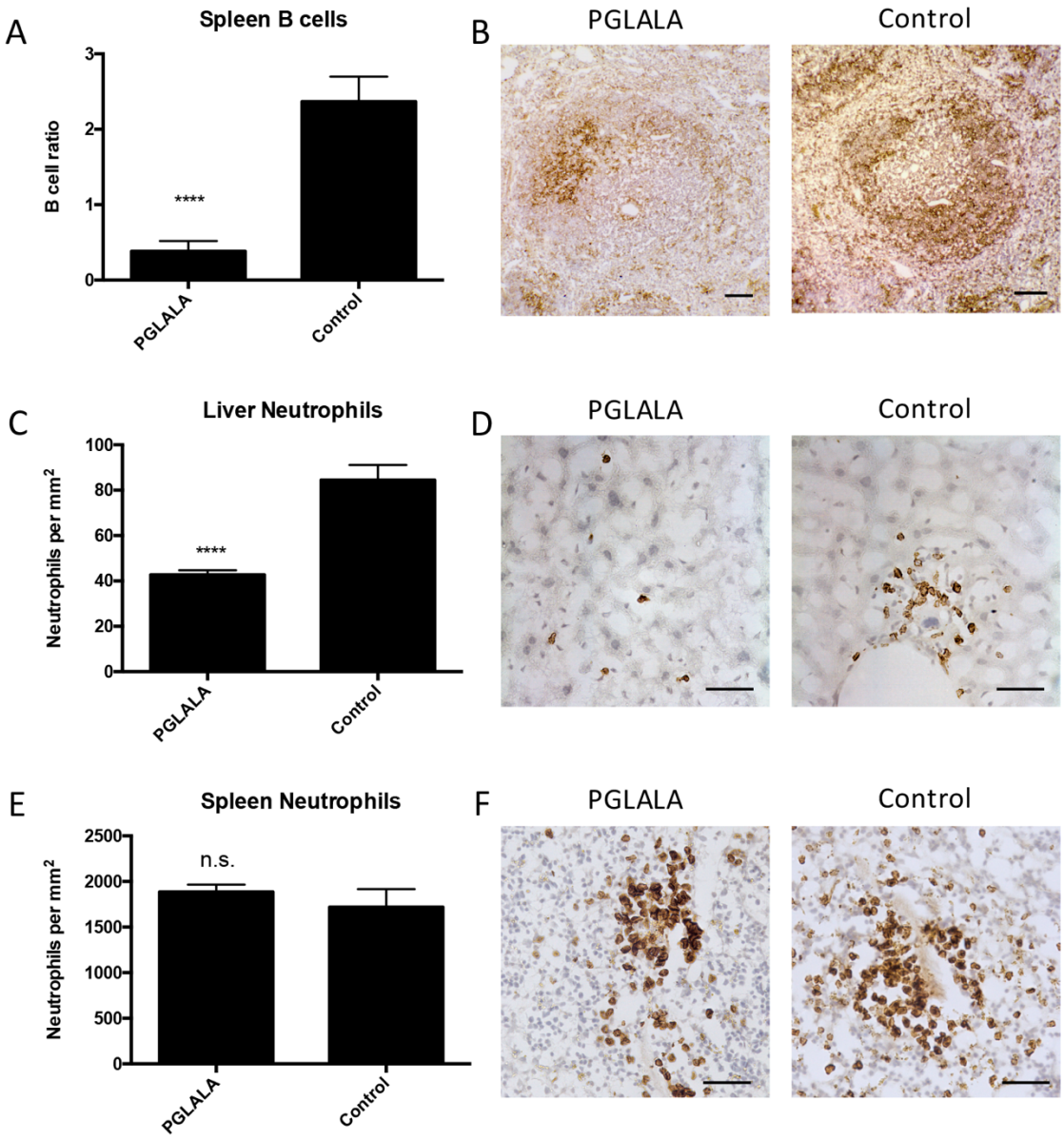


Figure 3.10 PGLALA depletes splenic B cells and liver neutrophils. Focal MS-like lesions were established in MOG-immunised huCD20 BALB/c mice by immunising the mice with MOG peptide and CFA on day -14, followed by an intrastriatal injection with heat-killed TB on day 0. Treatment with either PGLALA or a vehicle control started after 40 days. Immunohistochemistry was performed for B cells and neutrophils in spleens and livers. (A) PGLALA depletes splenic B cells. (B) Representative photomicrographs of B cells present in splenic follicles of treated and control mice. (C&E) PGLALA treatment reduces the number of neutrophils in the liver, but not in the spleen. (D&F) Representative photomicrographs of neutrophils in the liver and spleen respectively, of both treated mice and controls. Black scale bars represent 50μm. Data are mean +/-SEM; ****$p<0.0001$. Statistics: unpaired t test, n=8 mice. MOG myelin oligodendrocyte glycoprotein, CFA complete Freund's adjuvant, TB tuberculosis.

3.4 Discussion

Chronic progressive MS is a multifaceted inflammatory disease, and the capacity to target this inflammation within the CNS is likely to be key to future therapeutics. The majority of pre-clinical MS work in mice uses traditional relapse-remitting models of MS, such as EAE. Here, a novel focal model of MS has been developed in mice, and was used to study the potential of anti-LINGO-1 and anti-CD20 on MS-like lesions the brain behind an intact BBB. By using an intracranial injection of TB, on a background of MOG-CFA immunization, we produced a model with lesions that are characteristic of progressive MS; widespread microglial activation, activated astrocytes, infiltrating lymphocytes, demyelination, a granulomatous core, with transient BBB breakdown (Lassmann, Brück, and Lucchinetti 2007). The new model does not display the debilitating clinical paralysis that is normally observed in classical EAE, or resolution of the focal lesion within a couple of weeks as with other focal models (Kerschensteiner et al. 2004, Merkler et al. 2006, Hampton et al. 2008, Gardner et al. 2013). Although the model does require some surgical skills to set it up, once these skills are mastered, the model is reproducible, and allows for chronic studies with histological endpoints, which has not been possible with existing models in mice.

As demyelination and oligodendrocyte loss are key hallmarks of MS pathology, we sought to discover whether we could augment remyelination and promote oligodendrocyte proliferation in the new DTH-MOG model. Other studies have shown before that inhibiting LINGO-1 has a positive effect on myelination, and oligodendrocyte differentiation (Mi et al. 2007, Sun et al. 2015). Here we show that anti-LINGO-1, administered as an adjunct therapy, has a positive effect on both oligodendrocyte proliferation and myelination parameters in our model. Remyelination requires recruitment, proliferation, and differentiation of OPCs into myelinating oligodendrocytes (Plemel, Liu, and Yong 2017). Here, we showed an increase in the number of OPCs and immature oligodendrocytes after blocking LINGO-1, which is in line with previous

studies (Shao et al. 2017). Moreover, although mature, MBP^+ oligodendrocytes are hard to distinguish in brain sections, and have not been quantified in this study, we do show an increase of MBP mRNA expression after anti-LINGO-1 treatment compared to control treated animals. This can translate to either increased MBP expression by existing oligodendrocytes, or an increased number of mature oligodendrocytes.

LINGO-1 has been conserved throughout evolution and is expressed exclusively in the CNS (Mi et al. 2004). However, only 0.1% of circulating antibodies have been calculated to cross the BBB following peripheral intravenous administration, owing to the presence of the BBB, and the absence of any transport system (Freskgård and Urich 2016). Yet, multiple studies have shown that the small amount of BBB breakdown in EAE is enough to deliver efficacious amounts of anti-LINGO-1 into the CNS (Sun et al. 2015). Given the size and nature of the brain lesions seen in this cohort of animals, low level BBB breakdown was possible in these mice. However, it is difficult to ascertain what fraction of IgG would need to be transported in order to have a therapeutic effect. To increase drug delivery into the CNS, intrathecal administration can be considered.

Anti-LINGO-1 treatment also further reduced the extent of neuroinflammation in this model compared to Dex control treated animals, as shown by a reduction in the area of activated astrocytes and microglia. In previous studies, blocking LINGO-1 led to remyelination and amelioration of clinical symptoms in EAE, without affecting lymphocyte infiltration into the spinal cord (Mi et al. 2007, Mi et al. 2009). In MS patients, anti-LINGO-1 did not alter gene expression of inflammatory molecules in the blood or CSF, nor did it show any immunomodulatory effects on isolated T cells in vitro (Ranger et al. 2018). LINGO-1 inhibits myelination by forming a complex with NgR1 and p75 or TROY as coreceptor (Mi et al. 2004, Park et al. 2005, Shao et al. 2005). LINGO-1, NgR1, and TROY have all been shown to also be expressed by astrocytes and microglia (Satoh et al. 2007, Ukai et al. 2016). But the role of both

proteins in these cell types remains unknown (Figure 3.11). In a small study, LINGO-1 was shown to be downregulated in MS lesions, while TROY was upregulated (Satoh et al. 2007). To investigate whether mouse microglia and astrocytes express LINGO-1, and to assess whether the model or anti-LINGO-1 had an effect on this expression, brain sections could be double labelled for LINGO-1 and either microglia or astrocytes in future experiments.

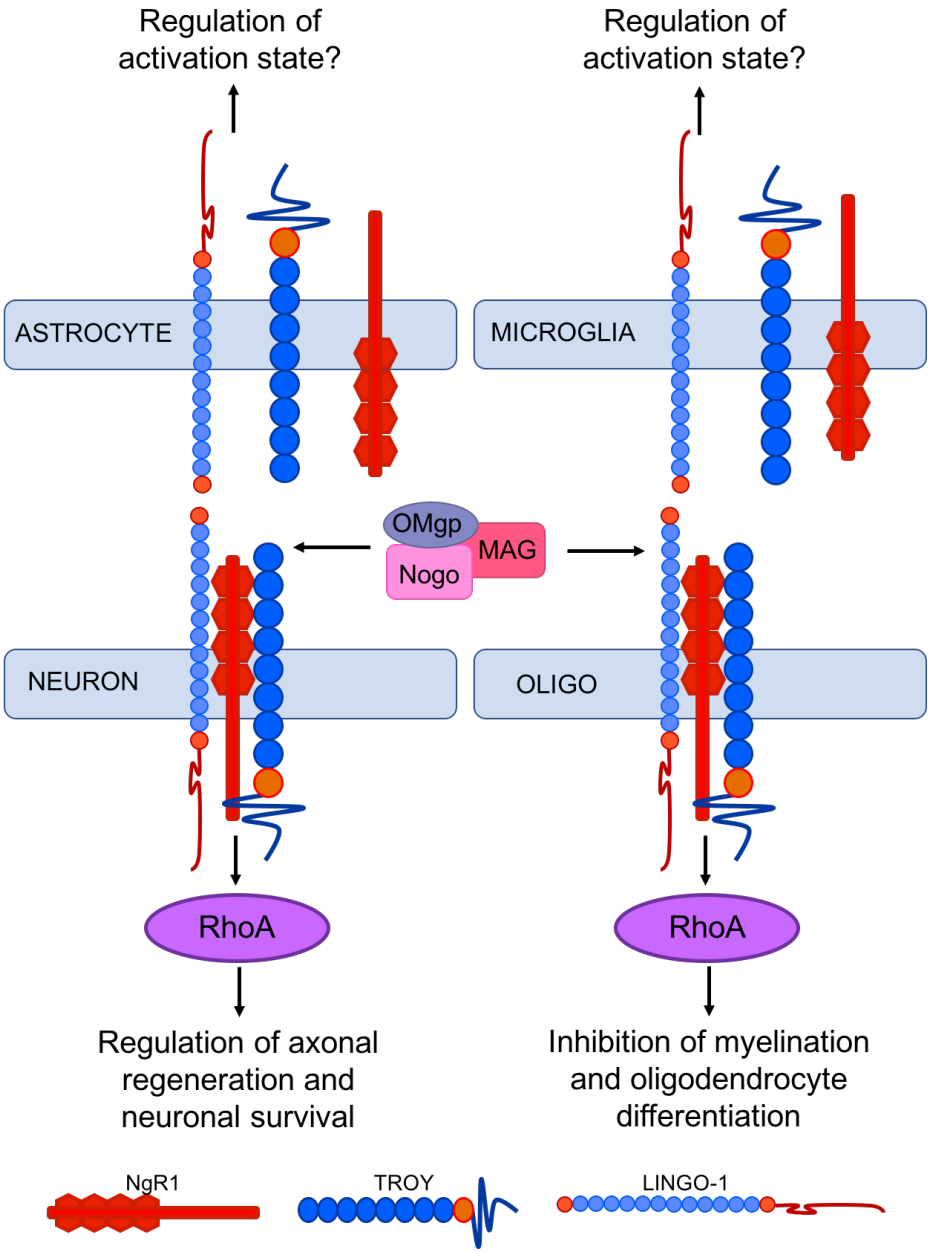


Figure 3.11 A simplified overview of the effects of LINGO-1 on CNS cell types. In Neurons and oligodendrocytes, LINGO-1 forms a complex with NgR1 and p75 or TROY, which activates RhoA, thereby inhibiting myelination and oligodendrocyte differentiation. The three components for the complex are present in microglia and astrocytes, but how they interact with each other is unknown.

The relationship between myelination and glial cells is complex. Clearance of myelin debris by both microglia and astrocytes is essential for remyelination, while at the same time oligodendrocytes can be stressed by proinflammatory cytokines secreted by glial cells (Plemel et al. 2013, Buntinx et al. 2004). In general, a 'contained' inflammatory environment seems to be important for remyelination (Zhao et al. 2005). Here we show that anti-LINGO-1, administered as adjunct therapy, improves MBP expression and proliferation and differentiation of oligodendrocytes, and reduces inflammation, compared to the steroid treatment alone. Whether anti-LINGO-1 therapy affects inflammation directly, or indirectly, via damage prevention, cannot be answered in this work. The positive effect on both aspects makes it a worthwhile target to study further, despite its poor efficacy. Future research should be focused on how to promote drug delivery to the target tissue, the CNS.

The DTH-MOG model was also used to study the B cell depleting anti-CD20 antibody. Anti-CD20 has been proven to be effective in different animal models of MS and in patients (Anthony, Dickens, et al. 2014, Hauser et al. 2016, Anwar Jagessar et al. 2013). However, how anti-CD20 improves MS remains unclear. In mice with EAE, the effect of anti-CD20 has been reported to have mixed effects, possibly due to the arguable involvement of B cells (Sefia et al. 2017, Monson et al. 2011). Here, we studied anti-CD20 therapy in a chronic focal model of MS that features brain B cells. First, we compared three different Fc variants of GA101: GA101, aglycosylated GA101, and PGLALA. We showed that all variants were very effective in the periphery, where all variants depleted splenic B cells equally well. In the lymph nodes, aglycosylated GA101 performed poorer than GA101. These experiments indicate that the direct cell-killing properties of GA101 are sufficient to induce splenic B cell depletion, while in the lymph nodes, ADCC or CDC activity increases B cell depletion even further. One of the advantages of using an antibody with lower or no ADCC/CDC activity is safety. Fc/FcγR or complement interactions can cause adverse effects, including infusion reactions and tissue damage and thus some loss of efficacy might be worthwhile (Schlothauer et al. 2016).

The improved safety profile of PGLALA was tested in a different study, where the treatment was compared with vehicle in the DTH-MOG model in huCD20 mice. Not only did we see B cell depletion in the periphery, but the number of B cells was also significantly reduced in the brain. What is interesting here is that the model features a non-leaky BBB at the time of treatment. We investigated the presence of endogenous IgG in the brains of these mice, which cannot cross the BBB, and no macroscopic signal could be observed. Even though the BBB was not leaking, anti-CD20 therapy was still effective at depleting brain B cells. As CD20 is not expressed by any CNS cells, the little anti-CD20 antibody that did enter the brain may have either been very effective, or the beneficial effect all arose from depletion of the peripheral target. The BBB is a barrier for 'toxins' and other molecules; however, cells can move across in both directions (Daneman and Engelhardt 2017). In fact, B cells residing in CNS compartments have been shown to mature in the cervical lymph nodes (Stern et al. 2014). It is possible trafficking B cells were targeted outside of the CNS. However, one of the arguments to explain the failure of therapy to suppress progressive disease is that antibody therapy cannot target CNS lymphocyte populations, owing to the BBB. Here, in the new DTH-MOG model, this does not seem to be the case.

The fact that B cells appear to play many roles in MS pathogenesis and this model, is evidenced by the anti-inflammatory effect of anti-CD20 therapy. B cells play many different roles, including antigen presentation, and cytokine production. As mentioned above, anti-CD20 therapy reduced the lesion size and reduced the area of activated microglia in this model. Similar results have been described in patients, where a reduction in the number of gadolinium enhanced lesions on MRI, and in animal models for MS is a feature of the therapy (Hauser et al. 2008, Hauser et al. 2016, Anthony, Dickens, et al. 2014, Kap et al. 2010, Weber et al. 2010). Astrocytes become reactive in MS lesions and this is reproduced in the new DTH-MOG model (Brosnan and Raine 2013). In our studies, we found that anti-CD20 therapy also reduced astrocyte reactivity in this model. In EAE, astrocytes have been shown to play a role in

autoimmunity, including disease activation. Moreover, blocking the NFκB pathway in astrocytes has been shown to reduce disease severity and CNS immune cell infiltration in EAE (Wang et al. 2005, Brambilla et al. 2009, Brambilla et al. 2014). How anti-CD20 affects astrocyte reactivity is unknown and no effects on astrocytes in a MS model have been reported previously. However, the decreased reactivity is likely a consequence of the less inflammatory CNS environment.

We also found that there was a reduction in brain T cells after anti-CD20 treatment in this model. This result has been described before, and led to the observation of $CD20^+$ T cells in the blood of individuals with MS and other autoimmune diseases (von Essen et al. 2019, Schuh et al. 2016, Schneider 2015, Anthony, Dickens, et al. 2014, Weber et al. 2010). This small subset of T cells are not the only T cells affected, $CD20^-$ T cells have also been reported to be decreased after anti-CD20 treatment (Monson et al. 2011, Schuh et al. 2016, Weber et al. 2010). B-T cell interactions are crucial in physiological immunity and autoimmunity. (Myelin-)reactive B cells can activate autoreactive T cells and promote their differentiation into a more proinflammatory profile (Weber et al. 2010). Furthermore, B cells secrete a variety of molecules, promoting T cell activation and proliferation. Depleting B cells evidently leads to a reduction in active and reactive T cells.

The suppression of inflammation-associated tissue destruction, by reducing the number of infiltrating lymphocytes, and the level of microglial and astrocyte reactivity (which is thought to be the cause of bystander tissue destruction) is the most likely way in which anti-CD20 therapy reduces damage in the DTH-MOG model, and possibly in MS patients. Another recurring and interesting observation is that anti-CD20 also lowers the number of neutrophils in the liver, but not in the lung or spleen. One of the reported adverse effects of anti-CD20 is that patients can become neutropenic (Shah et al. 2018, Pirich et al. 2018, Marcus et al. 2017). Moreover, people with MS and mice with EAE are known to have an increased number of neutrophils in their

liver, as is the case in mice with the DTH-MOG model (Campbell et al. 2010). Liver neutrophils, in turn, have been used as a surrogate marker for the acute phase response (APR) after acute brain lesions (Anthony et al. 2012). Viral or bacterial infections are associated with relapses in MS, and are also know to switch on the APR. Our lab has shown that EAE can be exacerbated by activating the APR in a model of bacterial infection (sepsis) (Mardiguian et al. 2017).

Anti-CD20 may contribute to the amelioration of MS pathology and MS models by altering the systemic inflammatory response, which accompanies the presence of any active CNS lesion. This could be mediated by the elimination of autoreactive B cells, or by other mechanisms of the antibody itself – possibly related to Fc binding. In chronic illnesses such as MS, or even in mice with DTH-MOG lesions, the ongoing hepatic response is not simply an extended acute response, but seems to change in nature in terms of the APPs that are expressed. Further work, beyond the scope of my thesis, is required to fully elucidate the role of the liver in the coordination of MS pathogenesis.

3.5 Conclusion from this chapter

This chapter describes the development of a new focal mouse model, the DTH-MOG model, that reproduces many of the histological hallmarks of progressive MS, including microglial activation, astrocyte reactivity, lymphocyte infiltration, and demyelination. The model distinguishes itself from others by the recruitment of B cells. Here we show two types of treatment that are able to rescue several aspects of the disease. First, we showed that inhibiting LINGO-1 improves myelination and oligodendrocyte differentiation, but also that it reduces the extent of astrocyte and microglial activation. Next, we studied different Fc variants of type II anti-CD20 antibody GA101 in the new model. We show that the antibody's direct-cell killing abilities are crucial in depleting B cells and suppressing the development of the MS-like lesions. Anti-CD20 therapy resulted in a reduction of microglial and astrocyte activation, and in a reduction in the number of CNS B and T cells. Finally, we showed that anti-CD20 reduces liver neutrophils, which may indicate a role for B cells in the potentiation of the hepatic response to the presence of CNS MS-like lesions.

4. Modelling tertiary lymphoid structures in a mouse model of multiple sclerosis.

4.1 Introduction and aims

In secondary progressive MS, patients can present with meningeal lymphoid-like structures, consisting of B cells, T cells, dendritic cells, and other stromal cells (Serafini et al. 2004, Corsiero et al. 2016). In SPMS, TLS are associated with a more aggressive disease progression, earlier age of disability, and earlier age of death (Magliozzi et al. 2007, Magliozzi et al. 2010, Howell et al. 2011). In MS, TLS-like structures tend to exist in the subarachnoid space, in the sulci of the brain, where larger blood and lymph vessels run, supplying the brain with nutrients, and draining it from waste (Magliozzi et al. 2007, Duvernoy, Delon, and Vannson 1981, Aspelund et al. 2015). One of the obvious differences between rodent and human brain is the lack of sulci in the rodent brain. There are still, however, cortical areas adjacent to these large blood and lymph vessels in the rodent brain, such as the piriform cortex (Antila et al. 2017, Xiong et al. 2017, Aspelund et al. 2015). The piriform cortex happens to be adjacent to the densely myelinated lateral olfactory tract, one of the few white matter structures on the brain surface (Hintiryan et al. 2012).

Increased meningeal inflammation, and especially TLS-like structures are associated with increased demyelination, neuronal damage, microglial activation, and damage to the glia limitans (Magliozzi et al. 2010, Howell et al. 2011). B cells in meningeal aggregates in SPMS are thought to contribute to disease pathology by the production of cytokines and other inflammatory mediators, and antigen-presentation (Molnarfi et al. 2013, Weber et al. 2010, Duddy et al. 2007). B cells in tertiary lymphoid structures in other chronic immune diseases, including rheumatoid arthritis and chronic hepatitis C, have been shown to contribute to disease pathogenesis (Sansonno et al. 2004, Murakami et al. 1999, Takemura et al. 2001).

New, or more accurate imaging and diagnostic tools are being investigated to study TLS in MS. Unfortunately, *in vivo* detection of TLS in MS using MRI remains challenging, and tissue samples scarce. These issues make it hard to study the formation and evolution of TLS in the brain. Therefore, relevant models are required to study this phenomenon.

The interest in the role of B cells in MS and anti-CD20 continues to grow. Although clinical trials with RTX did not reach their primary endpoint, RTX is often used to treat MS patients off-label (Hauser et al. 2008, Bar-Or et al. 2008, Salzer et al. 2016, Alping et al. 2016). Other type I anti-CD20 antibodies, such as RTX, are currently in clinical trials (ofatumumab), or have already been FDA approved (ocrelizumab), and have been shown to be effective in RRMS and PPMS (Montalban et al. 2016, Hauser et al. 2016, Bar-Or et al. 2018). GA101 is a type II antibody, and can be distinguished by its direct-cell killing abilities, while having lower CDC activity (Mössner et al. 2010). The two types of anti-CD20 antibodies have not been compared before in huCD20 mice, or in a rodent model of MS.

After developing a new B-cell mediated, focal model of MS, and with the interest in TLS in SPMS growing, we sought to develop a rodent model that could represent histological features of both SPMS and meningeal TLS. In this way, the model could serve as a platform to study mechanisms involved, and also potential treatments for the benefit of patients with this aggressive phenotype of SPMS. The aim was to model chronic, slowly expanding lesions, characterised by activated microglia and astrocytes, lymphocyte infiltration in the shape of vascular cuffing, demyelination, and the presence of B cell rich TLS-like structures in the parenchymal-meningeal interface, as seen in progressive MS (Machado-Santos et al. 2018, Frischer et al. 2009, Serafini et al. 2004, Magliozzi et al. 2007, Magliozzi et al. 2010, Howell et al. 2011). Existing focal models represent some of these features, but do not lend themselves for long-term studies, or for studies in mice (Chapter 3) (Gardner et al. 2013, Silva et al. 2018).

The principal aims of this chapter therefore were (1) to establish a focal model for progressive multiple sclerosis in mice, that features TLS, (2) to study the effect of anti-CD20 therapy in this model, (3) to compare the effect of type I and type II anti-CD20 antibodies in this model.

4.2 Methods

4.2.1 Induction of the DTH-TLS model

Humanised CD20 C57Bl/6 and their WT littermates were used for these studies. DTH-TLS lesions were established in mice as following: on day -12 animals were immunised with a MOG_{35-55}peptide (0.5mg/ml in saline) and CFA, topped up with heat-killed TB (5mg/ml in the total volume), as described in section 2.3.2. On day 0, mice received a stereotaxic injection of 1µl heat killed TB (8.8mg/ml in saline) in the piriform cortex (Bregma: AP +0.7, ML +2.7, DV-3.5), as described in 2.3.3.

4.2.2 Characterisation of the DTH-TLS model

To test for clinical signs, animals were tested for motor function with the inverted screen test and the fixed rods, as described in section 2.4.1 and 2.4.2 respectively. They were weighed and perfused afterwards on day 1, 6, 12, 28, and 60, as described in section 2.6. Four animals underwent MRI on day 100, before they were perfused. No behavioural data is available for this time point.

4.2.3 Treatment with anti-CD20

A different set of animals was used to test the effect of two different anti-CD20 antibodies on the lesions and the associated TLS-like structures. Treatment started on day 40 after lesion induction. Animals were treated with either a type II antibody, GA101 (10mg/kg) (n=11), a type I antibody with an overlapping binding site, RTX (10mg/kg) (n=8), or a vehicle control (n=13). Animals received three intravenous injections (100µl) via the tail vein spread over 15 days. Animals were perfused 17 days after starting treatment as described in section 2.6.

4.3 Results

4.3.1 Model characterisation

4.3.1.1 The DTH-TLS model does not cause weight loss, or loss of motor function.

Weight loss and loss of motor function are key clinical outcomes in EAE. Most focal models of MS are known not to have clinical symptoms. Focal DTH-TLS lesions were induced in mice by injecting heat-killed TB into the piriform cortex of MOG immunised mice. Mice were weighed and tested for motor function on day 1, 6, 12, 28, 40, and 60 after lesion induction, as no obvious clinical signs were visible (Figure 5.1). Compared to their pre-surgery body weight, the mice showed transient weight loss in the first week (-2.3±0.139% on day 1 and -1.1±1.502% on day 6), which was fully recovered by day 12 (+1.7±1.851%) (Figure 5.1A). After the initial small drop in weight, the mice started gaining weight again and no further periods of weight loss were observed. Instead, weights stayed stable until day 28 (+4.631±2.872%) and day 60 (+3.476±2.319%) (Figure 5.1A).

No loss of motor function or muscle strength was observed using the inverted screen test. Animals were placed on a wire mesh and the mesh was rotated upside down, ± 30cm above a padded surface. None of the animals in any of the groups fell off and thus all mice were assigned the maximum time of 60 seconds (Figure 5.1B).

Next, motor function was tested on the fixed rods. In this test, a two-way ANOVA revealed no significant interaction between the time points and size of the rod in how fast the mice oriented towards the bench ($F(4,25) = 0.9538$; $p=0.4499$), or how fast they returned to the bench ($F(4,26) = 0.07330$; $p=0.9896$). Instead, high variability and no significant differences were observed within and between the time points in the time it took for the mice to orient and return towards the bench (Figure 4.1C). The time it took for the mice to reach the bench was highly variable as well (Figure 4.1D). Mice at day 1 were the fastest to return on both the medium and the small rod (3.33±1.2 and 4.67±0.67 seconds respectively), followed by day 6 (22.75±2.06 and 8.75±1.76). Mice at day 12, 28, and 60 took longer on average to return to the bench (55.25±28.8, 52.250±25.24, and 49.33±35.63) on the medium rod and the time taken was highly variable. Similar to the mice on day 12, the mice at later time points, took less time to return to the bench on the small rod (34.0±18.68, 36.50±27.94, and 46.33±36.970 for day 12, 28, and 60 respectively). No significant differences were observed in returning to the bench. Taking a closer look at the videos of the behavioural tasks, the mice that took longer to return to the bench tended to exert more exploratory behaviour, including rearing.

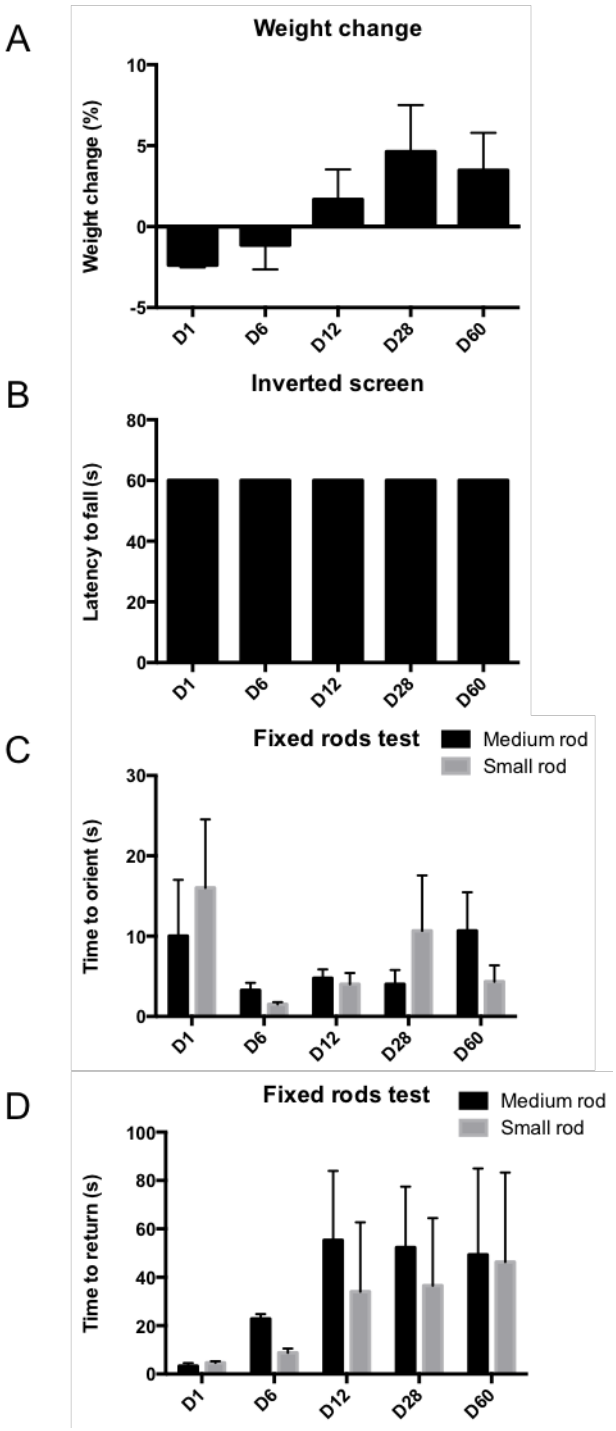


Figure 4.1 The DTH-TLS model caused transient weight loss, but no impairment in motor function. Focal brain lesions with TLS-like structures were established in mice. Mice were weighed and tested for motor function on day 1, 6, 12, 28, and 60. (A) Animals showed little, but transient weight loss in the first week after lesion induction. By day 12, all mice had gained weight, compared to their pre-surgery body weight. (B) The inverted screen test showed no loss of motor function, as all mice reached the maximum score in this test. The fixed rods showed great variability in the time to orient towards the table (C) and the time to return to the table (D). Data are mean ± SEM. Statistics: A&B one-way ANOVA with Tukey's comparisons test, C&D two-way ANOVA with Sidak's multiple comparisons test; n=4 mice per time point.

4.3.1.2 Chronically activated microglia and astrocytes spread out over time

Persistent and chronic inflammation is one of the hallmarks of MS. To determine the extent of microglial and astrocyte activation, immunohistochemistry was performed on brain sections for the two cell types (markers Iba-1 and GFAP respectively). As in MS, the DTH-TLS model displayed chronic, slowly expanding, microglial activation (Figure 4.2). Iba-1^+ cells proliferated in the piriform cortex and the meninges, and their number increased over time (Figure 4.2, 10x panel). From day 1 onwards, microglia adopted an activated phenotype, characterised by the amoeboid morphology and the retraction and thickening of their processes and this phenotype increased over time (Figure 4.2, 40x panel). The highly reactive morphology appeared with clustering and nodule formation of the microglia in the later time points, which indicates chronic inflammation. Microglial activation was the most intense around the hypercellular lesion cores, and around the lymphocyte aggregates on the meningeal interface (Figure 4.2, 10x panel). Staining with Iba-1 does not allow for distinction between microglia and other macrophages, thus these were stained in brown as well. Microglia (Iba-1^+ cells with a microglial morphology) did not tend to be part of the meningeal cell aggregates (Figure 4.2).

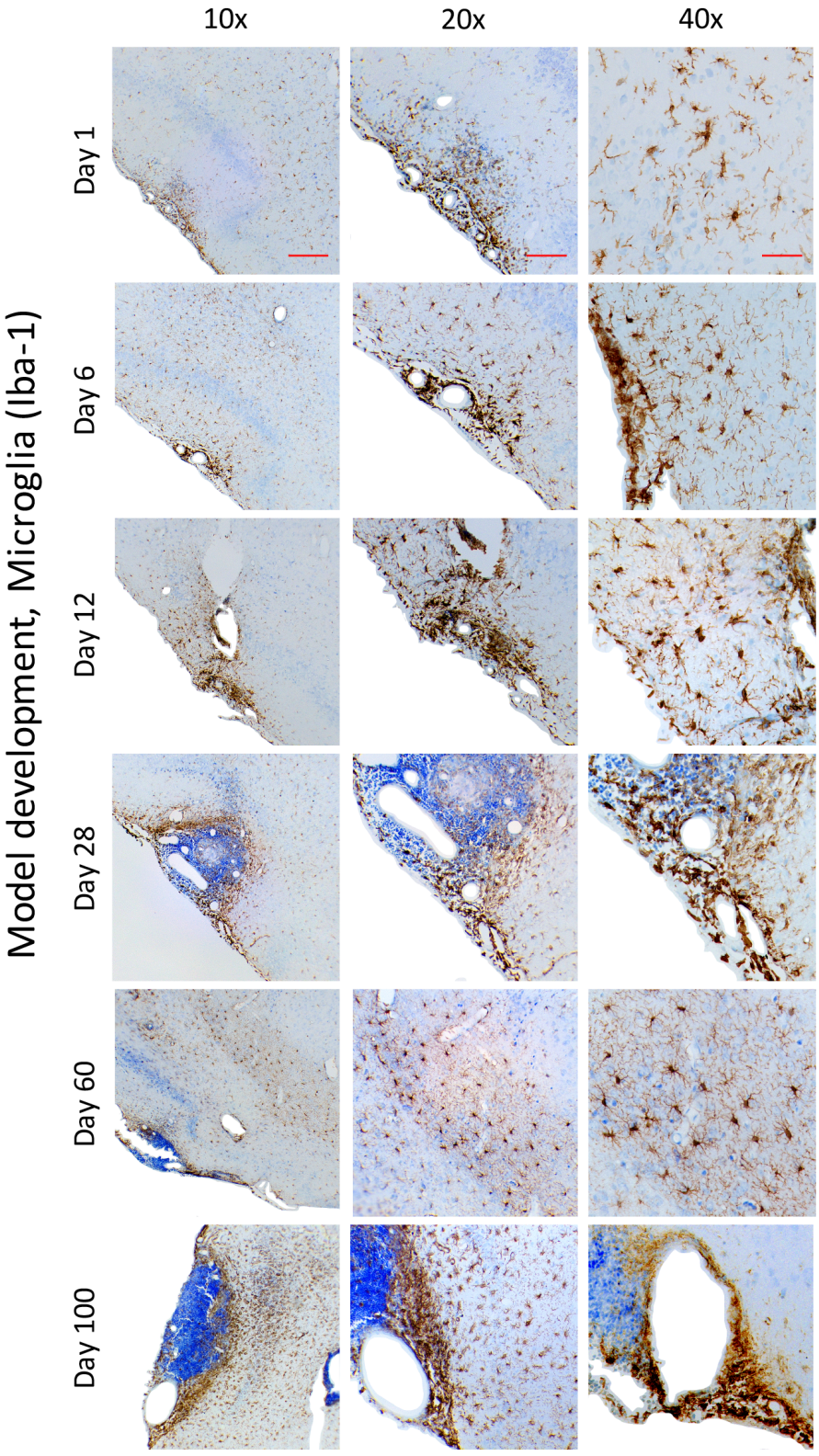


Figure 4.2 Spreading microglial activation in the DTH-TLS model. Focal brain lesions with TLS-like structures were established in mice. Mice were perfused on day 1, 6, 12, 28, 60, and 100. Microglia and macrophages were stained with anti-Iba-1 (brown) and cresyl violet was used as a nuclear stain (blue). Representative photomicrographs were taken for each time point. Microglial activation intensifies and spreads out over time. Red scale bars represent 200µm in the 100x pictures,100µm in the 20x pictures, and 50µm in the 40x pictures.

Astrocytes followed the microglial activation pattern in this model. The astrocytes adopted a reactive phenotype and the area covered by these cells increased over time, and tended to spread out even further than the area covered by microglia (Figure 4.3 10x panel). Reactive astrocytes could be recognised by their hypertrophied cell body and their thickened processes. Their density was highest around the lymphocytic cell aggregates and around inflamed blood vessels (Figure 4.3 10x panel). GFAP upregulation was seen on the edges of the lymphocyte aggregates in the later time points, day 60 and 100 (Figure 4.3). At these stages, the formation of glial scars was also evident, recognised by the elongated shape of the astrocytes, resembling a barrier between the lymphocytic aggregates and the brain parenchyma (Figure 4.3). As for the microglia, astrocytes were not part of the cellular aggregates in the meninges (Figure 4.3).

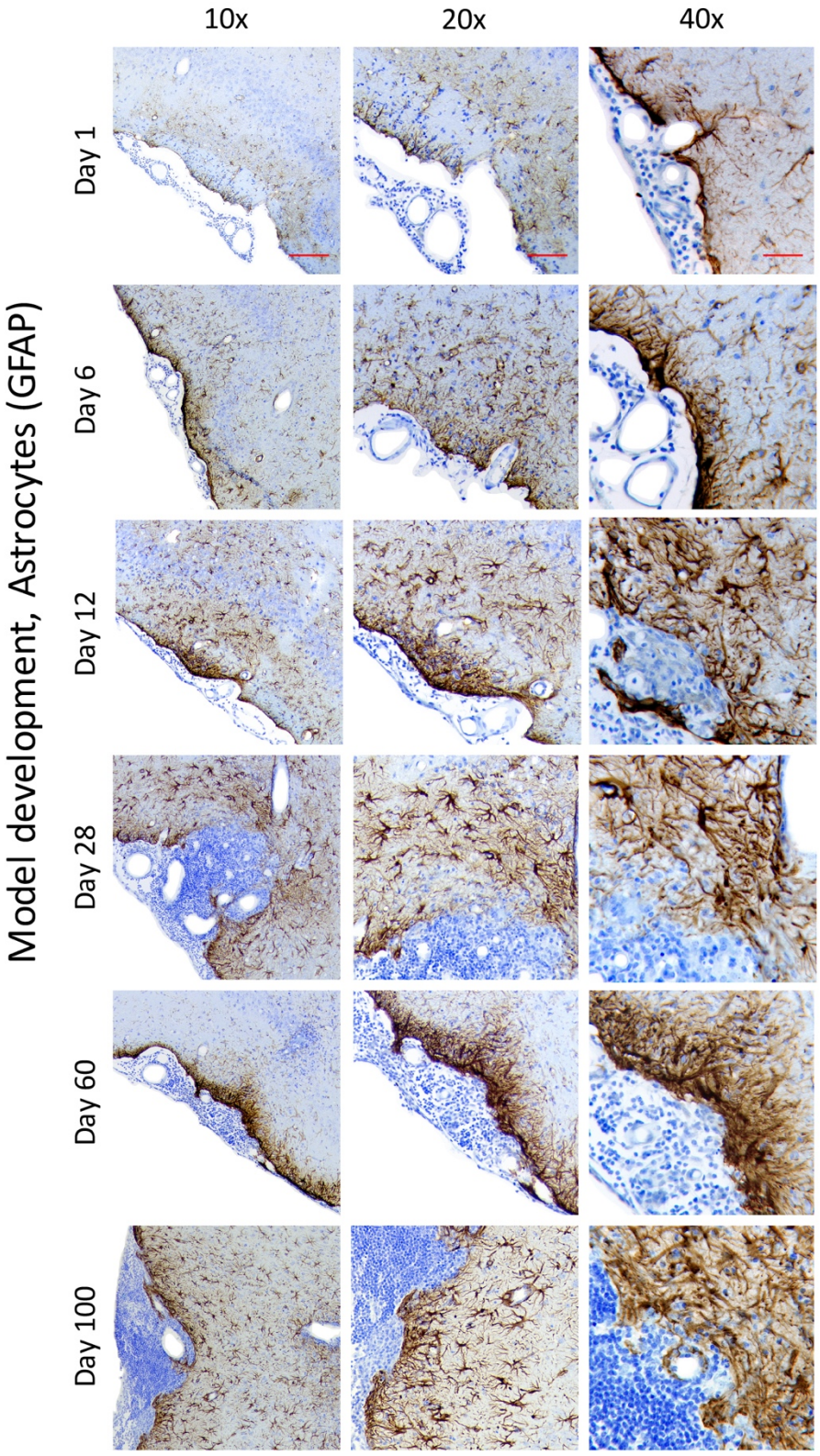


Figure 4.3 Spreading astrocyte activation in the DTH-TLS model. Focal brain lesions with TLS-like structures were established in mice. Mice were perfused on day 1, 6, 12, 28, 60, and 100. Astrocytes were stained with anti-GFAP (brown) and cresyl violet was used as a nuclear stain (blue). Representative photomicrographs were taken for each time point. Reactive astrocytes spread out and form glial scars over time. Red scale bars represent 200µm in the 10x pictures,100µm in the 20x pictures, and 50µm in the 40x pictures.

4.3.1.3 B and T cells increase in number over time and form TLS-like structures in the meninges in the DTH-TLS model.

After identifying lymphocytic meningeal aggregates by Nissl stain, anti-B220 and anti-CD3 were used to identify B and T cells within the aggregates. Both $B220^+$ B and $CD3^+$ T cells were present in the meninges even 1 day after lesion induction, and their numbers increased over time (Figure 4.4). B and T cells were not exclusively found in the meninges. In the brain parenchyma, both B and T cells were mostly present in the cuffs of large inflamed blood vessels. Some smaller cell clusters could be found throughout the parenchyma, often associated with the hypercellular lesion cores (data not shown). In the meninges, the proportion of B and T cells appeared similar in the earlier time points, but as time progressed, the proportion of B cells increased (relative to the T cells) (Figure 4.4). At the same time, the pattern of the meningeal aggregates changed – from lymphocytes being scattered randomly throughout the small meningeal aggregates, B and T cells started clustering together, and became compartmentalised from each other, which was especially evident on day 60 and day 100 (Figure 4.4). With the B and T cell compartmentalisation the meningeal aggregates started to resemble TLS. The lower magnification for day 100 displays the size of the TLS-like structures, with B and T cells in separate areas (Figure 4.4). As the model progressed, the TLS increased in size. No sign of shrinkage of the TLS-like structure, or reduction in the number of lymphocytes was observed (Figure 4.4).

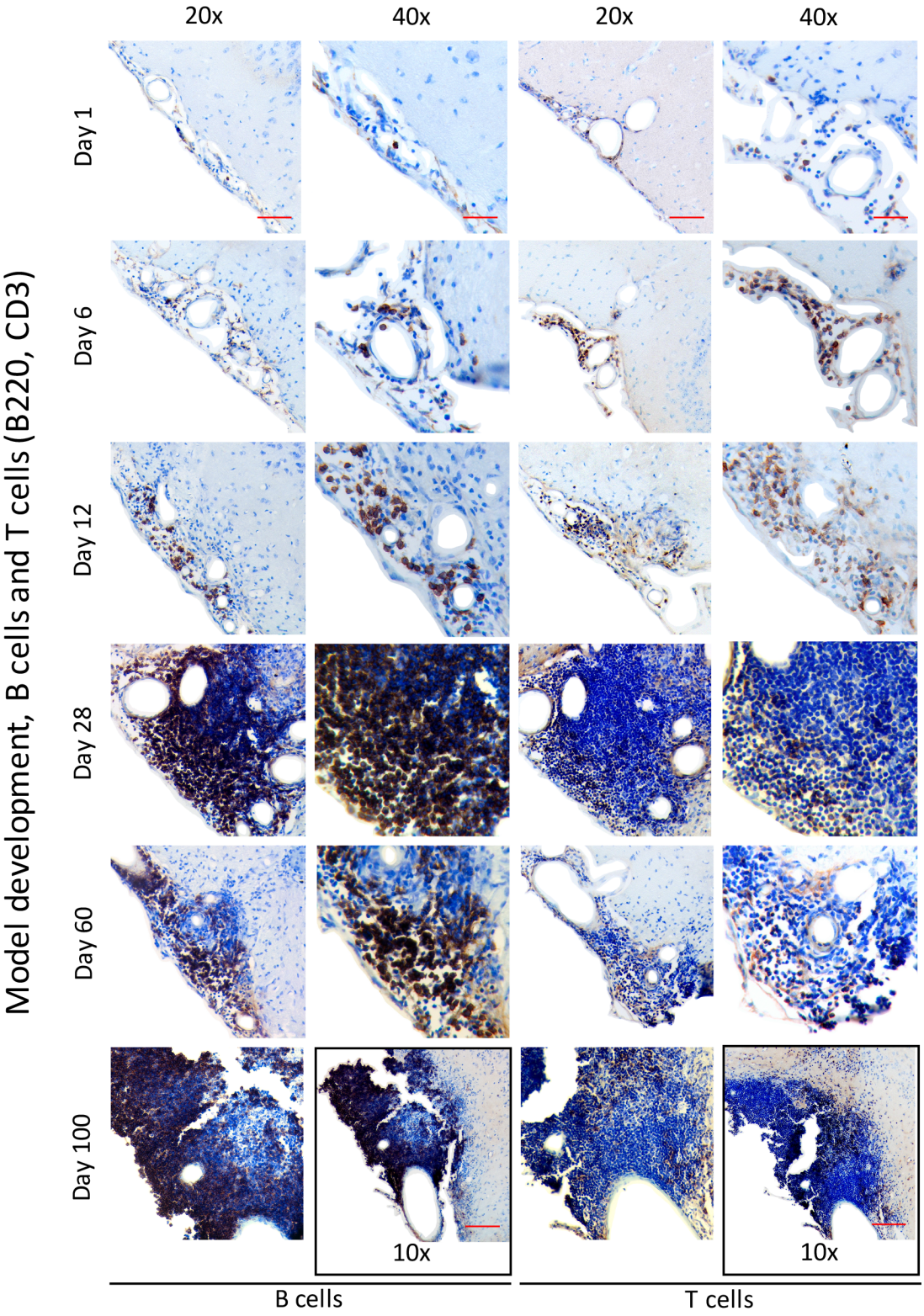


Figure 4.4 B and T cells increase in number over time and form TLS-like structures in the meninges. Focal brain lesions with TLS-like structures were established in mice. Mice were perfused on day 1, 6, 12, 28, 60, and 100. B and T cells were stained in brown with anti-B220 and anti-CD3 respectively and cresyl violet was used as a nuclear stain (blue). An increase in the number of both cell types can be seen as time progresses. Representative photomicrographs were taken for each time point. Red scale bars represent 200µm in the 10x pictures,100µm in the 20x pictures, and 50µm in the 40x pictures.

4.3.1.4 Proliferating B cells were present in meningeal TLS in the DTH-TLS model.

After observing the compartmentalisation of B and T cells in the meningeal TLS in the DTH-TLS mode, brain sections were double labelled for B cells (anti-B220) in red and a proliferation marker (anti-Ki67) in green using immunofluorescence. $Ki67^{+}B220^{+}$ cells were identified in the meningeal cell aggregates (Figure 4.5). The majority of the cells in these aggregates positive for the B-cell marker, of which some were also positive for the proliferation marker. The Ki67 stain colocalised with the cell nuclei stained in blue with DAPI.

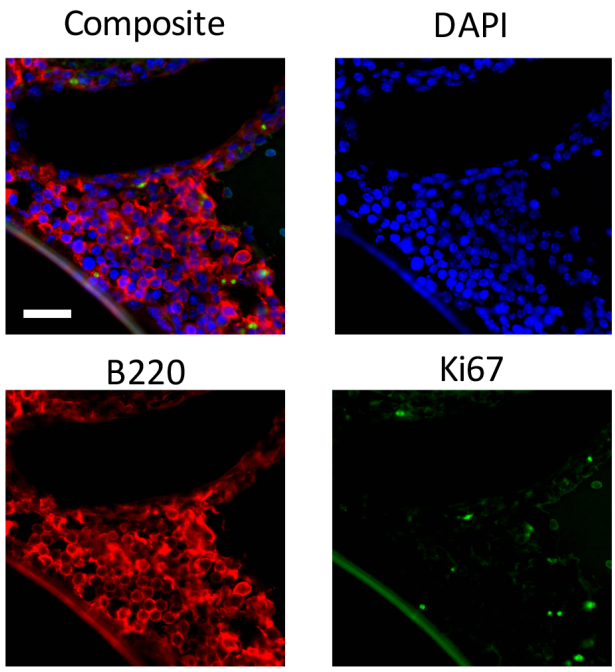


Figure 4.5 Proliferating B cells are present in meningeal TLS in the DTH-TLS model. Focal brain lesions with TLS-like structures were established in mice. Mice were perfused on day 28. Brain sections were stained for B cells (anti-B220 in red), proliferation marker (anti-Ki67 in green) and cell nuclei (Dapi, blue). Accumulaion of B cells was observed in the meningeal-parenchymal interface. Proliferatiting B cells were positive for both the B cell maker in red and the proliferation marker in green. Scale bar represents 50µm.

4.3.1.5 Worsening demyelination in the olfactory tract of mice with the DTH-TLS model.

To determine the extent of demyelination, an anti-MBP antibody was used to visualise the remaining myelin in the brains from mice with the DTH-TLS model. Not unexpectedly, with the increasing spread of inflammation and growth of TLS-like structures, demyelination became more prominent as time progressed (Figure 4.6). The lateral olfactory tract is a densely-myelinated track that runs along the piriform cortex in the brain. In the DTH-TLS model, the olfactory tract was in close proximity to the lesion core, as well as to the meningeal TLS-like structures, and the rim of activated microglia. As the lesions developed over time, they showed more characteristics of chronic inflammation, and clearer formation of the TLS-like structures, the demyelinated area of the olfactory tract kept expanding (Figure 4.6). From day 12, demyelination was clearly visible, and was associated with an increased number of cells in the cortex and the meninges surrounding the olfactory tract. Demyelination had worsened at day 28 and day 60. At day 100, very little MBP stain was found in the olfactory tract. The loss of myelin in this model was associated with the growth of the TLS-like structures in the meninges. As the TLS increased in size, the demyelinated area also grew (Figure 4.6).

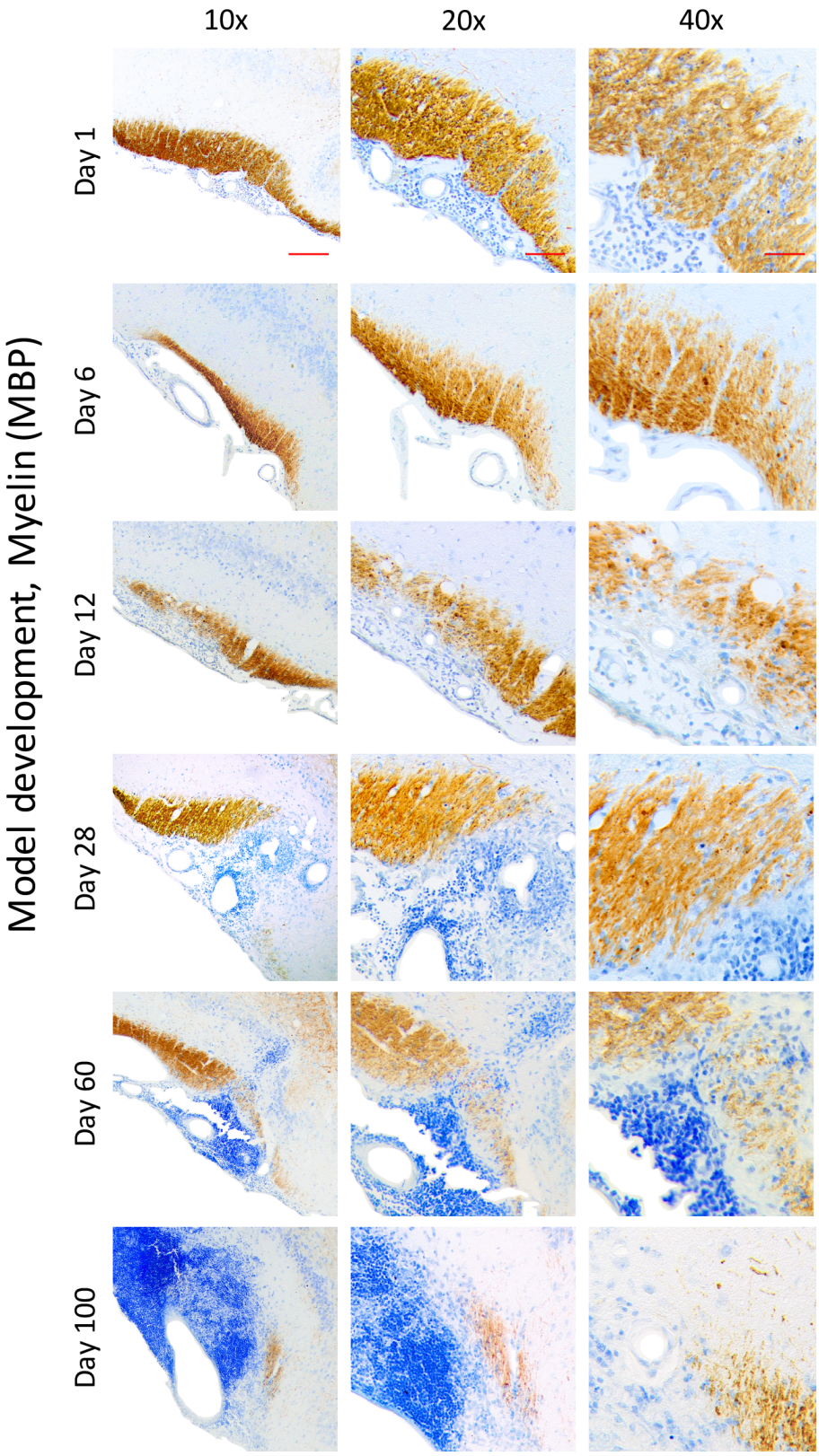


Figure 4.6 Progressing demyelination in the DTH-TLS model. Focal brain lesions with TLS-like structures were established in mice. Mice were perfused on day 1, 6, 12, 28, 60, and 100. Sections were stained for myelin in brown. The olfactory tract became more demeylinated as the model progressed. Representative photomicrographs were taken for each time point. Red scale bars represent 200µm in the 10x pictures,100µm in the 20x pictures, and 50µm in the 40x pictures.

4.3.1.6 No blood-brain barrier breakdown detected with gadolinium enhanced MRI in the DTH-TLS model on day 100.

To determine BBB permeability in the model, *in vivo* gadolinium enhanced MRI was performed. At day 100, postcontrast T1-weighted images revealed no gadolinium enhancement in the lesion area of any of the animals, indicating no BBB breakdown. Some enhancement could be seen in the simple difference between the pre-gadolinium and post-gadolinium images, but when corrected for movement, these changes were no longer present (Figure 4.7). Images from one mouse were not included, due to severe movement artefacts, which could not be corrected after the images were acquired.

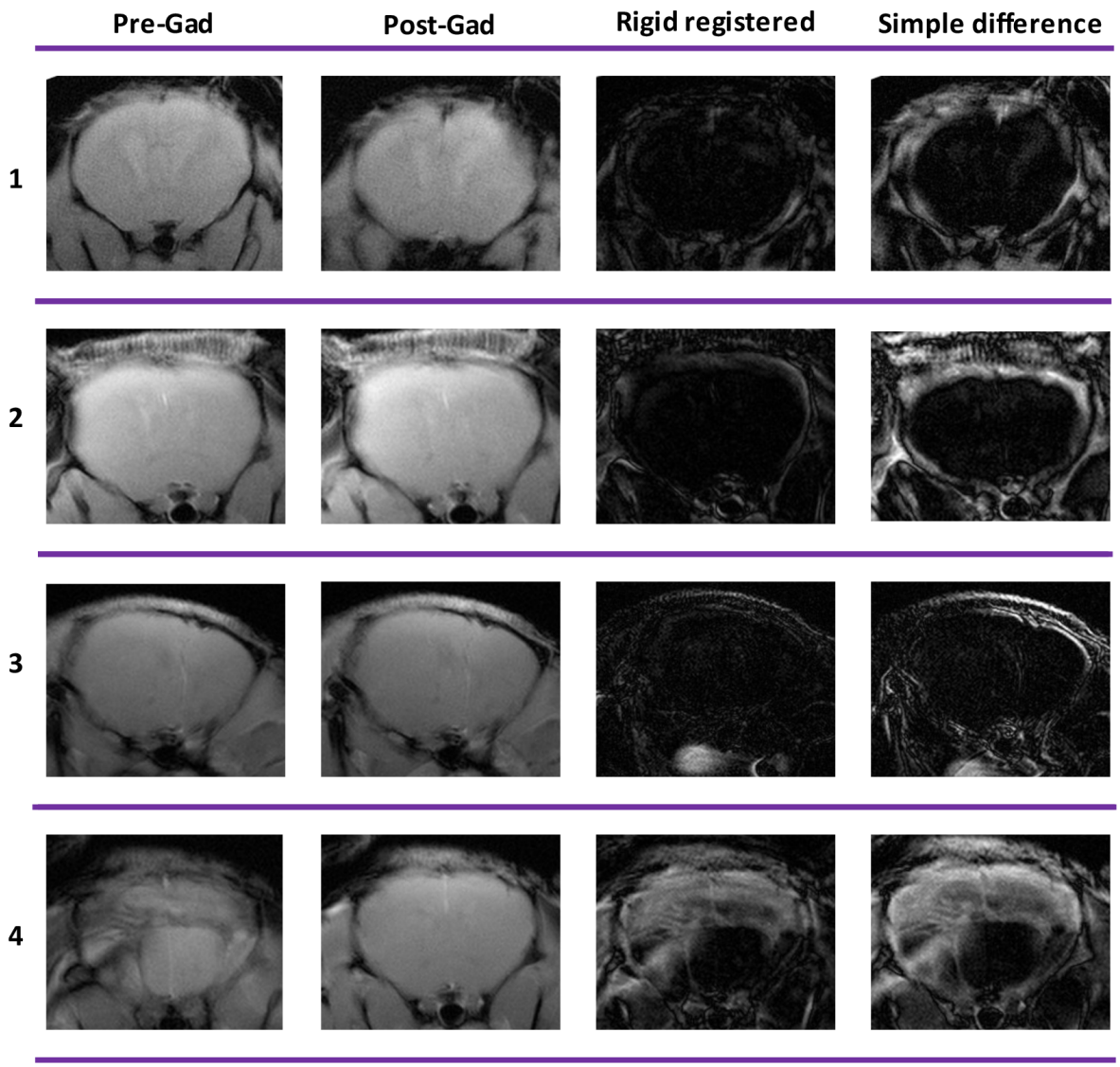


Figure 4.7 No blood-brain barrier breakdown observed with gadolinium enhanced MRI in the DTH-TLS model on day 100. Focal brain lesions with TLS-like structures were established in mice. Mice were imaged with gadolinium-enhanced MRI on day 100 (n=4). T1-weighted images were taken before and after intravenous administration of gadolinium. The simple difference was calculated between the two images, as well as the rigid registered image for a higher accuracy. No gadolinium enhancement, indicated by focal bright white areas, was seen in the brain. Pre-Gad= pre-gadolinium administration, Post-Gad = post-gadolinium administration.

4.3.2 Type I vs Type II anti-CD20 therapy

Focal DTH-TLS lesions were induced in mice by injecting heat-killed TB into the piriform cortex of MOG immunised mice. On day 40 after lesion induction, mice were treated with either a type II anti-CD20 antibody (GA101), a type I antibody (RTX), or a vehicle control. After 17 days of treatment, blood was collected, mice were perfused-fixed and organs were collected.

4.3.2.1 Anti-CD20 significantly depletes peripheral B cells.

To examine the effects of GA101 and RTX on B cell depletion, spleens and lymph nodes were stained for B cells (anti-B220). Both GA101 and RTX significantly reduced ($p<0.0001$) the ratio of the area covered by B cells compared to control mice in lymphoid follicles in the spleen (GA101: 0.1002±0.019, RTX: 0.6258±0.100 vs. Control:1.786±0.0761) (Figure 4.8A) and the lymph nodes (GA101:0.0514±0.0069, ($p<0.0001$); RTX:0.4724±0.0906 ($p=0.0090$) vs. Control:1.525±0.2810) (Figure 4.8B). In the spleen, GA101 depleted significantly more B cells than RTX ($p<0.0001$), though no significant difference was observed between the two drugs on lymph-node-B-cell depletion ($p=0.4602$). Treatment with both anti-CD20 antibodies resulted in a decrease in white blood cells in the blood (GA101: 2.285± 0.446, RTX: 2.648±0.652 vs control: 4.908±0.594 K/μl; $p=0.0052$ for GA101 and $p=0.0310$ for RTX) (Figure 4.8C). Assessment of the lymphocyte compartment specifically, an even larger reduction of cells was found after both treatments. GA101 reduced blood lymphocyte counts to 1.499±0.280 ($p=0.0006$) and RTX to 1.480±0.411 ($p=0.0015$) compared to 3.358±1.58K/μl in vehicle treated mice (Figure 4.8D). The reduction of lymphocytes and white blood cells was not different between the GA101 and the RTX treated mice ($p=0.9995$ and $p=0.9142$ respectively).

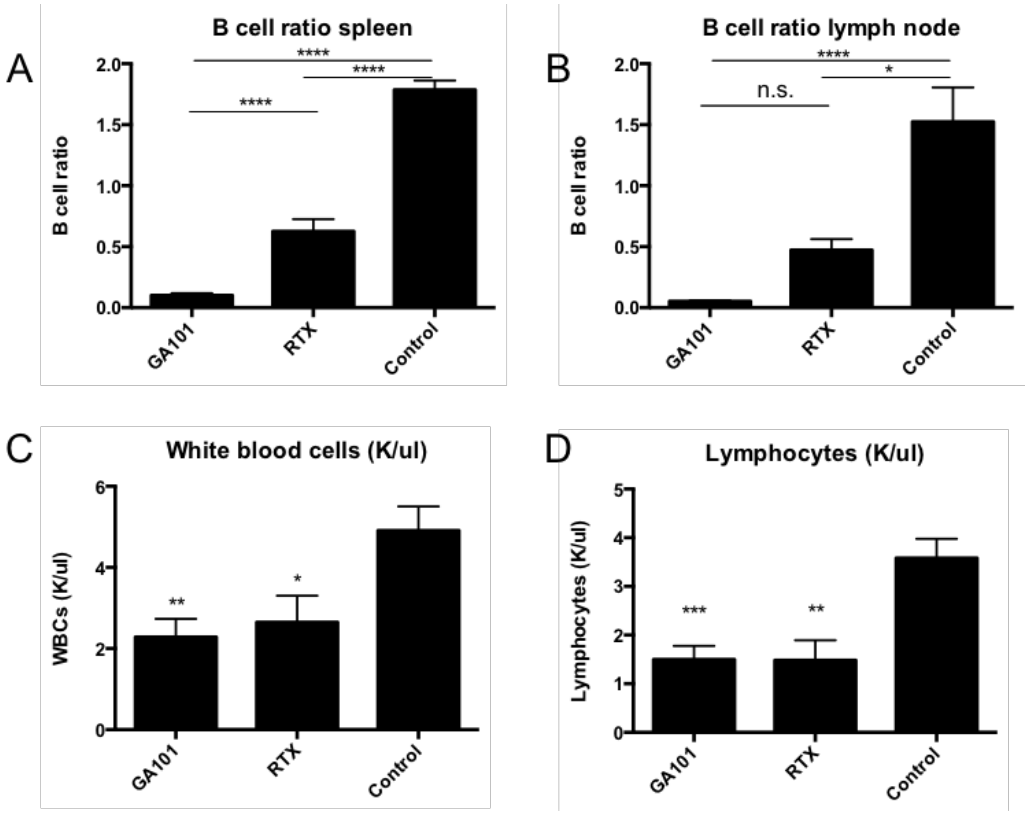


Figure 4.8 Anti CD20 depletes peripheral B cells in the DTH-TLS model. DTH-TLS lesions were established in huCD20 mice. 40 days after lesion induction, the mice were treated with either GA101, RTX or a vehicle control. 17 days after treatment, blood and organs were collected and analysed. (A) Both GA101 and RTX therapy caused a reduction in B cells in the spleen. GA101 depleted splenic B cells better than RTX. (B) The B cell proportion in the lymph nodes was also reduced after both GA101 and RTX treatment. There was no difference between the two treatment antibodies. (C) The number of white blood cells was reduced after anti-CD20 therapy. (D) GA101 and RTX both reduced the number of blood lymphocytes compared to controls. Data are mean ± SEM * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Statistics: one-way ANOVA with Tukey's multiple comparisons test (GA101 n= 11, RTX n=9. Control n= 13). RTX rituximab.

4.3.2.2 Anti-CD20 treatment reduces microglial and astrocyte activation in DTH-TLS lesions.

To compare the effect of the two drugs on the MS-like lesions, their effects on lesion size was analysed first. Staining for Iba-1 and GFAP allowed us to determine the extent of microglial and astrocyte activation. A camera lucida was used to draw around the area of activated microglia, to determine the lesion size. Anti-CD20 therapy significantly reduced the area of activated microglia. GA101 reduced the area of activated microglia to $1.895\pm0.249mm^2$ ($p=0.0002$), and RTX to $2.371\pm0.341mm^2$ ($p=0.0467$), compared to $3.222\pm0.153mm^2$ in the control treated mice (Figure 5.9A&C). No significant difference between the effects of the two antibodies was observed ($p=0.408$).

Astrocyte activation followed the pattern of microglial activation, but was more extensive. The area of activated astrocytes was determined the same way as with the microglia (Figure 5.8). GA101 reduced the area to $3.532\pm0.341mm^2$ ($p=0.012$), while RTX only reduced the area to $4.424\pm0.358\ mm^2$ ($p=0.370$), compared to control ($4.982\pm0.315\ mm^2$) (Figure 5.9B). In GA101 treated mice, the astrocytes tended to cluster focally at the parenchymal-meningeal interface, forming a glial scar, while in RTX and control treated mice, the astrocytes spread further out (Figure 4.9D).

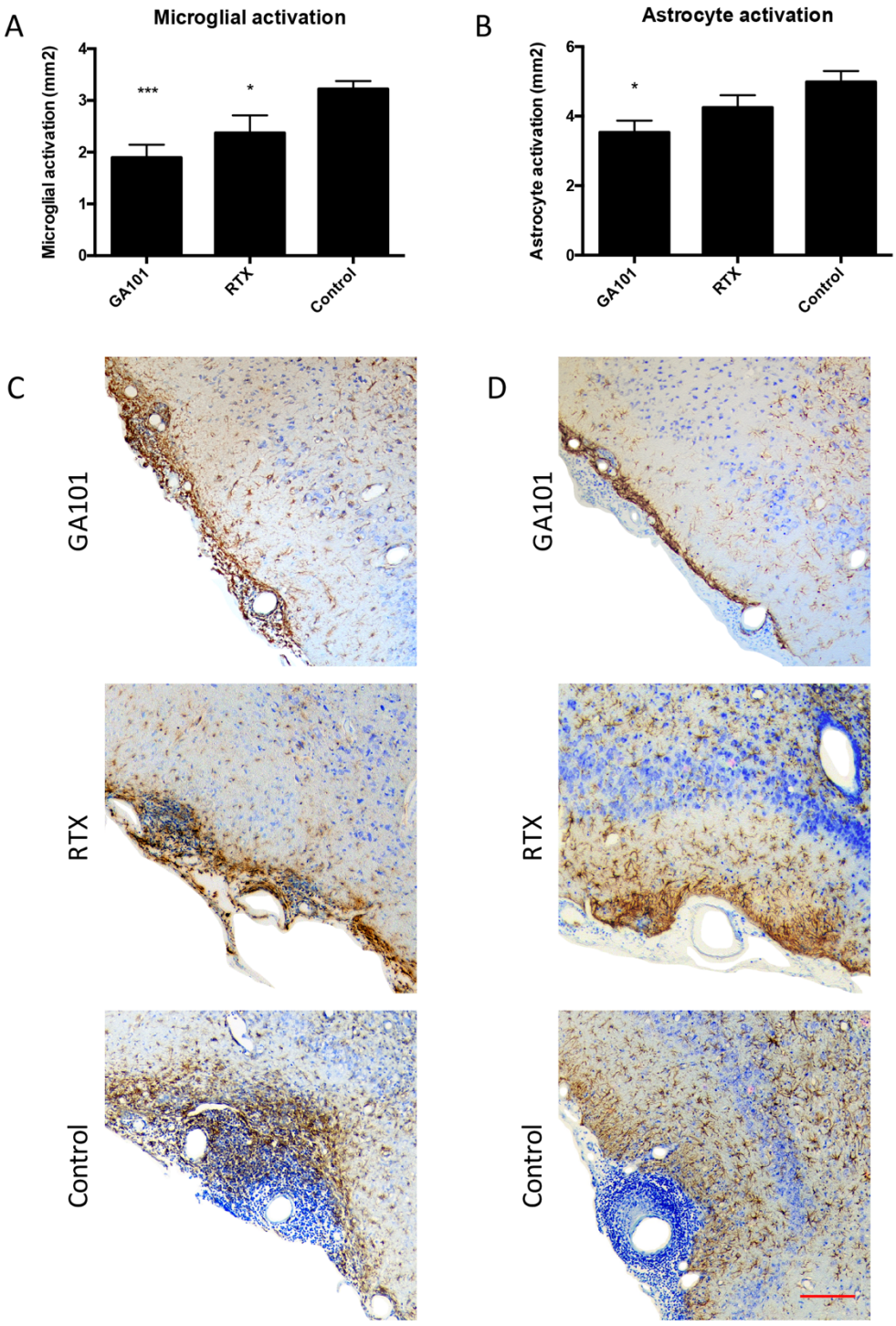


Figure 4.9 Anti-CD20 therapy reduces glial activation in DTH-TLS lesion. DTH-TLS lesions were established in huCD20 mice. 40 days after lesion induction, the mice were treated with either GA101, RTX or a vehicle control for 15 days. (A) Immunohistochemistry showed a reduction in the area of microglial activation after both anti-CD20 treatments. (B) Only GA101 reduced the area of astrocyte activation. (C) Photomicrographs show activated microglia in the lesions GA101 treated animals, RTX treated animals, and control animals. (D) Representative photomicrographs show astrocytes activation in GA101 treated animals, RTX treated animals, and control animals. Data represent mean ± SEM; *p<0.05,**p<0.01. Statistics: one-way ANOVA with Tukey's multiple comparisons test, vehicle n=8 for GA101, n= 6 for RTX, n= 11 for Control. Scale bars represents 100μm.

4.3.2.3 Anti-CD20 therapy significantly reduces the number of B and T cells in the brain.

The tertiary lymphoid-like structures featured in the DTH-TLS model are largely composed of B and T lymphocytes. To see the effect of the type I and type II anti-CD20 antibodies on the TLS structures in these mice, and on the number of B and T cells in general, immunohistochemistry was performed. Anti-B220 was used as a pan-B cell marker, and CD3 as a T cell marker. The total number of B cells and T cells was counted in a 10 μm section of the entire ipsilateral hemisphere of each animal. Complete shrinkage of the TLS-like structures was observed, with very few B cells left in the meninges (Figure 4.10C). Analysis showed that both anti-CD20 antibodies significantly reduced the total number o f B cells in the ipsilateral hemisphere (GA101: 31.78±9.260, $p<0.0001$; RTX: 82.60±36.63, $p<0.001$ vs Control: 1805±265.6) (Figure 5.9A). No significant difference between the type I and type II antibodies was observed in terms of depleting brain B cells (Figure 4.10A).

As for B cells, the number of T cells was also determined by immunohistochemistry. 10 μm sections were stained for CD3 and the $CD3^+$ cells were counted. Anti-CD20 treatment lowered the number of T cells in the meninges and brain parenchyma (Figure 4.10B&D). Only a small proportion of T cells were left in the meninges and around cuffed vessels in the brain parenchyma (Figure 4.10D). GA101 lowered the number of T cells to 116.1±26.71 ($p<0.0001$), while RTX lowered the T cell number to 270±63.89 ($p<0.01$) compared to vehicle treated animals (567±55.94) (Figure 5.9B). GA101 and RTX did not have significantly different effects on reducing the number of brain T cells ($p=0.266$) (Figure 4.10B).

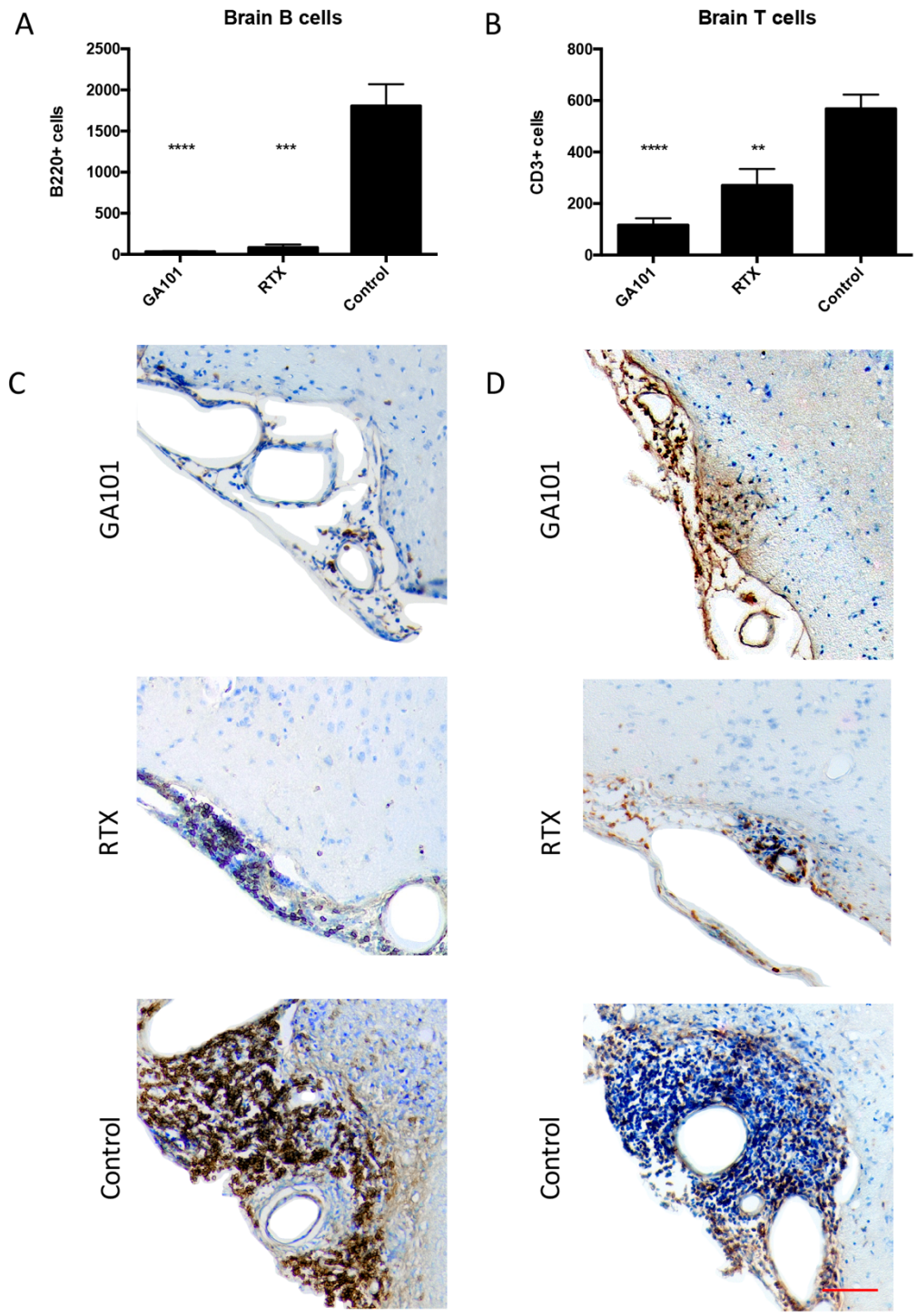


Figure 4.10 Anti-CD20 reduces the number of brain B and T cells. DTH-TLS lesions were established in huCD20 mice. Mice were treated with either GA101, RTX or a vehicle control. Brains were immunolabelled for B (B220) and T cells (CD3) and their numbers were counted. (A) GA101 and RTX both reduced the number of B cells in the brain. (B) Both anti-CD20 antibodies also lowered the number of T cells in the brain. (C) Representative photomicrographs of meningeal B cells in GA101, RTX, and vehicle treated mice. (D) Representative photomicrographs of meningeal T cells in GA101, RTX, and vehicle treated mice. Data are presented as mean ± SEM; **p<0.01, ***p<0.001, ****p<0.0001. Statistics: one-way ANOVA with Tukey's multiple comparisons test, vehicle n=8 for GA101, n= 6 for RTX, n= 11 for Control. Scale bar represents 100 μm.

4.3.2.4 GA101 protects neurons in the DTH-TLS model.

Although no behavioural changes were observed in this model, TLS-like structures are associated with increased axonal damage and neuronal loss. To determine the effect of anti-CD20 on neuronal survival, neurons were labelled with anti-NeuN using immunohistochemistry. Neuronal cell bodies were counted in the piriform cortex in the ipsilateral and contralateral hemisphere, and their ratio was calculated. The ratio between ipsilateral and contralateral was 1.001± 0.070 for GA101, 0.886±0.058 for RTX, and 0.850±0.014 for vehicle treated animals (Figure 4.11). Statistical analysis showed that mice treated with GA101 had a significantly higher ratio of ipsilateral vs contralateral neurons than control treated mice ($p=0.0480$). The effect of RTX was not significantly different from the vehicle treatment on neuronal survival ($p=0.876$), nor from GA101's effect ($p=0.249$) (Figure 4.11A).

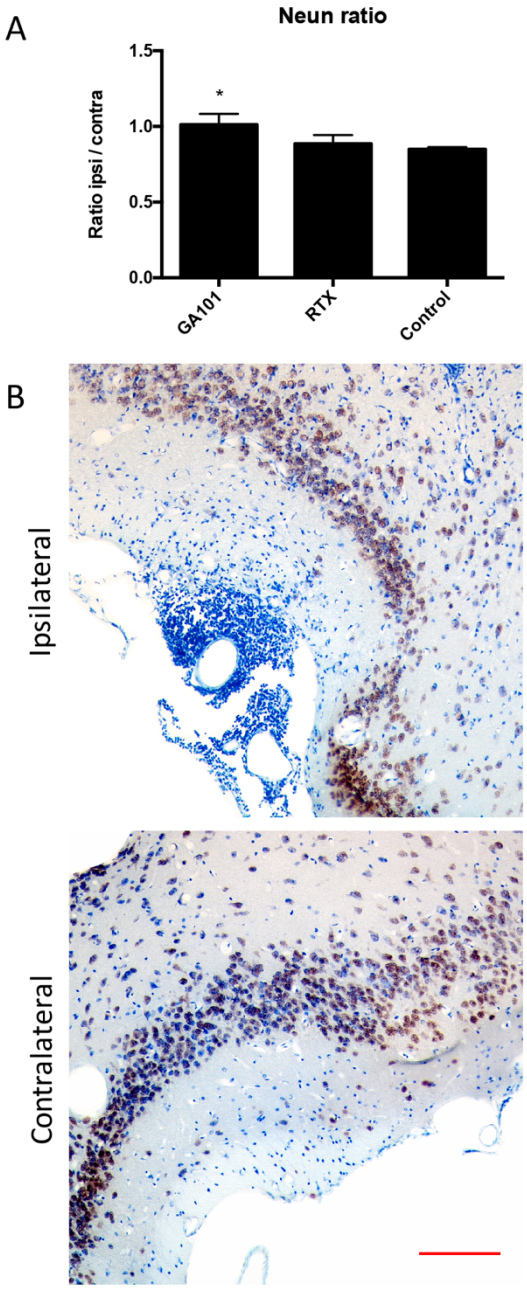


Figure 4.11 GA101 protects neurons in the DTH-TLS model. DTH-TLS lesions were established in huCD20 mice. Mice were treated with either GA101, RTX or a vehicle control. Neurons were immunolabelled (NeuN) and their numbers were counted in the piriform cortex in the ipsilateral and contralateral hemisphere. (A) GA101 improves the ratio of surviving neurons in the ipsilateral piriform cortex. (B) Representative photomicrographs of neurons stained in the ipsilateral and contralateral piriform cortex of a vehicle treated animals. Data are presented as mean ± SEM; *$p<0.05$. Statistics: one-way ANOVA with Tukey's multiple comparisons test, vehicle n=8 for GA101, n= 6 for RTX, n= 11 for Control. Scale bar represents 100 µm.

4.4 Discussion

In secondary progressive MS, tertiary lymphoid structures are associated with a more severe disease course (Serafini et al. 2004, Howell et al. 2011, Magliozzi et al. 2010). TLS form an understudied topic in MS, due to the lack of imaging tools and tissue samples. Efforts have been made to produce animal models that represent some of these features, but the models do not depict features of SPMS and TLS very well, nor do they lend themselves to long-term studies (Gardner et al. 2013, Silva et al. 2018, Pikor, Astarita, et al. 2015, Magliozzi et al. 2004). This chapters describes the development of a new model, the DTH-TLS model, based on the focal (DTH-MOG) model. As in the previous model, the focal lesions mimic MS histologically in terms of microglial activation, astrogliosis, demyelination and lymphocyte infiltration. Furthermore, the DTH-TLS model features structured accumulation of B and T cells in the meningeal-brain parenchymal interface, representing TLS.

MS patients can present with a range of clinical symptoms, depending on where the disease manifests itself topographically (Hauser and Oksenberg 2006). In many preclinical studies involving EAE, scoring weight changes and motor symptoms are the major parameters used to evaluate a drug's efficacy. Both weight loss and loss of motor function are highest during peak disease in EAE (Kerschensteiner et al. 2004, Kipp et al. 2016). In the DTH-TLS model we observed a brief, transient weight loss in the initial days after brain lesion induction, while animals returned to their normal weight after approximately a week. No motor impairments were found in this model either, as shown by a lack of difference in the inverted screen and static rod test. These findings may be due to the area targeted. The piriform cortex is involved in olfactory processing, learning, and memory (Mouly et al. 2001, Kay and Freeman 1998, Gottfried, Winston, and Dolan 2006). Further experiments are required to investigate whether olfactory or memory dysfunction occurs in this model, being two phenomena that MS patients suffer from (Good et al. 2017, Zivadinov et al. 1999, Atalar et al. 2018, Geurts et al. 2007,

González Torre et al. 2017). Some other behavioural aspects, such as anxiety may have been affected in the DTH-TLS model. Some of the older mice (d60) were not very active on the static rod. Variability and immobility may be attributed to the mice not being trained to perform the task. However, mice in earlier time points appeared to display more exploratory behaviour, exploring the room from the rod, even reared on their hind paws. Sickness behaviour is often associated with inflammatory disease or injury and includes a range of typical behavioural changes, including anhedonia, reduced social behaviour, hypersomnia, but also reduced motor and exploratory behaviour (Hart 1988, Maier and Watkins 1998). MS and rodent models of MS have been associated with increased sickness behaviour (Pollak et al. 2000, Campbell et al. 2010). The observations in this study suggest that the mice at later time points exhibited increased sickness behaviour, however, the variation and small group sizes in our characterisation study prevented us from finding any statistically significant differences between the study groups.

In MS lesions, microglia are one of the most common cell type, and they contribute to demyelination and cellular damage (Lassmann 2014a, Nylander and Hafler 2012, Fischer et al. 2012). As disease progresses, pathology becomes more diffuse, and so does the pattern of microglial activation. Active lesions in progressive MS contain activated microglia, and are associated with axonal and neuronal degeneration (Frischer et al. 2009, Prineas et al. 2001, Zrzavy et al. 2017, Frischer et al. 2015). These reactive microglia are present in DTH-TLS lesions, where they spread out over time, and maintain a dense ring/ridge of highly activated microglia surrounding the lesion core, similar to what is found in progressive MS (Frischer et al. 2015). Patients with progressive MS have variable disease severity, those with more active lesions tend to have a worse disease prognosis. Moreover, MS lesions with meningeal TLS are associated with a higher number of activated microglia, especially around TLS in the arachnoid space (Magliozzi et al. 2007, Magliozzi et al. 2010). This pattern was well-modelled in the DTH-TLS model, where dense microglial activation was found adjacent to the TLS. Astrocytes followed the microglial activation pattern in this model, they extended further as the lesion

progressed over time. Astrocytes are known to form glial scars in progressive MS lesions, a phenomenon represented in the DTH-TLS model in the late time points (Misu et al. 2013, Brosnan and Raine 2013, Markoullis et al. 2014). Both microglia and astrocytes surrounded, but were not found within the TLS.

The most important components of TLS are T and B cells. Meningeal TLS differ from meningeal inflammation by forming an organised structure (Serafini et al. 2004, Howell et al. 2011). In the DTH-TLS model we demonstrated that B and T cells are present in the meninges from day 1, and that their number keep increasing over time. From day 28 we started seeing compartmentalisation of the two cell types, a characteristic of lymphoid structures (Aloisi and Pujol-Borrell 2006, Corsiero et al. 2016). Moreover, we showed that B cells in the meningeal aggregates proliferated *in situ*, which is an important aspect of the meningeal TLS in SPMS (Magliozzi et al. 2007, Howell et al. 2011). Some B220-negative cells were positive for the proliferation marker as well.

Meningeal inflammation is not MS-specific, high numbers of B cells are also present in case of MS-like human demyelinating autoimmune encephalomyelitis, B cell lymphoma, or tuberculosis meningitis (Magliozzi et al. 2010, Höftberger et al. 2015, Machado-Santos et al. 2018, Fischer et al. 2013). Our model possibly overrepresented the B cell proportion, slightly, in the TLS, though studies on TLS in MS have reported that the number of B cells varies, and that the B cell proportion tends to be larger than the T cell proportion (Howell et al. 2011, Magliozzi et al. 2018, Serafini et al. 2004). Meninges are not only present on the outside of the brain, they line every surface and cavity of the brain. No TLS have yet been detected in the lateral ventricles, cerebellum, brain stem, and spinal cord of MS patients, though formation of TLS close to the third and fourth ventricles has not been ruled out (Howell et al. 2011, Magliozzi et al. 2007). TLS were found primarily adjacent to the lateral olfactory tract in the DTH-TLS model, though in some cases, TLS developed in the third ventricle. This usually occurred when the lesion core

appeared further caudal in the brain, possibly due the stereotaxic injection/lesion initiation being slightly further back. In other words, the location of the lesion determines the presence of TLS, which contradicts the hypothesis that cortical lesions are a consequence of meningeal inflammation and TLS (Howell et al. 2011). No TLS were formed in the lateral ventricles.

Next, we showed that TLS development and chronic inflammation were associated with perpetuating demyelination over time in the DHT-TLS model. Over the course of 100 days we found a gradual loss of myelin in the lateral olfactory tract, as well as in the adjacent cortex, which was associated with TLS formation microglial activation. Demyelination is an additional hallmark of MS, which affects both white and grey matter in both relapsing and progressive MS (Kidd et al. 1999, Prineas et al. 2001). Furthermore, SPMS patients with meningeal TLS, showed more severe demyelination, which corroborates what we observed in our model (Howell et al. 2011, Magliozzi et al. 2007).

As final part of the characterisation, we investigated the status of the BBB in the DTH-TLS model. In progressive MS, inflammatory lesions often evolve behind an intact BBB, and no gadolinium enhancement is visible on MRI (Hochmeister et al. 2006, Frischer et al. 2009, Correale et al. 2017, Filippi et al. 1995). No gadolinium enhancement was observed using MRI on the DTH-TLS lesions at day 100. We can therefore assume that there was no BBB breakdown at this time point. Although we did not image a positive control at the same time, it was clear that the gadolinium entered the system, as visualised by the intense bright coloured peripheral tissues.

In summary, based on the DTH-MOG model, a new MS-like model featuring TLS and neuronal loss is described (DTH-TLS). The two models share plenty of features, but exhibit differences as well. While lesions are restricted to the striatum in the DTH-MOG, lesions in the DTH-TLS model were occasionally found to disseminate, causing meningeal lymphocytic aggregates to form at multiple sites in the brain. The size of the lesion and the TLS can vary, depending on

the both the amount of TB and the location of the injection site. However, the focal TLS that forms close to the injection site is reproducible and amenable to histological endpoints in a manner that no other model can provide for.

The utility of the model was then investigated. We sought to discover how the lesions and associated TLS responded to different types of anti-CD20 therapy. More specifically, we compared the effects of a type II anti-CD20 antibody, GA101, with a type I antibody, rituximab (RTX), on the DTH-TLS model. We first showed that GA101 depletes peripheral B cells better than RTX, which is in line with previous studies comparing the two antibodies (Herter et al. 2013, Mössner et al. 2010). Whether full B cell depletion is required for beneficial effects remains unclear. Incomplete B cell depletion is associated with increased anti-drug antibodies and lower safety profile (Dunn et al. 2018).

We then showed that both anti-CD20 antibodies reduced the extent of microglial activation in this model, which has been shown before in focal models of MS (Anthony, Dickens, et al. 2014). This result is also in line with the decreased MRI activity in MS patients treated with anti-CD20, as microglial activation can be used, like MRI, as a surrogate marker for brain inflammation (Anthony, Dickens, et al. 2014, Montalban et al. 2016, Kappos et al. 2011, Hauser et al. 2008). Interestingly, only treatment with GA101reduced the extent of astrocyte activation, while RTX did not. As discussed before, astrocytes play a wide range of roles in physiology and disease, but their role in MS remains unclear. Why GA101 had an effect on astrocytes and RTX not, could be explained by the effects on brain B and T cells by the different antibodies. Once again, we showed a reduction in brain B cells after anti-CD20 treatment behind an intact BBB, and shrunk the TLS entirely, which impacted on the T cell population as well. Although there was no significant difference between the two treatment groups, GA101 depleted both B and T cells more effectively than RTX in the brain. Anti-CD20 antibodies have been shown to deplete B cells in both the parenchyma and the CSF of patients (Martin et al. 2009, Cross et al. 2006).

Finally, we showed, for the first time, that anti-CD20 (GA101) promoted neuronal survival in DTH-TLS animals, while treatment with RTX did not show significant protection compared to vehicle-treated animals.

Together, these results showed that anti-CD20 treatment reduces neuroinflammation, eliminates TLS, and augments neuronal survival in DTH-TLS mice. In these studies, more complete peripheral B cell depletion with GA101 was associated with a stronger effect on each of these factors compared to treatment with RTX. These results do not inform us whether more complete B cell depletion is necessary to achieve these results, or whether GA101 is a more effective antibody in terms of depleting B cells and exerting its effects on the brain. It is clear that anti-CD20 has impressive effects on neuroinflammation, TLS, and even neuronal survival in this model. Given its histological resemblance to progressive MS, these results show translational promise, although further validation is needed. The higher efficacy of GA101 could make the difference in clinical trials where other anti-CD20 antibodies failed (Hauser et al. 2008, Hawker et al. 2009).

Here, we have developed a model in place that resembles progressive MS with associated TLS. This model requires further characterisation, in terms of the cellular components of the lesion; it needs to be established whether other cell types, such plasma cells and dendritic cells are present in these TLS. Moreover, neuronal loss, or the type of demyelination require more extensive study. Further immunohistochemical analysis will better inform us more about structures and cell types, while other techniques, such as transcriptomics, can provide more information about the molecular events underlying these phenomena.

4.5 Conclusion from this chapter

This chapter describes the development of a new focal model of progressive MS. It models glial activation, as seen in MS, as well as demyelination, neuronal damage, and TLS formation, behind an intact BBB. This model forms a platform to study different aspects of MS and new potential treatments. Here we compare two different anti-CD20 antibodies that are used in the clinic and their effects on the TLS and inflammation in this model. We showed that GA101 causes a more complete peripheral B cell depletion than RTX, which is associated with a larger reduction of the extent of microglial and astrocyte activation, and TLS size in the brain. Finally, we show that GA101 prevents neuronal death in this model. Taken together, these results show that GA101 is likely to be a promising anti-CD20 antibody for the treatment of progressive multiple sclerosis.

5. The effect of anti-CD20 on the acute phase response after acute brain inflammation.

5.1 Introduction and aims

Recent clinical trial results have demonstrated that anti-CD20 therapy reduces new relapses in patients with MS and demonstrate that B cells play a role in the pathophysiology of (Hauser et al. 2016, Montalban et al. 2017). However, the precise mechanism of action remains unknown and it is becoming clearer that a number of unexpected consequences are associated with anti-CD20 therapy that could not have been predicted from our current knowledge of B cell biology. In particular, it has been observed that anti-CD20 therapy can result in neutropenia (Cartron et al. 2014, Chaiwatanatorn et al. 2003). In my own studies, I have discovered that anti-CD20 therapy reduced the number of neutrophils recruited to the liver in response to MS-like inflammatory lesions in the brain. MS patients and animals with EAE are known to have an increased number of neutrophils in the liver, which is thought to be associated with the acute phase response (APR) (Anthony et al. 2012, Campbell et al. 2010).

It is known that CNS injury is followed by a peripheral APR, coordinated primarily in the liver (Allan and Rothwell 2003, Anthony and Couch 2014a). The liver responds to (CNS) injury or infection by producing cytokines, chemokines and other acute phase proteins (Anthony et al. 2012, Campbell et al. 2010, Campbell et al. 2005). Models of stroke and traumatic brain injury are known to generate an APR (Villapol et al. 2015, Chapman et al. 2009). Moreover, Campbell et al. discovered that after a single, focal intracranial injection of IL-1β, an APR is switched on rapidly in the periphery, even prior to any apparent CNS inflammation. While injecting IL-1β into the striatum is a rather reductionist model of CNS inflammation, it has allowed for the dissection of different aspects of the effect of inflammation within the CNS, such as showing the age-dependent changes in the CNS inflammatory response, and the region-specific differences in CNS inflammation along the neuraxis (Anthony et al. 1997, Schnell, Fearn, Schwab, et al. 1999, Sá-Pereira et al. 2018). Furthermore, this model has demonstrated the importance of the hepatic chemokine response to CNS injury. Campbell et al. showed that the

liver produced and released CXCL1, CCL2, acute phase proteins, and other inflammatory mediators, while neutrophils were recruited to the liver, blood, and site of CNS injury in a dose-dependent manner (Campbell et al. 2003, Campbell et al. 2005, Wilcockson et al. 2002). By promoting neutrophil recruitment to the site of injury, the APR may worsen the injury, by contributing to further damage.

Multiple studies have previously shown that modulation of the APR can affect the outcome of acute CNS inflammation. Augmentation the APR can worsen injury, whereas blocking the APR can be protective (Campbell et al. 2003, Campbell, Zahid, et al. 2008, Campbell, Jiang, et al. 2007, Sá-Pereira et al. 2018, Hazelton et al. 2018). Thus, it was important to discover whether anti-CD20 therapy might have a positive effect on the outcome of acute brain injury by modulating the APR.

The principal aims of this chapter were (1) to study the effect of anti-CD20 therapy in a model of acute brain inflammation, (2) to study the effects of anti-CD20 therapy on the APR.

5.2 Methods

5.2.1 Treatment with anti-CD20

Adult huCD20 C57BL/6 mice were used for these studies. On day 0, mice were given a 100µl intravenous injection of either GA101(10mg/kg in saline), or saline via the tail vein under general anaesthesia, as described in section 2.3.4.

5.2.2 Intracranial injection of IL-1β

A week after anti-CD20 treatment, on day 7, all mice received a stereotaxic intracranial injection of IL-1β (10ng in 1µl saline) into the left striatum (Bregma: AP +0.5, ML +1.5, DV -1.5) as described in section 2.3.3.

5.2.3 Tissue collection

Animals were allowed to survive for 4 (n=3 per treatment group), or 24 hours (n=3 per treatment group) after the IL-1β injection, after which they were perfused as described in section 2.6. Histology and RT-qPCR were performed as described in section 2.8 and 2.9 respectively.

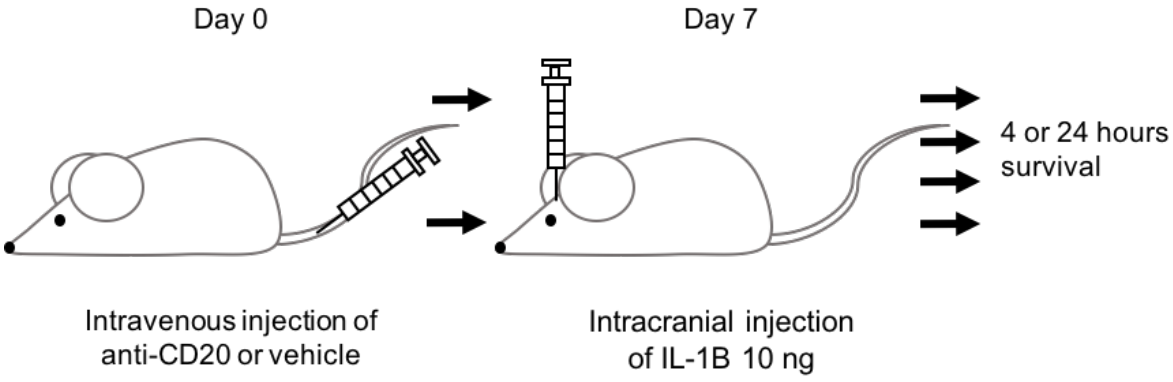


Figure 5.1 Treatment paradigm. HuCD20 mice were treated with GA101 or vehicle on day 0. On day 7, all mice received an intracranial injection of 10ng IL-1β and were allowed to survive for either 4 or 24 hours (n=3 per group).

5.3 Results

5.3.1 A single dose of GA101 depletes peripheral B cells.

To assess the effect of anti-CD20 on the APR after acute brain inflammation, huCD20 mice were treated with a single dose of either GA101 or saline, 7 days before receiving an intracranial injection of IL-1β to evoke an APR. B cells were visualised using immunohistochemistry for anti-B220. A two-way ANOVA with Sidak's post-hoc test revealed that a single dose of GA101 is sufficient to significantly deplete peripheral B cells (Figure 5.2). In the spleen, GA101 therapy significantly reduced the area covered by B cells in splenic germinal centres ($F(1,8) = 105.3$, $p<0.0001$) (Figure 5.2A). At 4 hours post intracranial IL-1β, the B cell area was reduced from $1.791mm^2$ to $0.5973\pm0.202mm^2$ ($p=0.0008$), and from $1.900\pm0.024mm^2$ to $0.1335\pm001mm^2$ at 24 hours post intracranial IL-1β. In the lymph nodes, similar results were observed, with a significant treatment effect ($F(1,8) = 177.1$, $p<0.0001$) (Figure 4.2B). B cell depletion was very similar in the lymph nodes decreasing from $0.6277\pm0.024mm^2$ to $0.1044\pm0.03mm^2$ at 4 hours ($p<0.0001$) and from $0.5160\pm0.063mm^2$ to $0.05585\pm0.003mm^2$ at 24 hours after intracranial IL-1β ($p<0.0001$) (Figure 5.2B).

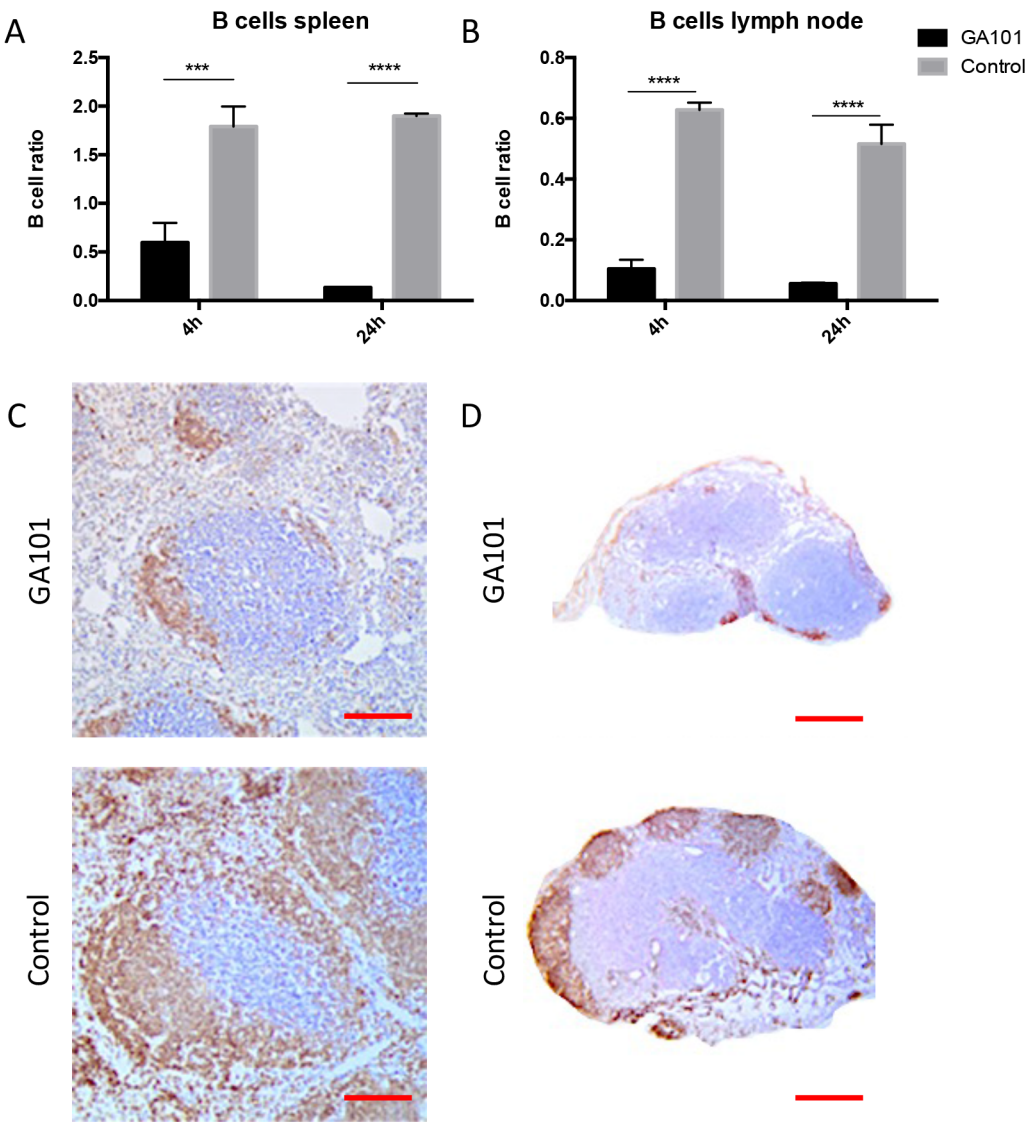


Figure 5.2 A single dose of GA101 significantly depletes peripheral B cells. HuCD20 mice were treated intravenously with either GA101 or a vehicle, followed by an intracranial injection of IL-1β (10ng) on day 7. Four or 24 hours after the intracranial injection, and mice were perfused. Spleen and lymph nodes were immunostained for B cells (anti-B220), and the proportion of B cells in the splenic germinal centres and in the lymph nodes was calculated. A single dose of GA101 caused a reduction in B cells in the spleen (A), and in the lymph nodes (B). (C) Representative photomicrographs of splenic B cells in treated and untreated mice, scale bars represent 200μm. (D) Representative photomicrographs of B cells in the lymph nodes of treated and untreated mice, scale bars represent 500μm. Data are mean ± SEM, ***$p<0.001$, ****$p<0.0001$. Statistics: two-way ANOVA with Sidak's multiple comparisons test (n=3 per time point, per treatment group).

5.3.2 GA101 reduces the number of liver neutrophils, but not Kupffer cells, associated with the hepatic acute phase response after acute brain inflammation.

To examine the effect of GA101 on the APR after brain inflammation, livers were stained for neutrophils (anti-MBS) and Kupffer cells (anti-Iba-1) as surrogate markers of the APR. Cell count analysis revealed that GA101 significantly reduced the number of liver neutrophils ($F(1,8) = 28.37$, $p=0.0007$) (Figure 5.3A). At 4 hours, GA101 reduced the number of neutrophils to $17.00 \pm 2.887/mm^2$ compared to $29.00 \pm 1.00/mm^2$ in control animals ($p=0.008$). At 24 hours, GA101 decreased the number of liver neutrophils to $17.00 \pm 2.646/mm^2$ compared to $27.67 \pm 0.667/mm^2$ in control animals ($p=0.015$) (Figure 5.3A). Furthermore, no reduction in the number of neutrophils was found in the blood or lungs of GA101 treated mice (data not shown).

GA101 did not have a significant effect on Kupffer cells in the liver ($F(1,8)$, $p=0.0923$) (Figure 5.3B). Four hours after intracranial injection of IL-1β, the number of Kupffer cells in GA101 treated animals was $168.33 \pm 5.364/mm^2$, and $202.33 \pm 16.83/mm^2$ in control mice ($p=0.43$). No difference was observed between the GA101 treated group and control group at the 24h time point either ($178.667 \pm 5.783/mm^2$ vs. $218 \pm 33.561/mm^2$ respectively, $p=0.34$) (Figure 5.3B).

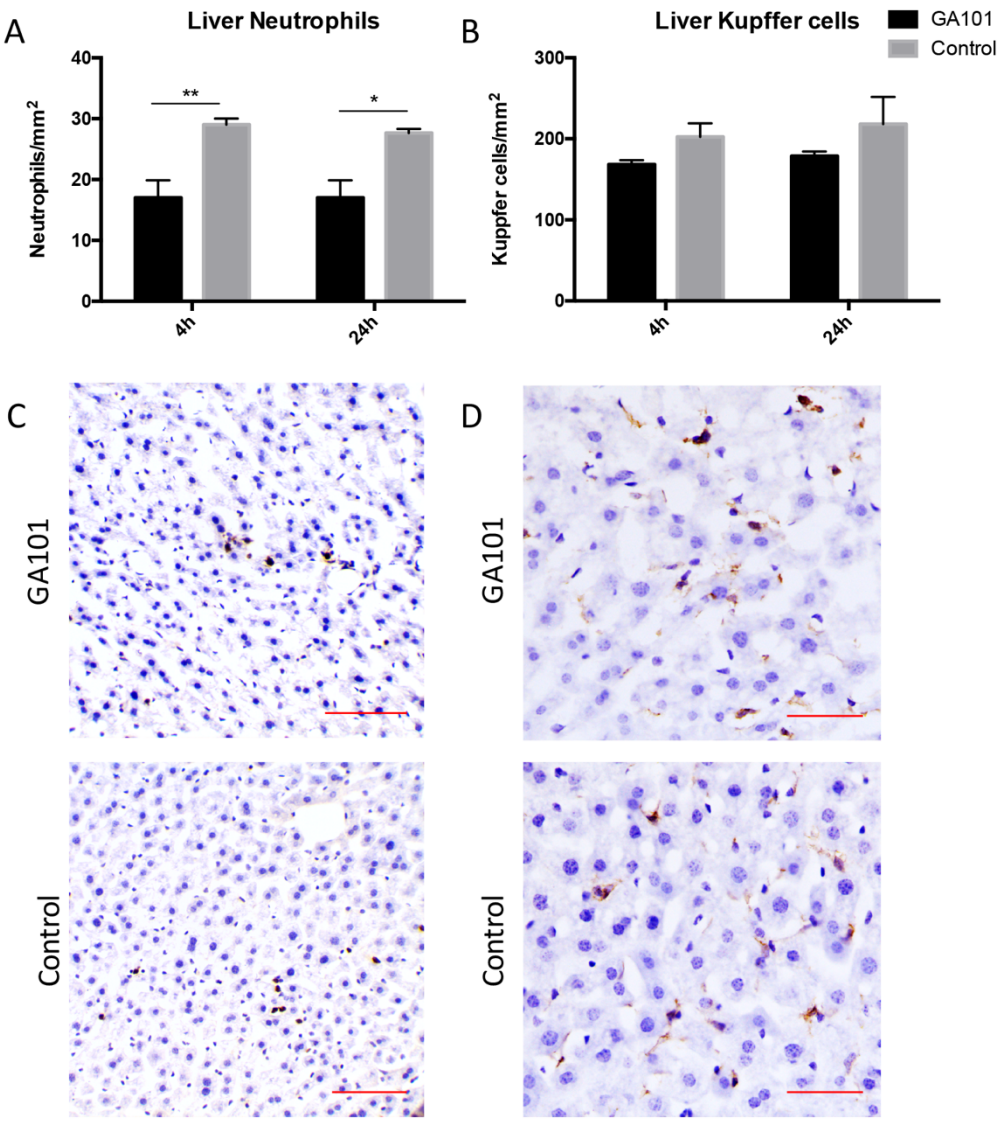


Figure 5.3 GA101 reduces the number of liver neutrophils, but not Kupffer cells, associated with the hepatic acute phase response after acute brain inflammation. HuCD20 mice were treated intravenously with either GA101 or a vehicle, followed by an intracranial injection of IL-1β (10ng) on day 7. Four or 24 hours after the intracranial injection, and mice were perfused. Livers were stained for neutrophils with anti-MBS and for Kupffer cells with anti-Iba-1. GA101 caused a reduction in the number of hepatic neutrophils (A), but not in Kupffer cells (B). (C) Representative photomicrographs of neutrophils in the livers of treated and untreated mice, scale bars represent 100µm. (D) Representative Kupffer cells of treated and untreated mice, scale bars represent 50µm. Data are mean ± SEM *$p<0.05$, **$p<0.01$. Statistics: two-way ANOVA with Sidak's multiple comparisons test (n=3 per time point, per treatment group).

5.3.3 GA101 affects IL1B mRNA expression in the liver after acute brain inflammation.

RT-qPCR was also performed for several inflammatory mediators. The mRNA expression of CXCL1, TNF, SAA, CXCL10, IL1B, CCL2, IL6, and IL10 was measured in snap frozen liver samples of the different treatment groups, and naïve mice. mRNA expression levels were presented as relative fold change compared to naïve mice.

Although only one significant difference between the treatment groups was found using two-way ANOVA with Tukey's post-hoc test (IL1B), multiple differences between the two time points were found. Chemokines CXCL1 and CXCL10 were downregulated at 24h, whereas SAA was upregulated compared to 4h (Figure 4.4). Firstly, there was no overall effect of GA101 treatment on liver IL1B expression ($F(1,7) = 5.154$, $p=0.058$), nor was there a difference between the two time points ($F(1,7(=4.407$, $p=0.074$) (Figure 5.4A). Examination of the treatment effect of GA101 revealed that IL1B mRNA expression was significantly upregulated at 4 hours post intracranial IL-1β compared to control mice (8.245 vs. 1.653 respectively, $p=0.038$). At 24h, there was no difference between the two treatment groups (GA101: 1.903 vs. control: 1.875, $p=0.99$). When comparing the two GA101 groups, IL1B was significantly downregulated at 24h compared to 4h ($p=0.045$) (Figure 5.4A).

Furthermore, CXCL1 was significantly downregulated at 24h compared to 4h ($F(1,7) = 20.20$, $p=0.0028$) (Figure 5.4B). This difference seemed mostly driven by the control groups, which showed a significant decrease in expression at 24h (3.80) compared to 4h (65.327, $p=0.014$). CXCL1 mRNA expression followed a similar pattern in GA101 treated mice (24h: 5.7 vs. 4h: 54.280, $p=0.064$). Treatment had no effect on CXLC1 mRNA expression at 4 or 24 hours ($p=0.81$ and $p=0.99$ respectively).

CXCL10 was also downregulated at 24h compared to 4h (F (1,7) =1.185, p=0.027) (Figure 5.4C). However, GA101 treatment had no effect on CXCL10 expression (F (1,7) =2390, p=0.17) (4h: 2.725 vs. 1.477 in controls, p=0.2264; 24h: 0.883 vs. 0.667 in controls, p=0.93).

Furthermore, GA101 had no effect on SAA expression (F (1,7) =1.368, p=0.28) (Figure 5.4D). No difference in SAA expression was observed between GA101 treated mice and control at 4h (129.715 vs. 149.347 respectively, p=0.98), or at 24h (582.613 vs. 360.680, p=0.18). As for time, SAA was significantly upregulated at 24h compared to 4h across the groups (F (1,7) =14.75, p=0.0064). The expression of SAA mRNA by the liver was increased in the GA101 treated mice (p=0.019), while it did not change in control mice, from 4h to 24h (p=0.21).

No differences were found in TNF mRNA expression between the different treatments (F (1,7) =4.508, p=0.071), or the different time points (F (1,7) =0.7149, p=0.26). GA101 had no effect at 4 hours (2.845 vs. 0.597 in controls, p=0.068), or at 24 hours (1.333 vs. 1.133 in controls, p=0.96) (Figure 5.4E). IL10 mRNA expression was not affected by GA101 either (F (1,7=3.419, p=0.11), and was not different between 4 and 24 hours post intracranial IL-1β (F (1,7) =3.969, p=0.09) (Figure 5.4F). At both time points, IL10 expression was not significantly different between GA101 and control mice (4h: 9.940 vs. 1.193 respectively, p=0.084; 24h: 0.853 vs. 0.810 respectively, p=0.99).

Hepatic IL6 expression was evaluated next, and was not affected by GA101 treatment (F (1,7) =1.20, p=0.30913) (Figure 5.4G). IL6 expression was not different in GA101 treated animals compared to the controls at either time point studied (4h: 0.915 vs. 0.63, p=0.43; 24h: 0.853 vs. 0.810, p=0.97). Finally, CCL2 expression was examined, which showed no differences between GA101 treated mice and controls (F (1,7) =2.269, p=0.18), or between time points (F (1,7) =20.0503, p=0.83) (Figure 5.4H). Again, GA101 did not affect the expression of this chemoattractant at either 4h or 24h compared to controls (4h: 2.090 vs. 0.128, p=0.1350; 24h: 1.193 vs. 1.300, p=0.99). In summary, only IL1B expression was affected by GA101.

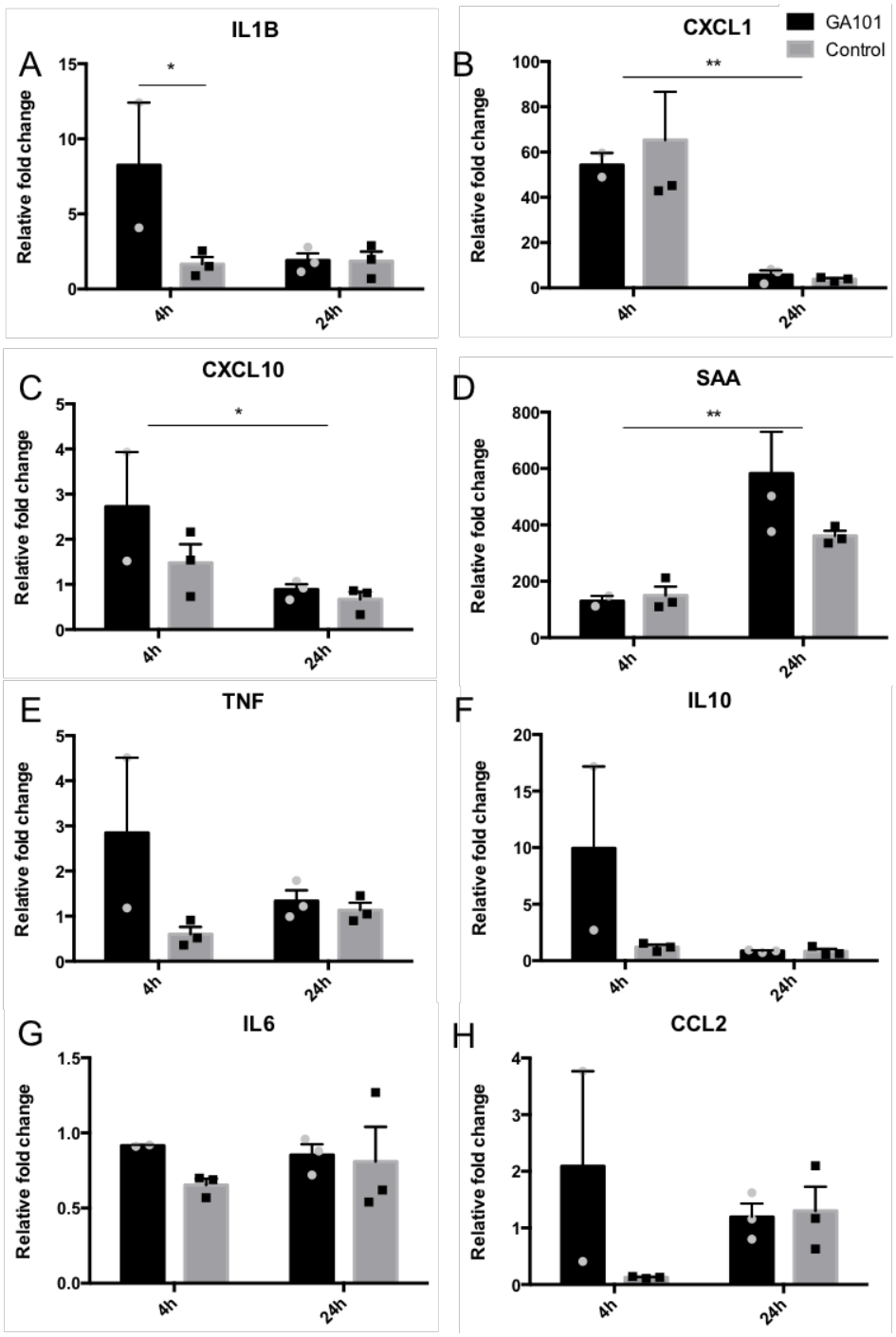


Figure 5.4 GA101 affects IL1B mRNA expression in the liver after acute brain inflammation. HuCD20 mice were treated intravenously with either GA101 or a vehicle, followed by an intracranial injection of IL-1β (10ng) on day 7. Four or 24 hours after the intracranial injection, mice were perfused, and organs were collected and analysed. CXCL1, TNF, CXCL10, SAA, CCL2, IL10, IL1B, AND IL6 mRNA was measured by RT-qPCR in the livers of mice that received 10ng of IL-1β in the brain after being treated with either GA101 or saline. GA101 only had an effect on IL1B expression. CXCL1 an CXCL10 expression was decreased at 24h compared to 4h, while SAA was increased at 24h. Data are represented as relative fold change from expression levels of naïve mice, and normalised to a housekeeping gene (Gapdh). Bars represent mean ± SEM *$p<0.05$, **$p<0.01$. Statistics: two-way ANOVA with Tukey's multiple comparisons test (n=2 for the GA101-4h group, and n=3 for the other groups).

5.3.4 Anti-CD20 does not affect neutrophil recruitment to the brain in an acute brain inflammation model.

Brains were stained for neutrophils (anti-MBS) in order to assess the effect of GA101 on brain inflammation. The number of neutrophils was determined in the striatum of the ipsilateral hemisphere of each animal, by calculating the average of two 10µm sections counted. A two-way ANOVA with Sidak's post-hoc test revealed no treatment effect ($F(1,8) = 0.1088$, $p=0.7500$), but showed an effect of time ($F(1,7) = 6.961$, $p=0.030$) (Figure 5.5A). GA101 did not have an effect on the number of brain infiltrated neutrophils at 4h ($12.333 \pm 3.844/mm^2$ vs. controls $12.667 \pm 3.930/mm^2$, $p=0.99$), or at 24h ($21.667 \pm 1.764/mm^2$ vs. controls $19.333 \pm 1.856/mm^2$, $p=0.84$). No significant difference in neutrophil number was observed within the treatment groups at the different time points (GA101, $p=0.1186$; control, $p=0.29$). A difference was observed in the distribution of the neutrophils at the different time points. At 4h, neutrophils were mainly present in and around blood vessels, whereas at 24h, neutrophils had extravasated the vessels and entered the brain parenchyma (Figure 5.5B&C).

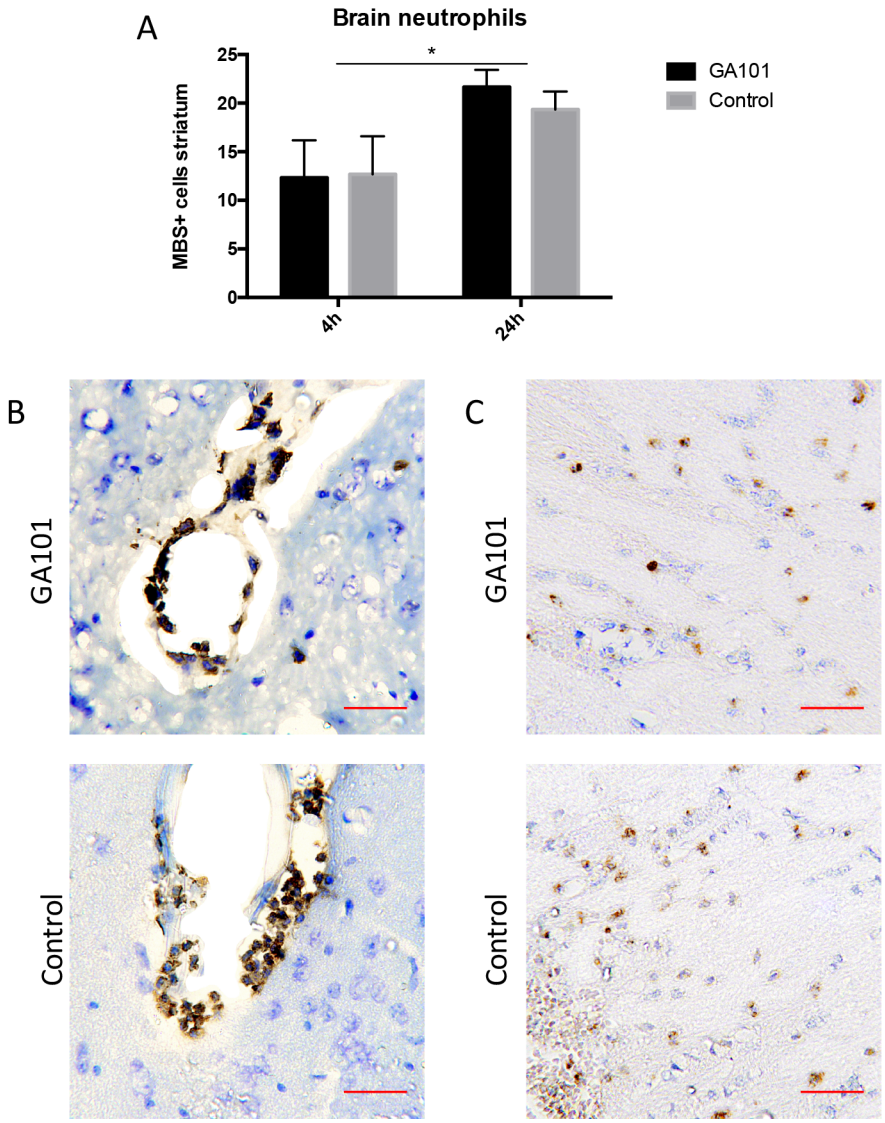


Figure 5.5 Anti-CD20 does not affect neutrophil recruitment in an acute brain inflammation model. HuCD20 mice were treated intravenously with either GA101 or a vehicle, followed by an intracranial injection of IL-1β (10ng) on day 7. Four or 24 hours after the intracranial injection, mice were perfused, and organs were collected and analysed. Brains were stained for neutrophils (anti-MBS) and the total number in the striatum was counted. (A) GA101 did not affect the number of neutrophils in the striatum, however, the number of neutrophils was higher at 24 hours after intracranial IL-1β compared to 4 hours. (B) Representative photomicrographs of brain neutrophils in GA101 treated and control treated animals at 4 hours after intracranial IL-1β. (C) Representative photomicrographs of brain neutrophils in GA101 treated and control treated animals at 24 hours after intracranial IL-1β. Note the neutrophils extravasated the blood vessels at 24 hours and infiltrated the brain parenchyma, scale bars represent 50µm. Data are mean ± SEM *$p<0.05$. Statistics: two-way ANOVA with Sidak's multiple comparisons test (n=3 per time point, per treatment group).

5.3.5 Anti-CD20 does not affect microglial activation in an acute brain inflammation model.

After observing a reduction in microglial activation with GA101 treatment in a focal model for MS, we sought to discover whether the effects were conserved in the acute model of brain inflammation. Coronal sections through the injection site were stained for microglia (anti-Iba1). The total number of activated microglia was counted in the striatum of the ipsilateral hemisphere in two 10μm sections of each animal. A two-way ANOVA with Sidak's post-hoc test revealed no treatment effect ($F(1,8) = 2.52$, $p = 0.15$), but did reveal a significant difference between the two time points ($F(1,8) = 19.03$, $p = 0.0024$) (Figure 5.6A). GA101 did not affect the number of microglia at 4h (221.083±16.873 vs. control: 173.167±36.770, $p = 0.72$), or at 24h (442.667±33.746 vs. control: 346.667±73.660, $p = 0.31$).

Not only had the total number of microglia increased between 4 and 24 hours post the intracranial IL-1β injection, but also more cells exhibited an activated morphology, characterised by retraction and thickening of their processes, and the appearance of an enlarged cell body, were observed at 24h compared to 4h (Figure 5.6B).

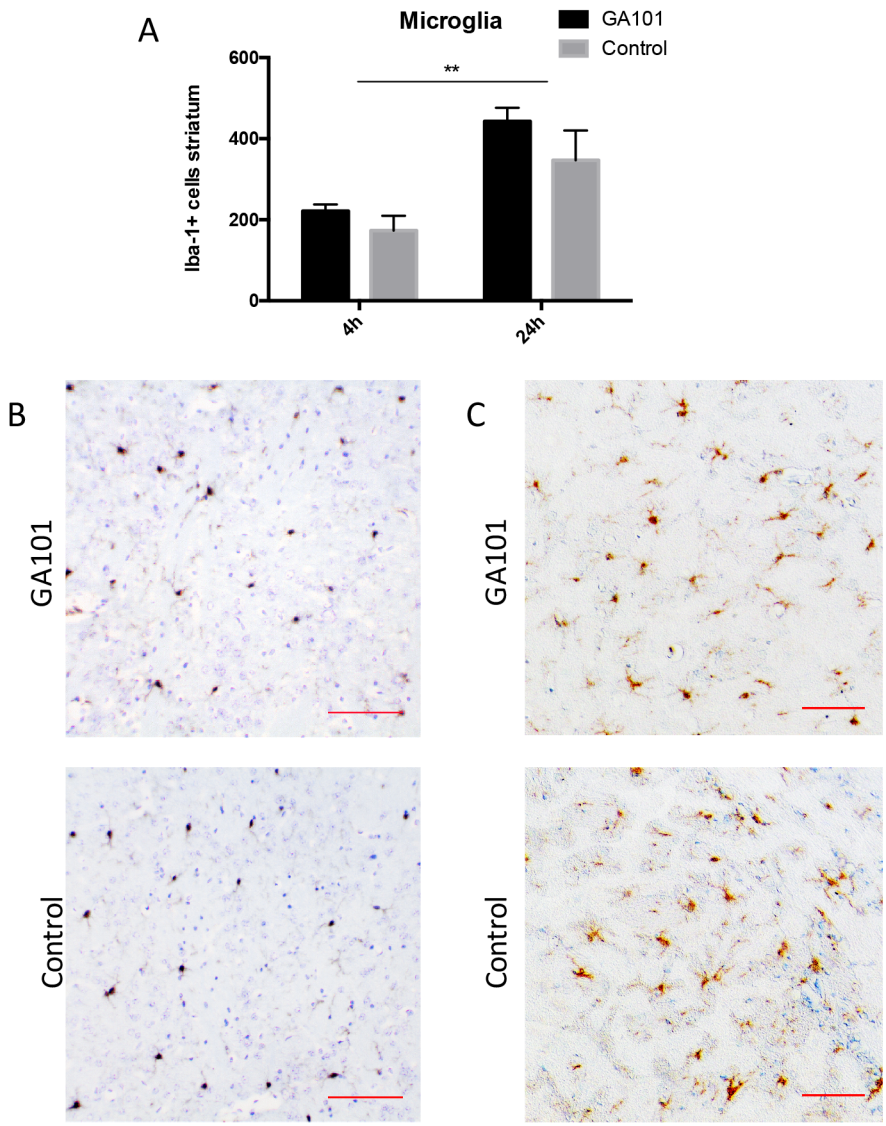


Figure 5.6 Anti-CD20 does not affect microglial activation in an acute brain inflammation model. HuCD20 mice were treated intravenously with either GA101 or a vehicle, followed by an intracranial injection of IL-1β (10ng) on day 7. Four or 24 hours after the intracranial injection, mice were perfused, and organs were collected and analysed. Brains were stained for microglia (anti-Iba1) and the total number in the striatum was counted. (A) GA101 did not affect the number of microglia in the striatum, however, their number was higher at 24 hours after intracranial IL-1β compared to 4 hours. (B) Representative photomicrographs of microglia in GA101 treated and control treated animals at 4 hours after intracranial IL-1β. (C) Representative photomicrographs of microglia in GA101 treated and control treated animals at 24 hours after intracranial IL-1β. Note the difference in microglial morphology between 4 and 24 hours, scale bars represent 100µm. Data are mean ± SEM *p<0.05. Statistics: two-way ANOVA with Sidak's multiple comparisons test (n=3 per time point, per treatment group).

5.4 Discussion

B cells are thought to play a range of roles in MS pathogenesis, including antibody production, antigen presentation, and cytokine production. Targeting B cells using anti-CD20 has been found to be successful in the treatment of MS. Exactly how B-cell depletion or anti-CD20 therapy benefits MS patients is not well-understood. Furthermore, Anti-CD20 is not the only approved therapy affecting B cells, other FDA-approved drugs, such as natalizumab and fingolimod, are also known to alter B cell function (Nakamura et al. 2014, Brown 2009, Putzki et al. 2010). However, simply inhibiting B cells is not necessarily effective: experimental drugs that primarily target B cells, such as atacicept, have been shown to worsen the outcome of MS patients (Kappos et al. 2014). Anti-CD20 therapy has interesting side effects, including neutropenia, and lowering the number of liver neutrophils (Shah et al. 2018, Parodis et al. 2018). This led me to the concept that anti-CD20 therapy may benefit MS patients by modulating the hepatic contribution to the development of chronic MS-like lesions, which is characterised by ongoing, elevated neutrophil recruitment to the liver.

Here, we sought to discover whether anti-CD20 therapy can modulate the APR induced by acute brain inflammation. The model used in these experiments is a well-established model of acute CNS inflammation and the related APR (Blond et al. 2002, Campbell et al. 2003, Campbell et al. 2005). This model was first described by Andersson et al., who were able to show that the inflammatory response in the brain is unique, illustrated by its resistance to leukocyte diapedesis. (Andersson, Perry, and Gordon 1992).

First, it was shown that a single dose of GA101 was efficient to significantly deplete B cells in both the spleen and the lymph nodes of huCD20 mice. In our previous studies, more frequent dosing regimens were used (Chapter 3). GA101 has been tested for B cell depletion in monkeys and in mouse cancer models, in all studies more than one injection dose was used (Dalle et al. 2011, Mössner et al. 2010, Herter et al. 2013, Awasthi et al. 2015). No reports of the effect of

GA101 on huCD20 mice spleen or lymph node depletion could be found in the literature. Our rate of lymph node and spleen B cell depletion is similar to those observed in monkeys that received 2 10mg/kg doses of GA101 (Mössner et al. 2010).

Next, analysis of liver neutrophils revealed that GA101 treatment reduced the number of liver neutrophils at both 4 and 24 hours after induction of CNS inflammation. These results are in line with results we observed before (Chapter 3), and were liver specific; we found no differences in the blood and lungs of GA101 treated animals compared to controls. Furthermore, the number of Kupffer cells, another cell type that increases in number during the APR, was not affected by GA101 therapy (Campbell et al. 2005, Campbell, Zahid, et al. 2008).

Neutrophils do not express CD20 and are thus not a direct target of GA101 therapy. However, neutrophils express Fcγ receptors CD32A and CD16B, which can bind the Fc part of antibodies, and are involved in neutrophil activation and phagocytosis (Golay et al. 2013, Rivas-Fuentes et al. 2010, Tsuboi et al. 2008, Chen et al. 2012, Nagarajan et al. 2000). Moreover, activated neutrophils may also degranulate and die (Dale, Boxer, and Liles 2008, Borregaard 2010). Anti-CD20 treatment could thus cause neutrophil death via Fc binding, which could explain our observation. However, the decrease in neutrophil number was liver specific, and no difference was observed in the blood or lungs of these mice. Moreover, this liver-specific reduction of neutrophils was also observed in mice treated with PGLALA, a variant of GA101 without any antibody-dependent cytotoxicity properties (Chapter 3) (Herter et al. 2018). In other words, activation and subsequent death of neutrophils by GA101 cannot explain the liver specific decrease of neutrophils, suggesting a role for the APR.

To study the effect of anti-CD20 on the APR, qPCR was performed on the livers of GA101 treated mice and controls that all received an intracranial IL-1β injection. The substantial upregulation in CXCL1 and SAA mRNA expression displayed that the APR indeed had been

activated (Uhlar and Whitehead 1999). GA101 had no effect on the mRNA for any of the genes tested, except for IL1B, which was increased in the GA101 group at 4h. Altogether, GA101 had little effect on the mRNA expression of acute phase response mediators. As these studies were underpowered, further experiments, studying more relevant genes for neutrophil recruitment, such as CXCL2, CXCL5, and ICAM1, could tell us whether anti-CD20 affects the other APPs (Campbell et al. 2003, Campbell et al. 2005, Sá-Pereira et al. 2018). Thus it remains unclear how and why GA101 (and other anti-CD20 antibodies) reduce the number of liver neutrophils after acute brain inflammation (and in the MS models).

As anti-CD20 therapy has been proven effective in reducing microglial activation and lymphocyte infiltration in our focal MS-like models, we sought to investigate the effect of this drug on acute brain inflammation (Chapter 3) (Anthony, Dickens, et al. 2014). At 4h after the intracranial IL-1β injection, the few neutrophils present in the brain, were mostly perivascular, while at 24h, many more neutrophils were present in the brain parenchyma. Activated microglia increased in number over time following the IL-1β injection. These results are in line with previous experiments using this model (Blond et al. 2002, Campbell et al. 2003, Campbell, Zahid, et al. 2008, Anthony et al. 1997, Campbell et al. 2002). However, GA101 had no effect on either neutrophil infiltration or microglial activation in this acute brain inflammation model.

Although anti-CD20 has been proven to be effective in chronic autoimmune diseases, including rheumatoid arthritis, B-cell targeting agents have shown mixed results in other models of acute CNS injury, including spinal cord injury, and stroke (Casili et al. 2016, Schuhmann et al. 2017, Monson et al. 2011). B cells are not generally thought to be major contributors to the outcome of acute injuries, though depleting them can be detrimental in acute inflammatory diseases, including sepsis (Kelly-Scumpia et al. 2011, Hotchkiss et al. 2000, Hotchkiss et al. 1999). Indeed, certain B cell populations have been shown to be protective against sepsis (Rauch et al. 2012). This illustrates the variety of different functions that B cells have. These results demonstrated

that anti-CD20 therapy has no effect on the outcome of the IL-1β-induced acute brain lesion. The pathogenic role of B cells in MS remains unclear, and these experiments do not answer the question whether it is B cell depletion, or the off-target effects of anti-CD20, induction of effector cell death for example, that are beneficial for MS patients and rodents with MS-like lesions.

The model used in this chapter has been well studied in terms of the associated APR, but it is not a model of MS. Furthermore, the chronic nature of MS, or even of the focal model described in Chapter 3, is not associated with an 'acute' phase response, though an elevated chemokine production may take place. Although chronic disease is associated with low-key inflammation, relapses can be triggered by activation of the peripheral immune system, during infection for example (Ascherio and Munger 2007, Panitch 1994). Our lab has shown that relapses can be induced in mice with chronic EAE, by giving them an intraperitoneal LPS injection, which switches on the APR (Mardiguian et al. 2017).

This leaves the question: how does anti-CD20 therapy benefit MS patients? Therapies for MS include multiple drugs affecting specific subsets of immune cells. The aggressive autologous haemopoietic stem-cell transplantation (AHSCT) therapy has shown promising results in a subset of patients in clinical trials, and works by completely resetting the immune system (Burt et al. 2009). Yet, depleting some cell types, including plasma cells, exacerbates MS (Kappos et al. 2014). It seems likely that disturbing the disturbed homeostatic balance in pathology, is only beneficial if it is disturbed 'the right way'.

5.5 Conclusions from this chapter

This chapter shows the effect of anti-CD20 (GA101) on the acute phase response, caused by acute brain inflammation. First, it was shown that one dose of GA101 was sufficient to deplete B cells in the spleen and lymph nodes, and that it causes a liver-specific reduction of the number of neutrophils. We then showed that GA101 had little effect on the hepatic APR, and no effect on the extent of microglial activation or neutrophil infiltration. These results show that anti-CD20 therapy has no effect on the outcome of an IL-1β-induced acute brain injury.

6. General discussion and future directions.

The work presented in this thesis describes the development of new focal models for MS, which can be used to investigate specific disease mechanisms associated with chronic demyelinating disease, as well as for an opportunity to explore the efficacy of new therapies. The principal findings of this thesis were as follows:

1. Focal lesions, recapitulating the histopathological hallmarks of progressive MS - including glial activation, demyelination, lymphocyte infiltration, and neuronal loss, can be established in rodents, and, depending on the injection site, such lesions can be associated with meningeal tertiary lymphoid structures.

2. Anti-LINGO1 and anti-CD20 therapy ameliorate the focal MS-like lesions by reducing inflammation, promoting oligodendrocyte or neuronal survival, and, given the results in this thesis, the data provides compelling evidence for their use as new treatment strategies for SPMS.

3. In support of the safety of anti-CD20 therapy, it was found that it does not affect the APR associated with acute brain inflammation, and the therapy does not impact neutrophil infiltration or microglial activation in an acute model.

Overall, the data in this thesis have demonstrated that SPMS-like lesions may be modelled in mice, and the lesions are susceptible to therapy. Thus the model provides a new platform on which to investigate new potential DMTs in SPMS. The pre-existing rodent models of MS do not recapitulate the histopathological features of progressive MS, nor do not lend themselves to the study of chronic disease because of the severity of the associated illness. The most common animal model to study MS remains EAE, and the most commonly used variant used is the classic $MOG_{peptide}$-CFA immunisation, followed by high doses of Pertussis toxin in C57BL/6 mice. Although this method is inexpensive and easy to scale, it merely presents a

monophasic $CD4^+$-T cell mediated disease that randomly targets the CNS (Lassmann and Bradl 2017, Nikić et al. 2011). In the new models described in this thesis, however, MS-like focal lesions are initiated at the site of focal injection of an antigen into a preselected site that allows for accurate lesion localisation. Other focal models of MS do exist, such as the DTH-model described by Matyszak and Perry in 1995, and the fEAE model by Kerschensteiner et al. in 2004 (Matyszak and Perry 1995, Kerschensteiner et al. 2004, Merkler et al. 2006). However, we found that these models do not work well in mice, which limits their use in most transgenic animals. Furthermore, the DTH model lacks an essential component of a progressive MS lesion, which is the presence of B cells (Machado-Santos et al. 2018, Anthony, Dickens, et al. 2014). The models described in this thesis are unique in that the lesions do recruit B cells and continue to evolve over time.

TLS resemble secondary lymphoid organs and are associated with a chronically inflamed environment, and have been associated with multiple autoimmune diseases, including rheumatoid arthritis, Sjögren's syndrome, myasthenia gravis, and cancer, but also in allograft rejections and chronic infection (Humby et al. 2009, Bombardieri and Pitzalis 2012, Ladányi et al. 2007, Dieu-Nosjean et al. 2008, Kerjaschki et al. 2004, Thaunat et al. 2005, Moyron-Quiroz et al. 2004) In MS, TLS are found in the subarachnoid space of SPMS patients, are associated with a more severe disease course, and are thought to contribute to cortical damage and neuronal loss. (Serafini et al. 2004, Magliozzi et al. 2007). Gardner et al. modified the fEAE model, by injecting the cytokine mixture into the subarachnoid space of Dark Agouti rats instead of the cortex, in an attempt to generate TLS in a MOG-immunised animal (Gardner et al. 2013, Kerschensteiner et al. 2004, Merkler et al. 2006). This model produced meningeal inflammation, microglial activation and cortical demyelination around the injection site. The pathology peaks at 7 days, but at 21 days post cytokine injection, the lesion had almost entirely resolved (Gardner et al. 2013). Lesions in the new DTH-TLS model, reported here for the first time, continue to

grow and evolve over time. Moreover, the associated TLS show B cell proliferation and T and B cell compartmentalisation, which are both essential features of the TLS (Magliozzi et al. 2010).

Histologically, the TLS in the DTH-TLS model appears to resemble those seen in SPMS. The model may overrepresent the proportion of B cells in the structure, however, in other autoimmune conditions, a larger B-cell proportion is associated with a more severe lesion (Christodoulou, Kapsogeorgou, and Moutsopoulos 2010). Other cell populations still need to be identified in the lesions of the models described in this thesis, but it is important to note that the loss of neurons underlying the TLS is also a feature of the new model and of the human pathology. The cell populations responsible for the neuronal death will be the subject of downstream investigations. Plasma cells and (follicular) dendritic cells have been shown to be present in the TLS in SPMS patients (Magliozzi et al. 2010, Howell et al. 2011). TLS have been better characterised in other autoimmune diseases such as Sjögren's syndrome, where they are known to also feature high endothelial venules, lymphatic vessels, and an array of chemoattractants and cell-adhesion molecules (Yoshida et al. 2002, Tang et al. 2017, Christodoulou, Kapsogeorgou, and Moutsopoulos 2010). These markers are yet to be defined in SPMS and in the models described in this thesis.

The models described in this thesis feature histological characteristics that are similar to SPMS, yet a decades-long disease can never be entirely mimicked in rodents. Unlike in SPMS, the lesions in our models are clinically silent. Focal models of MS do not generate obvious clinical symptoms, as they usually target clinically silent structures in the CNS (Matyszak and Perry 1995, Merkler et al. 2006). In MS, clinically silent lesions also exist (Okuda et al. 2009, Kantarci et al. 2016). However, the clinical symptoms are highly variable and the nature of the clinical symptoms is dependent on where the lesions appear in the CNS (Hauser and Oksenberg 2006). Patients with (motor)disabilities have many different lesions, whereas in focal models, rodents merely have a unilateral lesion, allowing for compensatory mechanisms to hide the effects of

neuronal loss. However, the focal and reproducible histological features of the model afford an opportunity to employ histological endpoints to investigate the impact of therapy in a way that is not possible with traditional EAE models.

Little is known about the role of TLS in SPMS, due to the limited availability of post-mortem tissue and suitable imaging techniques (Filippi et al. 2012, Absinta et al. 2015, Makshakov et al. 2017, Popescu et al. 2015, Kilsdonk, Schoonheim, and Wattjes 2017). At present, no imaging technique can offer the necessary resolution (PET, SPECT, or MRI). In other autoimmune diseases, the presence of TLS is associated with faster disease progression owning to the increased production of proinflammatory cytokines, altered production of survival factors, and pathogenic antibody production (Pipi et al. 2018, Pitzalis et al. 2014). In SPMS, TLS are thought to contribute to neuronal damage, by releasing proinflammatory molecules, such as cytokines, metalloproteinases, and reactive oxygen species (Howell et al. 2011, Magliozzi et al. 2018). The DTH-TLS model described in this thesis can be used in the future to investigate how the presence of TLS affect neuronal survival using modern *in vivo* imaging techniques for meningeal inflammation (2/3-photon in vivo microscopy for example). Such imaging techniques could be used to better understand the role of TLS in MS, as it remains unclear whether TLS are active contributors to a proinflammatory milieu, and are causative to the associated cortical damage, or whether they are only formed in areas where there is excessive inflammation and cortical cell death, and merely act as bystanders. In the future, it would be of interest to discover what the different subsets of TLS cell types are, what they produce, how they interact, and to find mechanisms through which they can contribute to MS pathology.

The work presented in this thesis showed that the formation of TLS in the brain is dependent on the location of the lesion. When MOG-immunised mice received an intracranial injection of heat-killed TB into the striatum, a MS-like lesion developed, but no TLS were observed. When the TB was injected into the piriform cortex, however, a similar MS-like lesion developed,

though this time in association with TLS. Interestingly, if the intrastriatal injection is moved further caudal, and towards the pallidum, meningeal TLS form in the third ventricle. This indicates that TLS will form in close proximity to an initial lesion, rather than in a more disseminated fashion. Though further experiments should be performed by injecting heat-killed TB in the subarachnoid space of MOG-immunised mice, to investigate whether meningeal TLS can induce a lesion in the adjacent cortex.

Following the characterisation of the models, I sought to discover how these new chronic rodent models would respond to therapy as part of the search for an effective DMT for SPMS. DMTs have been shown to only be partially effective in SPMS, though siponimod has recently been FDA-approved for the treatment of patients with *active* SPMS (Kappos et al. 2018, 1998, Panitch et al. 2004). Siponimod significantly reduced the confirmed disease progression compared to placebo in SPMS patients (Kappos et al. 2018). There is a variety of reasons why DMTs are less effective in SPMS. Firstly, the intact BBB is a barrier to most therapy to enter the CNS. In addition, efflux transporters actively pump antibody out of the CNS, and there is no know transporter responsible for the transit of antibodies into the CNS (Banks 2016, Freskgård and Urich 2017). Furthermore, lost brain cannot be replaced. The functional reserve of SPMS patients' brain has been exhausted, leaving no room to reverse the progressing disability, and leaving immunomodulators ineffective.

Two types of therapy were studied in this thesis: an immunomodulatory therapy, anti-CD20, and a restorative therapy, anti-LINGO-1. Anti-CD20 therapy targets the B cell lineage from pro-B cells to plasmablasts. B cells are thought to play a number of roles in MS pathogenesis, including antigen presentation, cytokine production, and (as plasma cells) antibody secretion (Fraussen, de Bock, and Somers 2016). Moreover, B cells from MS patients have been shown to have a more inflammatory expression profile (Bar-Or et al. 2010, Li, Rezk, Miyazaki, et al.

2015). Multiple anti-CD20 antibodies have been in clinical trials for MS, including rituximab (RTX), ocrelizumab (OCR), ofatumumab (OFA), and ublituximab (UBX).

Although trials with RTX did not reach their primary endpoint, anti-CD20 therapy (OCR) has been shown to be effective in RRMS and PPMS and has been FDA-approved (Hauser et al. 2008, Hawker et al. 2009, Hauser et al. 2016, Montalban et al. 2016). Even though the ONATARIO trial and the ongoing CONSONANCE trial have some SPMS patients on their trial as well, no clinical trial with an anti-CD20 therapy has been undertaken with SPMS patients exclusively (Hauser et al. 2016). Off-label treatment with RTX, has been reported to be beneficial for a proportion of SPMS patients (Rommer et al. 2011, Salzer et al. 2016).

The anti-CD20 antibody studied in this thesis was GA101, a novel type II antibody, which means that it has direct-cell killing abilities as well as ADCC and CDC mechanisms (Mössner et al. 2010, Herter et al. 2013). These direct cell-killing properties are especially beneficial when the target tissue does not support ADCC or CDC particularly well, as is the case in the CNS. Under physiological conditions, the synthesis of complement in the brain is very low or even undetectable. CDC is dependent on the classical complement pathway and triggered when C1q proteins bind to the Fc region of a target-bound antibody. This subsequently leads to the formation of C3 and C5, and eventually to the membrane attack complex, which causes target cell lysis (Tobinai et al. 2017). A downside is that complement activation is correlated with infusion toxicity / infusion reactions (Wang and Weiner 2008).

The impressive impact of the direct cell-killing properties was first demonstrated by the reduction of neuroinflammation in the DTH-MOG model after PGLALA therapy. PGLALA, a variant of GA101 has abolished ADCC and CDC activities, hence exclusively kills B cells by direct cell-death (Herter et al. 2018). In certain cancer models, PGLALA has been shown to be as effective as RTX in depleting B cells and anti-tumour activity (Herter et al. 2018). In this thesis PGLALA was shown to reduce the extent of microglial and astrocyte activation, and

lymphocyte infiltration in the DTH-MOG model. A reduction of microglial activation and T cell infiltration after anti-CD20 therapy has been reported in previous work from my laboratory in a rat focal model of MS (Anthony, Dickens, et al. 2014). The reduction in glial reactivity could be caused by downstream consequences of peripheral immunomodulatory effects of anti-CD20.

PGLALA was further shown to reduce the number of brain B cells. This is in keeping with the observation that anti-CD20 was able to decrease the number of CNS B cells in EAE (Monson et al. 2011). Finally, PGLALA was found to reduce astrocyte activation in this model, a finding that has not been observed or discussed in the literature.

In chapter 4, the utility of anti-CD20 therapy was further investigated by comparing the efficacy of the type II antibody GA101 with the type I antibody RTX in the DTH-TLS model. First, it was shown that GA101 depletes peripheral B cells in the spleen and lymph nodes better than RTX, which was in keeping with previous studies (Herter et al. 2013, Awasthi et al. 2015, Herting et al. 2014). In the brain, both antibodies reduced the extent of microglial activation, which was in agreement with the results in chapter 3 and previous studies (Anthony, Dickens, et al. 2014). Intriguingly, only GA101 managed to reduce the extent of astrocyte activation, whereas RTX did not. As mentioned before, the effect of anti-CD20 therapy on astrocyte behaviour has not been reported before. In MS, astrocytes are known to become reactive and secrete cytokines, including TNFα, IL-1β, IL-6, and CXCL12, contributing to a proinflammatory milieu and further activation of glial cells (Tanuma et al. 2006, Ludwin et al. 2016, Rothhammer and Quintana 2015, Selmaj et al. 1991). Furthermore, a previous study showed that blocking NFκB in astrocytes specifically, for example, caused amelioration of EAE (Brambilla et al. 2014). Although astrocytes also have roles in neuronal support and repairs as well, under the pathogenic conditions in MS astrocytes are less likely to perform these functions effectively, and to contribute to further damage instead (Moore, Milner, et al. 2011, Moore,

Abdullah, et al. 2011, Ludwin et al. 2016). Thus, the reduction in astrocyte activation after GA101 treatment is likely to be beneficial.

It was further shown that both anti-CD20 antibodies significantly reduced the number of B and T cells within the focal lesions in the brain. Even though there was no significant difference between the two antibodies, the magnitude of the response to GA101 was greater for lymphocyte reduction, and it shrunk the TLS almost entirely (visibly more B and T cells were present after RTX treatment). While anti-CD20 therapy has been shown to deplete CNS B cells in the past, the results that are achievable using RTX to target TLS in other diseases has been variable (Vos et al. 2007, Regard et al. 2018, Thaunat et al. 2008, Quartuccio et al. 2008, Martin et al. 2009). The superior effectiveness of GA101 was highlighted in its ability to promote neuronal survival in the DTH-TLS model. In this study, RTX therapy did not achieve this result. The ability of anti-CD20 to prevent neuronal loss has not been reported elsewhere. Though it could be argued that in humans, lack of disease progression, or a decreased rate of CNS atrophy after anti-CD20 therapy, could be viewed as neuroprotective (Hauser et al. 2016, Montalban et al. 2016). Perhaps, the more effective B-cell depleting capacities of GA101 will also be beneficial in other diseases featuring TLS.

One of the remaining questions is how exactly anti-CD20 therapy benefits patients. This thesis describes the reduction of glial activation, lymphocyte infiltration, and neuronal death after anti-CD20 therapy. Although the BBB shows no leakage in the models in this thesis, we still see an impressive reduction of both B and T cells in the brains of these mice. This could have to do with anti-CD20 therapy working systemically, depleting and altering the downstream functions of peripheral B cells. From studies on lymphoid tissues in animals, and on the blood of patients, we know that the subsets of B cells as well as their expression profile changes after anti-CD20 therapy (Monson et al. 2005, Häusler et al. 2018). Furthermore, B cells are known to be able to

travel between the CNS and the periphery (Eggers et al. 2017). This provides a rationale to study the effect of B cell depletion peripherally.

This thesis describes some unexpected peripheral effects of anti-CD20. In particular, we consistently found that the number of liver neutrophils was decreased after anti-CD20 treatment. People with MS, mice with EAE, and mice with the models described in this thesis, are known to have an increased number of liver neutrophils, which we might be associated with a '*chronic'* APR (Campbell, Zahid, et al. 2008, Mardiguian et al. 2017). To investigate the role of B cells or anti-CD20 therapy in the APR, mice were treated with anti-CD20 before receiving an intracranial IL-1β injection, a well-established model of acute brain inflammation. This model was chosen because my laboratory has extensively characterised the local and systemic APR to a focal injection of IL-1β into the brain. In my studies, a similar reduction in liver neutrophils was observed following anti-CD20 treatment. However, little difference was observed in the APR, or the brain lesions of anti-CD20 treated mice. These studies showed that anti-CD20 had no effect on the model of acute brain inflammation, and provided no further insight into how anti-CD20 causes a liver-specific decrease in the number of neutrophils.

Although there are no reports of the impact of anti-CD20 on the cellularity of the liver, anti-CD20 therapy is not the only drug known to cause neutropenia; for example, alemtuzumab has been reported to cause neutropenia (Gaitán, Ysrraelit, and Correale 2017, Tanaka, Park, and Tanaka 2013). To better understand the potential role of anti-CD20 in reducing the number of liver neutrophils in MS, the hepatic chemokine response should first be better characterised in MS, or in a model of MS, as this is likely to be very different from an APR generated by acute inflammation, or injury. Furthermore, different methods of depleting B cells, such as CART (chimeric antigen receptor T cells), could be used to investigate whether it is the lack of B cells, or the therapy *per se* that causes the reduction of liver neutrophils. The impact of anti-CD20 therapy on the liver neutrophil population remains entirely unexpected, as the proposed

mechanism of action of anti-CD20 in MS is still regarded, principally, as being a consequence of the depletion of autoreactive B cells.

The search for therapy to remyelinate the brain as a different approach to the treatment of MS, has existed since the development of the first MS models. Remyelination therapy has been more studied as a therapy than immunomodulation. This thesis describes the effects of anti-LINGO-1 in the DTH-MOG model. The drug works by inhibiting the tetramerisation of LINGO-1 into its active form, a negative regulator of myelination and oligodendrocyte differentiation, and gave promising results in EAE studies (Mi et al. 2004, Mi et al. 2005). As a consequence, the SYNERGY trial was established and anti-LINGO-1 became the first remyelinating therapy to be tested in humans (Tran et al. 2014). Although the SYNERGY trial did not reach its primary endpoint, another trial, AFFINITY, has been investigating the effect of anti-LINGO-1 as an add-on therapy to anti-inflammatory drugs for MS (Calabresi 2018, Biogen 2018).However, until the effect of anti-LINGO-1 was examined here, there were no studies in progressive-like disease animals or in humans.

In this thesis, it was shown that anti-LINGO-1 adjunct therapy improves myelin mRNA expression, and the proliferation and differentiation of oligodendrocytes in the presence of anti-inflammatory steroid therapy. LINGO-1 inhibition has previously been shown to promote myelination and oligodendrocyte differentiation in different rodent models (Mi et al. 2007, Sun et al. 2015). Furthermore, it has been shown to ameliorate EAE, but the mechanisms were unclear (Mi et al. 2007, Mi et al. 2009). Interestingly, the work in this thesis also showed that anti-LINGO-1 therapy further reduced neuroinflammation in this model, as demonstrated by a reduction in microglial and astrocyte activation in this model, compared to steroid treated, but anti-LINGO-1 naïve animals. Even though it is expressed on microglia and astrocytes, LINGO-1, to date, has not been reported by others to have the type of immunomodulatory functions that are reported in this thesis (Satoh et al. 2007, Ukai et al. 2016).

Whether anti-LINGO-1 therapy affects inflammation directly, or indirectly, by reducing primary tissue destruction, which, in turn, suppresses secondary, bystander damage, cannot be answered in this work. However, the positive effect on both myelin repair and inflammation makes anti-LINGO-1 therapy a worthwhile target for further study. Future research might now focus on how to promote drug delivery to the target tissue, the CNS. Improved CNS availability of the drug could be achieved by using a 'brain shuttle,' for example, or by intrathecal administration (Freskgård and Urich 2017, Niewoehner et al. 2014).

6.1 Concluding remarks

The data in this thesis describes the development of new focal models of SPMS, explores new therapies, and looks into how these therapies may benefit patients with SPMS. First, I produced a new focal rodent model that resembles SPMS histologically, featuring microglial and astrocyte activation, B and T cell infiltration, demyelination, and neuronal loss behind a closed BBB, which is unlike any previously published model. The focal model allows for exact preselection of the lesion site. It was then shown that the lesion site determines the presence of TLS, as lesions in the cortex were associated with meningeal TLS, whereas striatal lesions were not. The TLS associated with the focal MS-like lesions exhibited crucial TLS components, such as proliferating B cells and B and T cell compartmentalisation. These new models now provide a new platform on which to investigate mechanisms behind lesion progression, TLS formation, and new potential DMTs in SPMS.

Next, I used the models to investigate the effect of two different therapies – anti-LINGO-1, a remyelinating therapy, and anti-CD20, an immunomodulatory therapy. I showed that treatment with anti-LINGO-1 not only promoted myelination and oligodendrocyte differentiation, but I also showed, for the first time, that this treatment reduces neuroinflammation in the MS model. Future trials with anti-LINGO-1 should now also consider other outcomes, such as the number of gadolinium enhanced lesions.

Furthermore, anti-CD20 therapy was studied in the new MS models. It was shown that anti-CD20 therapy reduced glial activation, and drastically reduced the number of brain B and T cells. Moreover, TLS associated with these lesions, were almost entirely eliminated after anti-CD20 therapy. Furthermore, for the first time, I showed that anti-CD20 therapy worked as a neuroprotective in a model of SPMS. This data supports the consideration of a trial with anti-CD20 (GA101) for the treatment of SPMS.

Finally, I explored whether anti-CD20 on the APR after acute brain inflammation, as the number of liver neutrophils was consistently reduced after anti-CD20 therapy in mice with the MS model. These data showed that anti-CD20 had no effect on acute inflammatory brain lesions, or on the associated APR.

6.2 Bibliography

1998. "Placebo-controlled multicentre randomised trial of interferon beta-1b in treatment of secondary progressive multiple sclerosis. European Study Group on interferon beta-1b in secondary progressive MS." *Lancet* 352 (9139):1491-7.

Absinta, M., L. Vuolo, A. Rao, G. Nair, P. Sati, I. C. Cortese, J. Ohayon, K. Fenton, M. I. Reyes-Mantilla, D. Maric, P. A. Calabresi, J. A. Butman, C. A. Pardo, and D. S. Reich. 2015. "Gadolinium-based MRI characterization of leptomeningeal inflammation in multiple sclerosis." *Neurology* 85 (1):18-28. doi: 10.1212/WNL.0000000000001587.

Aguzzi, A., B. A. Barres, and M. L. Bennett. 2013. "Microglia: scapegoat, saboteur, or something else?" *Science* 339 (6116):156-61. doi: 10.1126/science.1227901.

Al-Izki, S., G. Pryce, J. K. O'Neill, C. Butter, G. Giovannoni, S. Amor, and D. Baker. 2012. "Practical guide to the induction of relapsing progressive experimental autoimmune encephalomyelitis in the Biozzi ABH mouse." *Mult Scler Relat Disord* 1 (1):29-38. doi: 10.1016/j.msard.2011.09.001.

Allan, S. M., and N. J. Rothwell. 2003. "Inflammation in central nervous system injury." *Philos Trans R Soc Lond B Biol Sci* 358 (1438):1669-77. doi: 10.1098/rstb.2003.1358.

Aloisi, F., and R. Pujol-Borrell. 2006. "Lymphoid neogenesis in chronic inflammatory diseases." *Nat Rev Immunol* 6 (3):205-17. doi: 10.1038/nri1786.

Alping, P., T. Frisell, L. Novakova, P. Islam-Jakobsson, J. Salzer, A. Björck, M. Axelsson, C. Malmeström, K. Fink, J. Lycke, A. Svenningsson, and F. Piehl. 2016. "Rituximab versus fingolimod after natalizumab in multiple sclerosis patients." *Ann Neurol* 79 (6):950-8. doi: 10.1002/ana.24651.

Altmann, D. R., B. Jasperse, F. Barkhof, K. Beckmann, M. Filippi, L. D. Kappos, P. Molyneux, C. H. Polman, C. Pozzilli, A. J. Thompson, K. Wagner, T. A. Yousry, and D. H. Miller. 2009. "Sample sizes for brain atrophy outcomes in trials for secondary progressive multiple sclerosis." *Neurology* 72 (7):595-601. doi: 10.1212/01.wnl.0000335765.55346.fc.

Alvarez, J. I., R. Cayrol, and A. Prat. 2011. "Disruption of central nervous system barriers in multiple sclerosis." *Biochim Biophys Acta* 1812 (2):252-64. doi: 10.1016/j.bbadis.2010.06.017.

Anderson, M. A., J. E. Burda, Y. Ren, Y. Ao, T. M. O'Shea, R. Kawaguchi, G. Coppola, B. S. Khakh, T. J. Deming, and M. V. Sofroniew. 2016. "Astrocyte scar formation aids central nervous system axon regeneration." *Nature* 532 (7598):195-200. doi: 10.1038/nature17623.

Andersson, P. B., V. H. Perry, and S. Gordon. 1992. "Intracerebral injection of proinflammatory cytokines or leukocyte chemotaxins induces minimal myelomonocytic cell recruitment to the parenchyma of the central nervous system." *J Exp Med* 176 (1):255-9.

Ando, T., R. R. Langley, Y. Wang, P. A. Jordan, A. Minagar, J. S. Alexander, and M. H. Jennings. 2007. "Inflammatory cytokines induce MAdCAM-1 in murine hepatic endothelial cells and mediate alpha-4 beta-7 integrin dependent lymphocyte endothelial adhesion in vitro." *BMC Physiol* 7:10. doi: 10.1186/1472-6793-7-10.

Anthony, and Yvonne Couch. 2014a. "The systemic response to CNS injury." *Experimental Neurology* 258:105-111. doi: http://dx.doi.org/10.1016/j.expneurol.2014.03.013.

Anthony, D. C., S. J. Bolton, S. Fearn, and V. H. Perry. 1997. "Age-related effects of interleukin-1 beta on polymorphonuclear neutrophil-dependent increases in blood-brain barrier permeability in rats." *Brain* 120 (Pt 3):435-44.

Anthony, D. C., and Y. Couch. 2014b. "The systemic response to CNS injury." *Exp Neurol* 258:105-11. doi: 10.1016/j.expneurol.2014.03.013.

Anthony, D. C., Y. Couch, P. Losey, and M. C. Evans. 2012. "The systemic response to brain injury and disease." *Brain Behav Immun* 26 (4):534-40. doi: 10.1016/j.bbi.2011.10.011.

Anthony, D. C., A. M. Dickens, N. Seneca, Y. Couch, S. Campbell, B. Checa, V. Kersemans, E. A. Warren, M. Tredwell, N. R. Sibson, V. Gouverneur, and D. Leppert. 2014. "Anti-CD20 inhibits T cell-mediated pathology and microgliosis in the rat brain." *Ann Clin Transl Neurol* 1 (9):659-69. doi: 10.1002/acn3.94.

Anthony, D. C., N. R. Sibson, P. Losey, D. P. Meier, and D. Leppert. 2014. "Investigation of immune and CNS-mediated effects of fingolimod in the focal delayed-type hypersensitivity multiple sclerosis model." *Neuropharmacology* 79:534-41. doi: 10.1016/j.neuropharm.2013.12.022.

Anthony, D. C., N. R. Sibson, M. A. McAteer, B. Davis, and R. P. Choudhury. 2011. "Detection of brain pathology by magnetic resonance imaging of iron oxide micro-particles." *Methods Mol Biol* 686:213-27. doi: 10.1007/978-1-60761-938-3_9.

Antila, S., S. Karaman, H. Nurmi, M. Airavaara, M. H. Voutilainen, T. Mathivet, D. Chilov, Z. Li, T. Koppinen, J. H. Park, S. Fang, A. Aspelund, M. Saarma, A. Eichmann, J. L. Thomas, and K. Alitalo. 2017. "Development and plasticity of meningeal lymphatic vessels." *J Exp Med* 214 (12):3645-3667. doi: 10.1084/jem.20170391.

Anwar Jagessar, S., Z. Fagrouch, N. Heijmans, J. Bauer, J. D. Laman, L. Oh, T. Migone, E. J. Verschoor, and B. A. 't Hart. 2013. "The different clinical effects of anti-BLyS, anti-APRIL and anti-CD20 antibodies point at a critical pathogenic role of γ-herpesvirus infected B cells in the marmoset EAE model." *J Neuroimmune Pharmacol* 8 (3):727-38. doi: 10.1007/s11481-013-9448-6.

Ascherio, A., and K. L. Munger. 2007. "Environmental risk factors for multiple sclerosis. Part I: the role of infection." *Ann Neurol* 61 (4):288-99. doi: 10.1002/ana.21117.

Ascherio, A., K. L. Munger, and K. C. Simon. 2010. "Vitamin D and multiple sclerosis." *Lancet Neurol* 9 (6):599-612. doi: 10.1016/S1474-4422(10)70086-7.

Aspelund, A., S. Antila, S. T. Proulx, T. V. Karlsen, S. Karaman, M. Detmar, H. Wiig, and K. Alitalo. 2015. "A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules." *J Exp Med* 212 (7):991-9. doi: 10.1084/jem.20142290.

Atalar, A., Y. Erdal, B. Tekin, M. Yıldız, Ö Akdoğan, and U. Emre. 2018. "Olfactory dysfunction in multiple sclerosis." *Mult Scler Relat Disord* 21:92-96. doi: 10.1016/j.msard.2018.02.032.

Attwell, D., A. M. Buchan, S. Charpak, M. Lauritzen, B. A. Macvicar, and E. A. Newman. 2010. "Glial and neuronal control of brain blood flow." *Nature* 468 (7321):232-43. doi: 10.1038/nature09613.

Awasthi, A., J. Ayello, C. Van de Ven, M. Elmacken, A. Sabulski, M. J. Barth, M. S. Czuczman, H. Islam, C. Klein, and M. S. Cairo. 2015. "Obinutuzumab (GA101) compared to rituximab significantly enhances cell death and antibody-dependent cytotoxicity and improves overall survival against CD20(+) rituximab-sensitive/-resistant Burkitt lymphoma (BL) and precursor B-acute lymphoblastic leukaemia (pre-B-ALL): potential targeted therapy in patients with poor risk CD20(+) BL and pre-B-ALL." *Br J Haematol* 171 (5):763-75. doi: 10.1111/bjh.13764.

Babiker, H. M., A. E. Glode, L. S. Cooke, and D. Mahadevan. 2018. "Ublituximab for the treatment of CD20 positive B-cell malignancies." *Expert Opin Investig Drugs* 27 (4):407-412. doi: 10.1080/13543784.2018.1459560.

Ballabh, P., A. Braun, and M. Nedergaard. 2004. "The blood-brain barrier: an overview: structure, regulation, and clinical implications." *Neurobiol Dis* 16 (1):1-13. doi: 10.1016/j.nbd.2003.12.016.

Banks, W. A. 2016. "From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery." *Nat Rev Drug Discov* 15 (4):275-92. doi: 10.1038/nrd.2015.21.

Bar-Or, A., P. A. Calabresi, D. Arnold, D. Arnlod, C. Markowitz, S. Shafer, L. H. Kasper, E. Waubant, S. Gazda, R. J. Fox, M. Panzara, N. Sarkar, S. Agarwal, and C. H. Smith. 2008. "Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial." *Ann Neurol* 63 (3):395-400. doi: 10.1002/ana.21363.

Bar-Or, A., L. Fawaz, B. Fan, P. J. Darlington, A. Rieger, C. Ghorayeb, P. A. Calabresi, E. Waubant, S. L. Hauser, J. Zhang, and C. H. Smith. 2010. "Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS?" *Ann Neurol* 67 (4):452-61. doi: 10.1002/ana.21939.

Bar-Or, A., R. A. Grove, D. J. Austin, J. M. Tolson, S. A. VanMeter, E. W. Lewis, F. J. Derosier, M. C. Lopez, S. T. Kavanagh, A. E. Miller, and P. S. Sorensen. 2018. "Subcutaneous ofatumumab in patients with relapsing-remitting multiple sclerosis: The MIRROR study." *Neurology* 90 (20):e1805-e1814. doi: 10.1212/WNL.0000000000005516.

Barr, T. A., P. Shen, S. Brown, V. Lampropoulou, T. Roch, S. Lawrie, B. Fan, R. A. O'Connor, S. M. Anderton, A. Bar-Or, S. Fillatreau, and D. Gray. 2012. "B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells." *J Exp Med* 209 (5):1001-10. doi: 10.1084/jem.20111675.

Batista, F. D., and N. E. Harwood. 2009. "The who, how and where of antigen presentation to B cells." *Nat Rev Immunol* 9 (1):15-27. doi: 10.1038/nri2454.

Beecham, A. H., N. A. Patsopoulos, D. K. Xifara, M. F. Davis, A. Kemppinen, C. Cotsapas, T. S. Shah, C. Spencer, D. Booth, A. Goris, A. Oturai, J. Saarela, B. Fontaine, B. Hemmer, C. Martin, F. Zipp, S. D'Alfonso, F. Martinelli-Boneschi, B. Taylor, H. F. Harbo, I. Kockum, J. Hillert, T. Olsson, M. Ban, J. R. Oksenberg, R. Hintzen, L. F. Barcellos, C. Agliardi, L. Alfredsson, M. Alizadeh, C. Anderson, R. Andrews, H. B. Søndergaard, A. Baker, G. Band, S. E. Baranzini, N. Barizzone, J. Barrett, C. Bellenguez, L. Bergamaschi, L. Bernardinelli, A. Berthele, V. Biberacher, T. M. Binder, H. Blackburn, I. L. Bomfim, P. Brambilla, S. Broadley, B. Brochet, L. Brundin, D. Buck, H. Butzkueven, S. J. Caillier, W. Camu, W. Carpentier, P. Cavalla, E. G. Celius, I. Coman, G. Comi, L. Corrado, L. Cosemans, I. Cournu-Rebeix, B. A. Cree, D. Cusi, V. Damotte, G. Defer, S. R. Delgado, P. Deloukas, A. di Sapio, A. T. Dilthey, P. Donnelly, B. Dubois, M. Duddy, S. Edkins, I. Elovaara, F. Esposito, N. Evangelou, B. Fiddes, J. Field, A. Franke, C. Freeman, I. Y. Frohlich, D. Galimberti, C. Gieger, P. A. Gourraud, C. Graetz, A. Graham, V. Grummel, C. Guaschino, A. Hadjixenofontos, H. Hakonarson, C. Halfpenny, G. Hall, P. Hall, A. Hamsten, J. Harley, T. Harrower, C. Hawkins, G. Hellenthal, C. Hillier, J. Hobart, M. Hoshi, S. E. Hunt, M. Jagodic, I. Jelčić, A. Jochim, B. Kendall, A. Kermode, T. Kilpatrick, K. Koivisto, I. Konidari, T. Korn, H. Kronsbein, C. Langford, M. Larsson, M. Lathrop, C. Lebrun-Frenay, J. Lechner-Scott, M. H. Lee, M. A. Leone, V. Leppä, G. Liberatore, B. A. Lie, C. M. Lill, M. Lindén, J. Link, F. Luessi, J. Lycke, F. Macciardi, S. Männistö, C. P. Manrique, R. Martin, V. Martinelli, D. Mason, G. Mazibrada, C. McCabe, I. L. Mero, J. Mescheriakova, L. Moutsianas, K. M. Myhr, G. Nagels, R. Nicholas, P. Nilsson, F. Piehl, M. Pirinen, S. E. Price, H. Quach, M. Reunanen, W. Robberecht, N. P. Robertson, M. Rodegher, D. Rog, M. Salvetti, N. C. Schnetz-Boutaud, F. Sellebjerg, R. C. Selter, C. Schaefer, S. Shaunak, L. Shen, S. Shields, V. Siffrin, M. Slee, P. S. Sorensen, M. Sorosina, M. Sospedra, A. Spurkland, A. Strange, E. Sundqvist, V. Thijs, J. Thorpe, A. Ticca, P. Tienari, C. van Duijn, E. M. Visser, S. Vucic, H. Westerlind, J. S. Wiley, A. Wilkins, J. F. Wilson, J. Winkelmann, J. Zajicek, E. Zindler, J. L. Haines, M. A. Pericak-Vance, A. J. Ivinson, G. Stewart, D. Hafler, S. L. Hauser, A. Compston, G. McVean, P. De Jager, S. J. Sawcer, J. L. McCauley, International Multiple Sclerosis Genetics Consortium (IMSGC), Wellcome Trust Case Control Consortium 2 (WTCCC2), and International IBD Genetics Consortium (IIBDGC). 2013. "Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis." *Nat Genet* 45 (11):1353-60. doi: 10.1038/ng.2770.

Bender, S. J., and S. R. Weiss. 2010. "Pathogenesis of murine coronavirus in the central nervous system." *J Neuroimmune Pharmacol* 5 (3):336-54. doi: 10.1007/s11481-010-9202-2.

Bennett, M. L., F. C. Bennett, S. A. Liddelow, B. Ajami, J. L. Zamanian, N. B. Fernhoff, S. B. Mulinyawe, C. J. Bohlen, A. Adil, A. Tucker, I. L. Weissman, E. F. Chang, G. Li, G. A. Grant, M. G. Hayden Gephart, and B. A. Barres. 2016. "New tools for studying microglia in the mouse and human CNS." *Proc Natl Acad Sci U S A* 113 (12):E1738-46. doi: 10.1073/pnas.1525528113.

Bernard, C. C. 1976. "Experimental autoimmune encephalomyelitis in mice: genetic control of susceptibility." *J Immunogenet* 3 (4):263-74.

Bettelli, E., M. Pagany, H. L. Weiner, C. Linington, R. A. Sobel, and V. K. Kuchroo. 2003. "Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis." *J Exp Med* 197 (9):1073-81. doi: 10.1084/jem.20021603.

Beum, P. V., A. D. Kennedy, M. E. Williams, M. A. Lindorfer, and R. P. Taylor. 2006. "The shaving reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes." *J Immunol* 176 (4):2600-9.

Bevan, R. J., R. Evans, L. Griffiths, L. M. Watkins, M. I. Rees, R. Magliozzi, I. Allen, G. McDonnell, R. Kee, M. Naughton, D. C. Fitzgerald, R. Reynolds, J. W. Neal, and O. W. Howell. 2018. "Meningeal inflammation and cortical demyelination in acute multiple sclerosis." *Ann Neurol* 84 (6):829-842. doi: 10.1002/ana.25365.

Biasi, G., A. Facchinetti, G. Monastra, S. Mezzalira, S. Sivieri, B. Tavolato, and P. Gallo. 1997. "Protection from experimental autoimmune encephalomyelitis (EAE): non-depleting anti-CD4 mAb treatment induces peripheral T-cell tolerance to MBP in PL/J mice." *J Neuroimmunol* 73 (1-2):117-23.

Bieber, A. J., S. Kerr, and M. Rodriguez. 2003. "Efficient central nervous system remyelination requires T cells." *Ann Neurol* 53 (5):680-4. doi: 10.1002/ana.10578.

Bin, J. M., S. N. Harris, and T. E. Kennedy. 2016. "The oligodendrocyte-specific antibody 'CC1' binds Quaking 7." *J Neurochem* 139 (2):181-186. doi: 10.1111/jnc.13745.

Biogen. 2018. "Efficacy and Safety of BIIB033 (Opicinumab) as an Add-on Therapy to Disease-Modifying Therapies (DMTs) in Relapsing Multiple Sclerosis (MS)." accessed 29/12/2018. https://clinicaltrials.gov/ct2/show/NCT03222973.

Bjartmar, C., G. Kidd, S. Mörk, R. Rudick, and B. D. Trapp. 2000. "Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients." *Ann Neurol* 48 (6):893-901.

Blond, D., S. J. Campbell, A. G. Butchart, V. H. Perry, and D. C. Anthony. 2002. "Differential induction of interleukin-1beta and tumour necrosis factor-alpha may account for specific patterns of leukocyte recruitment in the brain." *Brain Res* 958 (1):89-99.

Bloomgren, G., S. Richman, C. Hotermans, M. Subramanyam, S. Goelz, A. Natarajan, S. Lee, T. Plavina, J. V. Scanlon, A. Sandrock, and C. Bozic. 2012. "Risk of natalizumab-associated progressive multifocal leukoencephalopathy." *N Engl J Med* 366 (20):1870-80. doi: 10.1056/NEJMoa1107829.

Bologna, L., E. Gotti, F. Da Roit, T. Intermesoli, A. Rambaldi, M. Introna, and J. Golay. 2013. "Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy." *J Immunol* 190 (1):231-9. doi: 10.4049/jimmunol.1202645.

Bombardieri, M., and C. Pitzalis. 2012. "Ectopic lymphoid neogenesis and lymphoid chemokines in Sjogren's syndrome: at the interplay between chronic inflammation, autoimmunity and lymphomagenesis." *Curr Pharm Biotechnol* 13 (10):1989-96.

Bomprezzi, R. 2015. "Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview." *Ther Adv Neurol Disord* 8 (1):20-30. doi: 10.1177/1756285614564152.

Borregaard, N. 2010. "Neutrophils, from marrow to microbes." *Immunity* 33 (5):657-70. doi: 10.1016/j.immuni.2010.11.011.

Bradl, M., and H. Lassmann. 2010. "Oligodendrocytes: biology and pathology." *Acta Neuropathol* 119 (1):37-53. doi: 10.1007/s00401-009-0601-5.

Brambilla, R., P. D. Morton, J. J. Ashbaugh, S. Karmally, K. L. Lambertsen, and J. R. Bethea. 2014. "Astrocytes play a key role in EAE pathophysiology by orchestrating in the CNS the inflammatory response of resident and peripheral immune cells and by suppressing remyelination." *Glia* 62 (3):452-67. doi: 10.1002/glia.22616.

Brambilla, R., T. Persaud, X. Hu, S. Karmally, V. I. Shestopalov, G. Dvoriantchikova, D. Ivanov, L. Nathanson, S. R. Barnum, and J. R. Bethea. 2009. "Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation." *J Immunol* 182 (5):2628-40. doi: 10.4049/jimmunol.0802954.

Bramow, S., J. M. Frischer, H. Lassmann, N. Koch-Henriksen, C. F. Lucchinetti, P. S. Sørensen, and H. Laursen. 2010. "Demyelination versus remyelination in progressive multiple sclerosis." *Brain* 133 (10):2983-98. doi: 10.1093/brain/awq250.

Brosnan, C. F., B. Cannella, L. Battistini, and C. S. Raine. 1995. "Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species." *Neurology* 45 (6 Suppl 6):S16-21.

Brosnan, C. F., and C. S. Raine. 2013. "The astrocyte in multiple sclerosis revisited." *Glia* 61 (4):453-65. doi: 10.1002/glia.22443.

Brown, A. M., and D. E. McFarlin. 1981. "Relapsing experimental allergic encephalomyelitis in the SJL/J mouse." *Lab Invest* 45 (3):278-84.

Brown, B. A. 2009. "Natalizumab in the treatment of multiple sclerosis." *Ther Clin Risk Manag* 5 (3):585-94.

Brück, W., P. Porada, S. Poser, P. Rieckmann, F. Hanefeld, H. A. Kretzschmar, and H. Lassmann. 1995. "Monocyte/macrophage differentiation in early multiple sclerosis lesions." *Ann Neurol* 38 (5):788-96. doi: 10.1002/ana.410380514.

Buljevac, D., H. Z. Flach, W. C. Hop, D. Hijdra, J. D. Laman, H. F. Savelkoul, F. G. van Der Meché, P. A. van Doorn, and R. Q. Hintzen. 2002. "Prospective study on the relationship between infections and multiple sclerosis exacerbations." *Brain* 125 (Pt 5):952-60.

Buntinx, M., E. Gielen, P. Van Hummelen, J. Raus, M. Ameloot, P. Steels, and P. Stinissen. 2004. "Cytokine-induced cell death in human oligodendroglial cell lines. II: Alterations in gene expression induced by interferon-gamma and tumor necrosis factor-alpha." *J Neurosci Res* 76 (6):846-61. doi: 10.1002/jnr.20117.

Burt, R. K., Y. Loh, B. Cohen, D. Stefoski, D. Stefosky, R. Balabanov, G. Katsamakis, Y. Oyama, E. J. Russell, J. Stern, P. Muraro, J. Rose, A. Testori, J. Bucha, B. Jovanovic, F. Milanetti, J. Storek, J. C. Voltarelli, and W. H. Burns. 2009. "Autologous non-myeloablative haemopoietic stem cell transplantation in relapsing-remitting multiple sclerosis: a phase I/II study." *Lancet Neurol* 8 (3):244-53. doi: 10.1016/S1474-4422(09)70017-1.

Byravan, S., L. M. Foster, T. Phan, A. N. Verity, and A. T. Campagnoni. 1994. "Murine oligodendroglial cells express nerve growth factor." *Proc Natl Acad Sci U S A* 91 (19):8812-6.

Bénardais, K., V. Gudi, L. Gai, J. Neßler, V. Singh, C. K. Prajeeth, T. Skripuletz, and M. Stangel. 2014. "Long-term impact of neonatal inflammation on demyelination and remyelination in the central nervous system." *Glia* 62 (10):1659-70. doi: 10.1002/glia.22706.

Bø, L., C. A. Vedeler, H. I. Nyland, B. D. Trapp, and S. J. Mørk. 2003a. "Subpial demyelination in the cerebral cortex of multiple sclerosis patients." *J Neuropathol Exp Neurol* 62 (7):723-32.

Bø, L., C. A. Vedeler, H. Nyland, B. D. Trapp, and S. J. Mørk. 2003b. "Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration." *Mult Scler* 9 (4):323-31. doi: 10.1191/1352458503ms917oa.

Cadavid, D., L. Balcer, S. Galetta, O. Aktas, T. Ziemssen, L. Vanopdenbosch, J. Frederiksen, M. Skeen, G. J. Jaffe, H. Butzkueven, F. Ziemssen, L. Massacesi, Y. Chai, L. Xu, S. Freeman, and RENEW Study Investigators. 2017. "Safety and efficacy of opicinumab in acute optic neuritis (RENEW): a randomised, placebo-controlled, phase 2 trial." *Lancet Neurol* 16 (3):189-199. doi: 10.1016/S1474-4422(16)30377-5.

Cader, S., A. Cifelli, Y. Abu-Omar, J. Palace, and P. M. Matthews. 2006. "Reduced brain functional reserve and altered functional connectivity in patients with multiple sclerosis." *Brain* 129 (Pt 2):527-37. doi: 10.1093/brain/awh670.

Calabrese, M., M. Filippi, and P. Gallo. 2010. "Cortical lesions in multiple sclerosis." *Nat Rev Neurol* 6 (8):438-44. doi: 10.1038/nrneurol.2010.93.

Calabrese, M., V. Poretto, A. Favaretto, S. Alessio, V. Bernardi, C. Romualdi, F. Rinaldi, P. Perini, and P. Gallo. 2012. "Cortical lesion load associates with progression of disability in multiple sclerosis." *Brain* 135 (Pt 10):2952-61. doi: 10.1093/brain/aws246.

Calabresi, P. A. 2018. "Advances in multiple sclerosis: from reduced relapses to remedies." *Lancet Neurol* 17 (1):10-12. doi: 10.1016/S1474-4422(17)30417-9.

Campbell, S. J., D. C. Anthony, F. Oakley, H. Carlsen, A. M. Elsharkawy, R. Blomhoff, and D. A. Mann. 2008. "Hepatic nuclear factor kappa B regulates neutrophil recruitment to the injured brain." *J Neuropathol Exp Neurol* 67 (3):223-30. doi: 10.1097/NEN.0b013e3181654957.

Campbell, S. J., R. M. Deacon, Y. Jiang, C. Ferrari, F. J. Pitossi, and D. C. Anthony. 2007. "Overexpression of IL-1beta by adenoviral-mediated gene transfer in the rat brain causes a prolonged hepatic chemokine response, axonal injury and the suppression of spontaneous behaviour." *Neurobiol Dis* 27 (2):151-63. doi: 10.1016/j.nbd.2007.04.013.

Campbell, S. J., P. M. Hughes, J. P. Iredale, D. C. Wilcockson, S. Waters, F. Docagne, V. H. Perry, and D. C. Anthony. 2003. "CINC-1 is an acute-phase protein induced by focal brain injury causing leukocyte mobilization and liver injury." *FASEB J* 17 (9):1168-70. doi: 10.1096/fj.02-0757fje.

Campbell, S. J., Y. Jiang, A. E. Davis, R. Farrands, J. Holbrook, D. Leppert, and D. C. Anthony. 2007. "Immunomodulatory effects of etanercept in a model of brain injury act through attenuation of the acute-phase response." *J Neurochem* 103 (6):2245-55. doi: 10.1111/j.1471-4159.2007.04928.x.

Campbell, S. J., U. Meier, S. Mardiguian, Y. Jiang, E. T. Littleton, A. Bristow, J. Relton, T. J. Connor, and D. C. Anthony. 2010. "Sickness behaviour is induced by a peripheral CXC-chemokine also expressed in multiple sclerosis and EAE." *Brain Behav Immun* 24 (5):738-46. doi: 10.1016/j.bbi.2010.01.011.

Campbell, S. J., V. H. Perry, F. J. Pitossi, A. G. Butchart, M. Chertoff, S. Waters, R. Dempster, and D. C. Anthony. 2005. "Central nervous system injury triggers hepatic CC and CXC chemokine expression that is associated with leukocyte mobilization and recruitment to both the central nervous system and the liver." *Am J Pathol* 166 (5):1487-97. doi: 10.1016/S0002-9440(10)62365-6.

Campbell, S. J., D. C. Wilcockson, A. G. Butchart, V. H. Perry, and D. C. Anthony. 2002. "Altered chemokine expression in the spinal cord and brain contributes to differential interleukin-1beta-induced neutrophil recruitment." *J Neurochem* 83 (2):432-41.

Campbell, S. J., I. Zahid, P. Losey, S. Law, Y. Jiang, M. Bilgen, N. van Rooijen, D. Morsali, A. E. Davis, and D. C. Anthony. 2008. "Liver Kupffer cells control the magnitude of the inflammatory response in the injured brain and spinal cord." *Neuropharmacology* 55 (5):780-7. doi: 10.1016/j.neuropharm.2008.06.074.

Cartron, G., S. de Guibert, M. S. Dilhuydy, F. Morschhauser, V. Leblond, J. Dupuis, B. Mahe, R. Bouabdallah, G. Lei, M. Wenger, E. Wassner-Fritsch, and M. Hallek. 2014. "Obinutuzumab (GA101) in relapsed/refractory chronic lymphocytic leukemia: final data from the phase 1/2 GAUGUIN study." *Blood* 124 (14):2196-202. doi: 10.1182/blood-2014-07-586610.

Casili, G., D. Impellizzeri, M. Cordaro, E. Esposito, and S. Cuzzocrea. 2016. "B-Cell Depletion with CD20 Antibodies as New Approach in the Treatment of Inflammatory and Immunological Events Associated with Spinal Cord Injury." *Neurotherapeutics* 13 (4):880-894. doi: 10.1007/s13311-016-0446-2.

Cayrol, R., K. Wosik, J. L. Berard, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. S. Haqqani, K. Kreymborg, S. Krug, R. Moumdjian, A. Bouthillier, B. Becher, N. Arbour, S. David, D. Stanimirovic, and A. Prat. 2008. "Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system." *Nat Immunol* 9 (2):137-45. doi: 10.1038/ni1551.

Cepok, S., B. Rosche, V. Grummel, F. Vogel, D. Zhou, J. Sayn, N. Sommer, H. P. Hartung, and B. Hemmer. 2005. "Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis." *Brain* 128 (Pt 7):1667-76. doi: 10.1093/brain/awh486.

Chaiwatanatorn, K., N. Lee, A. Grigg, R. Filshie, and F. Firkin. 2003. "Delayed-onset neutropenia associated with rituximab therapy." *Br J Haematol* 121 (6):913-8.

Chamberlain, K. A., S. E. Nanescu, K. Psachoulia, and J. K. Huang. 2016. "Oligodendrocyte regeneration: Its significance in myelin replacement and neuroprotection in multiple sclerosis." *Neuropharmacology* 110 (Pt B):633-643. doi: 10.1016/j.neuropharm.2015.10.010.

Chapman, K. Z., V. Q. Dale, A. Dénes, G. Bennett, N. J. Rothwell, S. M. Allan, and B. W. McColl. 2009. "A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke." *J Cereb Blood Flow Metab* 29 (11):1764-8. doi: 10.1038/jcbfm.2009.113.

Charcot, J. M., and B. Ball. 1868. *Leçons sur les maladies des vieillards et les maladies chroniques*. Paris: A. Delahaye.

Chaudhary, P., G. Marracci, D. Galipeau, E. Pocius, B. Morris, and D. Bourdette. 2015. "Lipoic acid reduces inflammation in a mouse focal cortical experimental autoimmune encephalomyelitis model." *J Neuroimmunol* 289:68-74. doi: 10.1016/j.jneuroim.2015.10.011.

Chen, K., H. Nishi, R. Travers, N. Tsuboi, K. Martinod, D. D. Wagner, R. Stan, K. Croce, and T. N. Mayadas. 2012. "Endocytosis of soluble immune complexes leads to their clearance by FcγRIIIB but induces neutrophil extracellular traps via FcγRIIA in vivo." *Blood* 120 (22):4421-31. doi: 10.1182/blood-2011-12-401133.

Choi, S. R., O. W. Howell, D. Carassiti, R. Magliozzi, D. Gveric, P. A. Muraro, R. Nicholas, F. Roncaroli, and R. Reynolds. 2012. "Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis." *Brain* 135 (Pt 10):2925-37. doi: 10.1093/brain/aws189.

Christodoulou, M. I., E. K. Kapsogeorgou, and H. M. Moutsopoulos. 2010. "Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome." *J Autoimmun* 34 (4):400-7. doi: 10.1016/j.jaut.2009.10.004.

Chu, A. B., J. L. Sever, D. L. Madden, M. Iivanainen, M. Leon, W. Wallen, B. R. Brooks, Y. J. Lee, and S. Houff. 1983. "Oligoclonal IgG bands in cerebrospinal fluid in various neurological diseases." *Ann Neurol* 13 (4):434-9. doi: 10.1002/ana.410130410.

Ciotti, J. R., and A. H. Cross. 2018. "Disease-Modifying Treatment in Progressive Multiple Sclerosis." *Curr Treat Options Neurol* 20 (5):12. doi: 10.1007/s11940-018-0496-3.

Cohen, J. A., F. Barkhof, G. Comi, H. P. Hartung, B. O. Khatri, X. Montalban, J. Pelletier, R. Capra, P. Gallo, G. Izquierdo, K. Tiel-Wilck, A. de Vera, J. Jin, T. Stites, S. Wu, S. Aradhye, L. Kappos, and TRANSFORMS Study Group. 2010. "Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis." *N Engl J Med* 362 (5):402-15. doi: 10.1056/NEJMoa0907839.

Cohen, J. A., A. J. Coles, D. L. Arnold, C. Confavreux, E. J. Fox, H. P. Hartung, E. Havrdova, K. W. Selmaj, H. L. Weiner, E. Fisher, V. V. Brinar, G. Giovannoni, M. Stojanovic, B. I. Ertik, S. L. Lake, D. H. Margolin, M. A. Panzara, D. A. Compston, and CARE-MS I investigators. 2012. "Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial." *Lancet* 380 (9856):1819-28. doi: 10.1016/S0140-6736(12)61769-3.

Comi, G., M. Radaelli, and P. Soelberg Sørensen. 2017. "Evolving concepts in the treatment of relapsing multiple sclerosis." *Lancet* 389 (10076):1347-1356. doi: 10.1016/S0140-6736(16)32388-1.

Compston, A. 1988. "The 150th anniversary of the first depiction of the lesions of multiple sclerosis." *J Neurol Neurosurg Psychiatry* 51 (10):1249-52.

Compston, A., and A. Coles. 2008. "Multiple sclerosis." *Lancet* 372 (9648):1502-17. doi: 10.1016/S0140-6736(08)61620-7.

Confavreux, C., S. Vukusic, and P. Adeleine. 2003. "Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process." *Brain* 126 (Pt 4):770-82.

Confavreux, C., S. Vukusic, T. Moreau, and P. Adeleine. 2000. "Relapses and progression of disability in multiple sclerosis." *N Engl J Med* 343 (20):1430-8. doi: 10.1056/NEJM200011163432001.

Constantinescu, C. S., B. Hilliard, E. Ventura, M. Wysocka, L. Showe, E. Lavi, T. Fujioka, P. Scott, G. Trinchieri, and A. Rostami. 2001. "Modulation of susceptibility and resistance to an autoimmune model of multiple sclerosis in prototypically susceptible and resistant strains by neutralization of interleukin-12 and interleukin-4, respectively." *Clin Immunol* 98 (1):23-30. doi: 10.1006/clim.2000.4944.

Correale, J. 2014. "The role of microglial activation in disease progression." *Mult Scler* 20 (10):1288-95. doi: 10.1177/1352458514533230.

Correale, J., M. Fiol, and W. Gilmore. 2006. "The risk of relapses in multiple sclerosis during systemic infections." *Neurology* 67 (4):652-9. doi: 10.1212/01.wnl.0000233834.09743.3b.

Correale, J., M. I. Gaitán, M. C. Ysrraelit, and M. P. Fiol. 2017. "Progressive multiple sclerosis: from pathogenic mechanisms to treatment." *Brain* 140 (3):527-546. doi: 10.1093/brain/aww258.

Corsiero, E., A. Nerviani, M. Bombardieri, and C. Pitzalis. 2016. "Ectopic Lymphoid Structures: Powerhouse of Autoimmunity." *Front Immunol* 7:430. doi: 10.3389/fimmu.2016.00430.

Couch, Y., N. Akbar, J. Roodselaar, M. C. Evans, C. Gardiner, I. Sargent, I. A. Romero, A. Bristow, A. M. Buchan, N. Haughey, and D. C. Anthony. 2017. "Circulating endothelial cell-derived extracellular vesicles mediate the acute phase response and sickness behaviour associated with CNS inflammation." *Sci Rep* 7 (1):9574. doi: 10.1038/s41598-017-09710-3.

Cross, A. H., R. McCarron, D. E. McFarlin, and C. S. Raine. 1987. "Adoptively transferred acute and chronic relapsing autoimmune encephalomyelitis in the PL/J mouse and observations on altered pathology by intercurrent virus infection." *Lab Invest* 57 (5):499-512.

Cross, A. H., and C. S. Raine. 1990. "Serial adoptive transfer of murine experimental allergic encephalomyelitis: successful transfer is dependent on active disease in the donor." *J Neuroimmunol* 28 (1):27-37.

Cross, A. H., J. L. Stark, J. Lauber, M. J. Ramsbottom, and J. A. Lyons. 2006. "Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients." *J Neuroimmunol* 180 (1-2):63-70. doi: 10.1016/j.jneuroim.2006.06.029.

Cyster, J. G., K. M. Ansel, K. Reif, E. H. Ekland, P. L. Hyman, H. L. Tang, S. A. Luther, and V. N. Ngo. 2000. "Follicular stromal cells and lymphocyte homing to follicles." *Immunol Rev* 176:181-93.

Dale, D. C., L. Boxer, and W. C. Liles. 2008. "The phagocytes: neutrophils and monocytes." *Blood* 112 (4):935-45. doi: 10.1182/blood-2007-12-077917.

Dalle, S., L. Reslan, T. Besseyre de Horts, S. Herveau, F. Herting, A. Plesa, T. Friess, P. Umana, C. Klein, and C. Dumontet. 2011. "Preclinical studies on the mechanism of action and the anti-lymphoma activity of the novel anti-CD20 antibody GA101." *Mol Cancer Ther* 10 (1):178-85. doi: 10.1158/1535-7163.MCT-10-0385.

Daneman, R., and B. Engelhardt. 2017. "Brain barriers in health and disease." *Neurobiol Dis* 107:1-3. doi: 10.1016/j.nbd.2017.05.008.

Daneman, R., and A. Prat. 2015. "The blood-brain barrier." *Cold Spring Harb Perspect Biol* 7 (1):a020412. doi: 10.1101/cshperspect.a020412.

Daneman, R., L. Zhou, A. A. Kebede, and B. A. Barres. 2010. "Pericytes are required for blood-brain barrier integrity during embryogenesis." *Nature* 468 (7323):562-6. doi: 10.1038/nature09513.

De Groot, C. J., E. Bergers, W. Kamphorst, R. Ravid, C. H. Polman, F. Barkhof, and P. van der Valk. 2001. "Post-mortem MRI-guided sampling of multiple sclerosis brain lesions: increased yield of active demyelinating and (p)reactive lesions." *Brain* 124 (Pt 8):1635-45.

De Jager, P. L., X. Jia, J. Wang, P. I. de Bakker, L. Ottoboni, N. T. Aggarwal, L. Piccio, S. Raychaudhuri, D. Tran, C. Aubin, R. Briskin, S. Romano, S. E. Baranzini, J. L. McCauley, M. A. Pericak-Vance, J. L. Haines, R. A. Gibson, Y. Naeglin, B. Uitdehaag, P. M. Matthews, L. Kappos, C. Polman, W. L. McArdle, D. P. Strachan, D. Evans, A. H. Cross, M. J. Daly, A. Compston, S. J. Sawcer, H. L. Weiner, S. L. Hauser, D. A. Hafler, J. R. Oksenberg, and International MS Genetics Consortium. 2009. "Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci." *Nat Genet* 41 (7):776-82. doi: 10.1038/ng.401.

De Keyser, J., N. Wilczak, R. Leta, and C. Streetland. 1999. "Astrocytes in multiple sclerosis lack beta-2 adrenergic receptors." *Neurology* 53 (8):1628-33.

De Stefano, N., A. Giorgio, M. Battaglini, M. Rovaris, M. P. Sormani, F. Barkhof, T. Korteweg, C. Enzinger, F. Fazekas, M. Calabrese, D. Dinacci, G. Tedeschi, A. Gass, X. Montalban, A. Rovira, A. Thompson, G. Comi, D. H. Miller, and M. Filippi. 2010. "Assessing brain atrophy rates in a large population of untreated multiple sclerosis subtypes." *Neurology* 74 (23):1868-76. doi: 10.1212/WNL.0b013e3181e24136.

Deacon, R. M. 2013. "Measuring motor coordination in mice." *J Vis Exp* (75):e2609. doi: 10.3791/2609.

DeLuca, G. C., K. Williams, N. Evangelou, G. C. Ebers, and M. M. Esiri. 2006. "The contribution of demyelination to axonal loss in multiple sclerosis." *Brain* 129 (Pt 6):1507-16. doi: 10.1093/brain/awl074.

Dendrou, C. A., L. Fugger, and M. A. Friese. 2015. "Immunopathology of multiple sclerosis." *Nat Rev Immunol* 15 (9):545-58. doi: 10.1038/nri3871.

Denic, A., A. J. Johnson, A. J. Bieber, A. E. Warrington, M. Rodriguez, and I. Pirko. 2011. "The relevance of animal models in multiple sclerosis research." *Pathophysiology* 18 (1):21-9. doi: 10.1016/j.pathophys.2010.04.004.

Deshmukh, V. A., V. Tardif, C. A. Lyssiotis, C. C. Green, B. Kerman, H. J. Kim, K. Padmanabhan, J. G. Swoboda, I. Ahmad, T. Kondo, F. H. Gage, A. N. Theofilopoulos, B. R. Lawson, P. G. Schultz, and L. L. Lairson. 2013. "A regenerative approach to the treatment of multiple sclerosis." *Nature* 502 (7471):327-332. doi: 10.1038/nature12647.

Dhaeze, T., E. Peelen, A. Hombrouck, L. Peeters, B. Van Wijmeersch, N. Lemkens, P. Lemkens, V. Somers, S. Lucas, B. Broux, P. Stinissen, and N. Hellings. 2015. "Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis." *J Immunol* 195 (3):832-40. doi: 10.4049/jimmunol.1500759.

Dickens, A. M., L. B. Tovar-Y-Romo, S. W. Yoo, A. L. Trout, M. Bae, M. Kanmogne, B. Megra, D. W. Williams, K. W. Witwer, M. Gacias, N. Tabatadze, R. N. Cole, P. Casaccia, J. W. Berman, D. C. Anthony, and N. J. Haughey. 2017. "Astrocyte-shed extracellular vesicles regulate the peripheral leukocyte response to inflammatory brain lesions." *Sci Signal* 10 (473). doi: 10.1126/scisignal.aai7696.

Dieu-Nosjean, M. C., M. Antoine, C. Danel, D. Heudes, M. Wislez, V. Poulot, N. Rabbe, L. Laurans, E. Tartour, L. de Chaisemartin, S. Lebecque, W. H. Fridman, and J. Cadranel. 2008. "Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures." *J Clin Oncol* 26 (27):4410-7. doi: 10.1200/JCO.2007.15.0284.

Dougherty, K. D., C. F. Dreyfus, and I. B. Black. 2000. "Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury." *Neurobiol Dis* 7 (6 Pt B):574-85. doi: 10.1006/nbdi.2000.0318.

Drayton, D. L., S. Liao, R. H. Mounzer, and N. H. Ruddle. 2006. "Lymphoid organ development: from ontogeny to neogenesis." *Nat Immunol* 7 (4):344-53. doi: 10.1038/ni1330.

Duddy, M., M. Niino, F. Adatia, S. Hebert, M. Freedman, H. Atkins, H. J. Kim, and A. Bar-Or. 2007. "Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis." *J Immunol* 178 (10):6092-9.

Dulamea, A. O. 2017. "Role of Oligodendrocyte Dysfunction in Demyelination, Remyelination and Neurodegeneration in Multiple Sclerosis." *Adv Exp Med Biol* 958:91-127. doi: 10.1007/978-3-319-47861-6_7.

Dunn, N., A. Juto, M. Ryner, A. Manouchehrinia, L. Piccoli, K. Fink, F. Piehl, and A. Fogdell-Hahn. 2018. "Rituximab in multiple sclerosis: Frequency and clinical relevance of anti-drug antibodies." *Mult Scler* 24 (9):1224-1233. doi: 10.1177/1352458517720044.

Duvernoy, H. M., S. Delon, and J. L. Vannson. 1981. "Cortical blood vessels of the human brain." *Brain Res Bull* 7 (5):519-79.

Ebers, G. C., A. D. Sadovnick, and N. J. Risch. 1995. "A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group." *Nature* 377 (6545):150-1. doi: 10.1038/377150a0.

Eggers, E. L., B. A. Michel, H. Wu, S. Z. Wang, C. J. Bevan, A. Abounasr, N. S. Pierson, A. Bischof, M. Kazer, E. Leitner, A. L. Greenfield, S. Demuth, M. R. Wilson, R. G. Henry, B. A. Cree, S. L. Hauser, and H. C. von Büdingen. 2017. "Clonal relationships of CSF B cells in treatment-naive multiple sclerosis patients." *JCI Insight* 2 (22). doi: 10.1172/jci.insight.92724.

Engell, T. 1989. "A clinical patho-anatomical study of clinically silent multiple sclerosis." *Acta Neurol Scand* 79 (5):428-30.

Evans , Karleyton C., Robert Naismith , Douglas L. Arnold , Alexey Boyko , Nikos Evangelou , Martin Valis , Elizabeth Fisher , Nancy Richert , Jie Li , Lei Xu , Diego Cadavid , and Michelle Mellion. 2017.

"Exploratory MRI Biomarkers of Opicinumab (Anti-LINGO-1) Show Stabilization of Pre-existing T2 Lesions in Relapsing Multiple Sclerosis: Results from the Phase 2b SYNERGY Trial (P2.100)." *Neurology* 88 (16 Supplement):P2.100.

Farina, C., F. Aloisi, and E. Meinl. 2007. "Astrocytes are active players in cerebral innate immunity." *Trends Immunol* 28 (3):138-45. doi: 10.1016/j.it.2007.01.005.

Ferguson, B., M. K. Matyszak, M. M. Esiri, and V. H. Perry. 1997. "Axonal damage in acute multiple sclerosis lesions." *Brain* 120 (Pt 3):393-9.

Ferrara, C., S. Grau, C. Jäger, P. Sondermann, P. Brünker, I. Waldhauer, M. Hennig, A. Ruf, A. C. Rufer, M. Stihle, P. Umaña, and J. Benz. 2011. "Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose." *Proc Natl Acad Sci U S A* 108 (31):12669-74. doi: 10.1073/pnas.1108455108.

Ferrari, C. C., A. M. Depino, F. Prada, N. Muraro, S. Campbell, O. Podhajcer, V. H. Perry, D. C. Anthony, and F. J. Pitossi. 2004. "Reversible demyelination, blood-brain barrier breakdown, and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain." *Am J Pathol* 165 (5):1827-37. doi: 10.1016/S0002-9440(10)63438-4.

Filippi, M., A. Campi, V. Martinelli, B. Colombo, T. Yousry, N. Canal, G. Scotti, and G. Comi. 1995. "Comparison of triple dose versus standard dose gadolinium-DTPA for detection of MRI enhancing lesions in patients with primary progressive multiple sclerosis." *J Neurol Neurosurg Psychiatry* 59 (5):540-4.

Filippi, M., M. A. Rocca, F. Barkhof, W. Brück, J. T. Chen, G. Comi, G. DeLuca, N. De Stefano, B. J. Erickson, N. Evangelou, F. Fazekas, J. J. Geurts, C. Lucchinetti, D. H. Miller, D. Pelletier, B. F. Popescu, H. Lassmann, and Attendees of the Correlation between Pathological MRI findings in MS workshop. 2012. "Association between pathological and MRI findings in multiple sclerosis." *Lancet Neurol* 11 (4):349-60. doi: 10.1016/S1474-4422(12)70003-0.

Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. "B cells regulate autoimmunity by provision of IL-10." *Nat Immunol* 3 (10):944-50. doi: 10.1038/ni833.

Fischer, M. T., R. Sharma, J. L. Lim, L. Haider, J. M. Frischer, J. Drexhage, D. Mahad, M. Bradl, J. van Horssen, and H. Lassmann. 2012. "NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury." *Brain* 135 (Pt 3):886-99. doi: 10.1093/brain/aws012.

Fischer, M. T., I. Wimmer, R. Höftberger, S. Gerlach, L. Haider, T. Zrzavy, S. Hametner, D. Mahad, C. J. Binder, M. Krumbholz, J. Bauer, M. Bradl, and H. Lassmann. 2013. "Disease-specific molecular events in cortical multiple sclerosis lesions." *Brain* 136 (Pt 6):1799-815. doi: 10.1093/brain/awt110.

Fisniku, L. K., D. T. Chard, J. S. Jackson, V. M. Anderson, D. R. Altmann, K. A. Miszkiel, A. J. Thompson, and D. H. Miller. 2008. "Gray matter atrophy is related to long-term disability in multiple sclerosis." *Ann Neurol* 64 (3):247-54. doi: 10.1002/ana.21423.

Fox, Edward. 2018. "Phase 2 Study of Ublituximab in Patients With Relapsing Forms of Multiple Sclerosis." accessed 30 December 2018. https://clinicaltrials.gov/ct2/show/NCT02738775.

Fox, R. J., C. S. Coffey, R. Conwit, M. E. Cudkowicz, T. Gleason, A. Goodman, E. C. Klawiter, K. Matsuda, M. McGovern, R. T. Naismith, A. Ashokkumar, J. Barnes, D. Ecklund, E. Klingner, M. Koepp, J. D. Long, S. Natarajan, B. Thornell, J. Yankey, R. A. Bermel, J. P. Debbins, X. Huang, P. Jagodnik, M. J. Lowe, K. Nakamura, S. Narayanan, K. E. Sakaie, B. Thoomukuntla, X. Zhou, S. Krieger, E. Alvarez, M. Apperson, K. Bashir, B. A. Cohen, P. K. Coyle, S. Delgado, L. D. Dewitt, A. Flores, B. S. Giesser, M. D. Goldman, B. Jubelt, N. Lava, S. G. Lynch, H. Moses, D. Ontaneda, J. S. Perumal, M. Racke, P. Repovic, C. S. Riley, C. Severson, S. Shinnar, V. Suski, B. Weinstock-Guttman, V. Yadav, A. Zabeti, and NN102/SPRINT-MS Trial Investigators. 2018. "Phase 2 Trial of Ibudilast in Progressive Multiple Sclerosis." *N Engl J Med* 379 (9):846-855. doi: 10.1056/NEJMoa1803583.

Fox, R. J., D. H. Miller, J. T. Phillips, M. Hutchinson, E. Havrdova, M. Kita, M. Yang, K. Raghupathi, M. Novas, M. T. Sweetser, V. Viglietta, K. T. Dawson, and CONFIRM Study Investigators. 2012. "Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis." *N Engl J Med* 367 (12):1087-97. doi: 10.1056/NEJMoa1206328.

Fraussen, J., L. de Bock, and V. Somers. 2016. "B cells and antibodies in progressive multiple sclerosis: Contribution to neurodegeneration and progression." *Autoimmun Rev* 15 (9):896-9. doi: 10.1016/j.autrev.2016.07.008.

Freedman, M. S., A. Bar-Or, J. Oger, A. Traboulsee, D. Patry, C. Young, T. Olsson, D. Li, H. P. Hartung, M. Krantz, L. Ferenczi, T. Verco, and MAESTRO-01 Investigators. 2011. "A phase III study evaluating the efficacy and safety of MBP8298 in secondary progressive MS." *Neurology* 77 (16):1551-60. doi: 10.1212/WNL.0b013e318233b240.

Freskgård, P. O., and E. Urich. 2016. "Antibody therapies in CNS diseases." *Neuropharmacology*. doi: 10.1016/j.neuropharm.2016.03.014.

Freskgård, P. O., and E. Urich. 2017. "Antibody therapies in CNS diseases." *Neuropharmacology* 120:38-55. doi: 10.1016/j.neuropharm.2016.03.014.

FREUND, J. 1956. "The mode of action of immunologic adjuvants." *Bibl Tuberc* (10):130-48.

Friese, M. A., B. Schattling, and L. Fugger. 2014. "Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis." *Nat Rev Neurol* 10 (4):225-38. doi: 10.1038/nrneurol.2014.37.

Frischer, J. M., S. Bramow, A. Dal-Bianco, C. F. Lucchinetti, H. Rauschka, M. Schmidbauer, H. Laursen, P. S. Sorensen, and H. Lassmann. 2009. "The relation between inflammation and neurodegeneration in multiple sclerosis brains." *Brain* 132 (Pt 5):1175-89. doi: 10.1093/brain/awp070.

Frischer, J. M., S. D. Weigand, Y. Guo, N. Kale, J. E. Parisi, I. Pirko, J. Mandrekar, S. Bramow, I. Metz, W. Brück, H. Lassmann, and C. F. Lucchinetti. 2015. "Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque." *Ann Neurol* 78 (5):710-21. doi: 10.1002/ana.24497.

Fritz, R. B., C. H. Chou, and D. E. McFarlin. 1983a. "Induction of experimental allergic encephalomyelitis in PL/J and (SJL/J x PL/J)F1 mice by myelin basic protein and its peptides: localization of a second encephalitogenic determinant." *J Immunol* 130 (1):191-4.

Fritz, R. B., C. H. Chou, and D. E. McFarlin. 1983b. "Relapsing murine experimental allergic encephalomyelitis induced by myelin basic protein." *J Immunol* 130 (3):1024-6.

Frohman, E. M., M. K. Racke, and C. S. Raine. 2006. "Multiple sclerosis--the plaque and its pathogenesis." *N Engl J Med* 354 (9):942-55. doi: 10.1056/NEJMra052130.

Fu, Y. X., and D. D. Chaplin. 1999. "Development and maturation of secondary lymphoid tissues." *Annu Rev Immunol* 17:399-433. doi: 10.1146/annurev.immunol.17.1.399.

Fünfschilling, U., L. M. Supplie, D. Mahad, S. Boretius, A. S. Saab, J. Edgar, B. G. Brinkmann, C. M. Kassmann, I. D. Tzvetanova, W. Möbius, F. Diaz, D. Meijer, U. Suter, B. Hamprecht, M. W. Sereda, C. T. Moraes, J. Frahm, S. Goebbels, and K. A. Nave. 2012. "Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity." *Nature* 485 (7399):517-21. doi: 10.1038/nature11007.

Gabay, C., and I. Kushner. 1999. "Acute-phase proteins and other systemic responses to inflammation." *N Engl J Med* 340 (6):448-54. doi: 10.1056/NEJM199902113400607.

Gaitán, M. I., M. C. Ysrraelit, and J. Correale. 2017. "Neutropenia in Patients With Multiple Sclerosis Treated With Alemtuzumab." *JAMA Neurol* 74 (9):1143-1144. doi: 10.1001/jamaneurol.2017.1456.

Gardner, C., R. Magliozzi, P. F. Durrenberger, O. W. Howell, J. Rundle, and R. Reynolds. 2013. "Cortical grey matter demyelination can be induced by elevated pro-inflammatory cytokines in the subarachnoid space of MOG-immunized rats." *Brain* 136 (Pt 12):3596-608. doi: 10.1093/brain/awt279.

Gentile, A., A. Musella, S. Bullitta, D. Fresegna, F. De Vito, R. Fantozzi, E. Piras, F. Gargano, G. Borsellino, L. Battistini, A. Schubart, G. Mandolesi, and D. Centonze. 2016. "Siponimod (BAF312) prevents synaptic neurodegeneration in experimental multiple sclerosis." *J Neuroinflammation* 13 (1):207. doi: 10.1186/s12974-016-0686-4.

Geurts, J. J., L. Bö, P. J. Pouwels, J. A. Castelijns, C. H. Polman, and F. Barkhof. 2005. "Cortical lesions in multiple sclerosis: combined postmortem MR imaging and histopathology." *AJNR Am J Neuroradiol* 26 (3):572-7.

Geurts, J. J., L. Bö, S. D. Roosendaal, T. Hazes, R. Daniëls, F. Barkhof, M. P. Witter, I. Huitinga, and P. van der Valk. 2007. "Extensive hippocampal demyelination in multiple sclerosis." *J Neuropathol Exp Neurol* 66 (9):819-27. doi: 10.1097/nen.0b013e3181461f54.

Gilli, F., L. Li, S. J. Campbell, D. C. Anthony, and A. R. Pachner. 2015. "The effect of B-cell depletion in the Theiler's model of multiple sclerosis." *J Neurol Sci* 359 (1-2):40-7. doi: 10.1016/j.jns.2015.10.012.

Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov, and M. Merad. 2010. "Fate mapping analysis reveals that adult microglia derive from primitive macrophages." *Science* 330 (6005):841-5. doi: 10.1126/science.1194637.

Giuliani, F., C. G. Goodyer, J. P. Antel, and V. W. Yong. 2003. "Vulnerability of human neurons to T cell-mediated cytotoxicity." *J Immunol* 171 (1):368-79.

Glennie, M. J., R. R. French, M. S. Cragg, and R. P. Taylor. 2007. "Mechanisms of killing by anti-CD20 monoclonal antibodies." *Mol Immunol* 44 (16):3823-37. doi: 10.1016/j.molimm.2007.06.151.

Golay, J., F. Da Roit, L. Bologna, C. Ferrara, J. H. Leusen, A. Rambaldi, C. Klein, and M. Introna. 2013. "Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab." *Blood* 122 (20):3482-91. doi: 10.1182/blood-2013-05-504043.

Gold, R., L. Kappos, D. L. Arnold, A. Bar-Or, G. Giovannoni, K. Selmaj, C. Tornatore, M. T. Sweetser, M. Yang, S. I. Sheikh, K. T. Dawson, and DEFINE Study Investigators. 2012. "Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis." *N Engl J Med* 367 (12):1098-107. doi: 10.1056/NEJMoa1114287.

Goldenberg, M. M. 2012. "Multiple sclerosis review." *P T* 37 (3):175-84.

Gonzalez, D., W. L. Dees, J. K. Hiney, S. R. Ojeda, and R. P. Saneto. 1990. "Expression of beta-nerve growth factor in cultured cells derived from the hypothalamus and cerebral cortex." *Brain Res* 511 (2):249-58.

González Torre, J. A., ÁJ Cruz-Gómez, A. Belenguer, C. Sanchis-Segura, C. Ávila, and C. Forn. 2017. "Hippocampal dysfunction is associated with memory impairment in multiple sclerosis: A volumetric and functional connectivity study." *Mult Scler* 23 (14):1854-1863. doi: 10.1177/1352458516688349.

Good, K. P., I. A. Tourbier, P. Moberg, J. L. Cuzzocreo, R. J. Geckle, D. M. Yousem, D. L. Pham, and R. L. Doty. 2017. "Unilateral olfactory sensitivity in multiple sclerosis." *Physiol Behav* 168:24-30. doi: 10.1016/j.physbeh.2016.10.017.

Goodin, D. S. 2012. "The genetic and environmental bases of complex human-disease: extending the utility of twin-studies." *PLoS One* 7 (12):e47875. doi: 10.1371/journal.pone.0047875.

Goodin, D. S. 2014. "The epidemiology of multiple sclerosis: insights to disease pathogenesis." *Handb Clin Neurol* 122:231-66. doi: 10.1016/B978-0-444-52001-2.00010-8.

Gotot, J., C. Gottschalk, S. Leopold, P. A. Knolle, H. Yagita, C. Kurts, and I. Ludwig-Portugall. 2012. "Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells in vivo." *Proc Natl Acad Sci U S A* 109 (26):10468-73. doi: 10.1073/pnas.1201131109.

Gottfried, J. A., J. S. Winston, and R. J. Dolan. 2006. "Dissociable codes of odor quality and odorant structure in human piriform cortex." *Neuron* 49 (3):467-79. doi: 10.1016/j.neuron.2006.01.007.

Green, A. J., J. M. Gelfand, B. A. Cree, C. Bevan, W. J. Boscardin, F. Mei, J. Inman, S. Arnow, M. Devereux, A. Abounasr, H. Nobuta, A. Zhu, M. Friessen, R. Gerona, H. C. von Büdingen, R. G. Henry, S. L. Hauser, and J. R. Chan. 2017. "Clemastine fumarate as a remyelinating therapy for multiple sclerosis (ReBUILD): a randomised, controlled, double-blind, crossover trial." *Lancet* 390 (10111):2481-2489. doi: 10.1016/S0140-6736(17)32346-2.

Greenfield, A. L., and S. L. Hauser. 2018. "B-cell Therapy for Multiple Sclerosis: Entering an era." *Ann Neurol* 83 (1):13-26. doi: 10.1002/ana.25119.

Gresle, M. M., Y. Liu, T. J. Kilpatrick, D. Kemper, Q. Z. Wu, B. Hu, Q. L. Fu, K. F. So, G. Sheng, G. Huang, B. Pepinsky, H. Butzkueven, and S. Mi. 2016. "Blocking LINGO-1 in vivo reduces degeneration and enhances regeneration of the optic nerve." *Mult Scler J Exp Transl Clin* 2:2055217316641704. doi: 10.1177/2055217316641704.

Griffiths, I., M. Klugmann, T. Anderson, D. Yool, C. Thomson, M. H. Schwab, A. Schneider, F. Zimmermann, M. McCulloch, N. Nadon, and K. A. Nave. 1998. "Axonal swellings and degeneration in mice lacking the major proteolipid of myelin." *Science* 280 (5369):1610-3.

Grossman, R. I., F. Gonzalez-Scarano, S. W. Atlas, S. Galetta, and D. H. Silberberg. 1986. "Multiple sclerosis: gadolinium enhancement in MR imaging." *Radiology* 161 (3):721-5. doi: 10.1148/radiology.161.3.3786722.

Group, GBD 2015 Neurological Disorders Collaborator. 2017. "Global, regional, and national burden of neurological disorders during 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015." *Lancet Neurol* 16 (11):877-897. doi: 10.1016/S1474-4422(17)30299-5.

Hampton, D. W., J. Anderson, G. Pryce, K. A. Irvine, G. Giovannoni, J. W. Fawcett, A. Compston, R. J. Franklin, D. Baker, and S. Chandran. 2008. "An experimental model of secondary progressive multiple sclerosis that shows regional variation in gliosis, remyelination, axonal and neuronal loss." *J Neuroimmunol* 201-202:200-11. doi: 10.1016/j.jneuroim.2008.05.034.

Hart, B. L. 1988. "Biological basis of the behavior of sick animals." *Neurosci Biobehav Rev* 12 (2):123-37.

Hauser, S. L., A. Bar-Or, G. Comi, G. Giovannoni, H. P. Hartung, B. Hemmer, F. Lublin, X. Montalban, K. W. Rammohan, K. Selmaj, A. Traboulsee, J. S. Wolinsky, D. L. Arnold, G. Klingelschmitt, D. Masterman, P. Fontoura, S. Belachew, P. Chin, N. Mairon, H. Garren, L. Kappos, and OPERA I and OPERA II Clinical Investigators. 2016. "Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis." *N Engl J Med*. doi: 10.1056/NEJMoa1601277.

Hauser, S. L., D. M. Dawson, J. R. Lehrich, M. F. Beal, S. V. Kevy, R. D. Propper, J. A. Mills, and H. L. Weiner. 1983. "Intensive immunosuppression in progressive multiple sclerosis. A randomized, three-arm study of high-dose intravenous cyclophosphamide, plasma exchange, and ACTH." *N Engl J Med* 308 (4):173-80. doi: 10.1056/NEJM198301273080401.

Hauser, S. L., T. H. Doolittle, R. Lincoln, R. H. Brown, and C. A. Dinarello. 1990. "Cytokine accumulations in CSF of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6." *Neurology* 40 (11):1735-9.

Hauser, S. L., and J. R. Oksenberg. 2006. "The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration." *Neuron* 52 (1):61-76. doi: 10.1016/j.neuron.2006.09.011.

Hauser, S. L., E. Waubant, D. L. Arnold, T. Vollmer, J. Antel, R. J. Fox, A. Bar-Or, M. Panzara, N. Sarkar, S. Agarwal, A. Langer-Gould, C. H. Smith, and HERMES Trial Group. 2008. "B-cell depletion with rituximab in relapsing-remitting multiple sclerosis." *N Engl J Med* 358 (7):676-88. doi: 10.1056/NEJMoa0706383.

Hawker, K., P. O'Connor, M. S. Freedman, P. A. Calabresi, J. Antel, J. Simon, S. Hauser, E. Waubant, T. Vollmer, H. Panitch, J. Zhang, P. Chin, C. H. Smith, and OLYMPUS trial group. 2009. "Rituximab in patients with primary progressive multiple sclerosis: results of a randomized double-blind placebo-controlled multicenter trial." *Ann Neurol* 66 (4):460-71. doi: 10.1002/ana.21867.

Hazelton, I., A. Yates, A. Dale, J. Roodselaar, N. Akbar, M. J. Ruitenberg, D. C. Anthony, and Y. Couch. 2018. "Exacerbation of Acute Traumatic Brain Injury by Circulating Extracellular Vesicles." *J Neurotrauma* 35 (4):639-651. doi: 10.1089/neu.2017.5049.

Hellings, N., M. Barée, C. Verhoeven, M. B. D'hooghe, R. Medaer, C. C. Bernard, J. Raus, and P. Stinissen. 2001. "T-cell reactivity to multiple myelin antigens in multiple sclerosis patients and healthy controls." *J Neurosci Res* 63 (3):290-302. doi: 10.1002/1097-4547(20010201)63:3<290::AID-JNR1023>3.0.CO;2-4.

Herter, S., F. Herting, O. Mundigl, I. Waldhauer, T. Weinzierl, T. Fauti, G. Muth, D. Ziegler-Landesberger, E. Van Puijenbroek, S. Lang, M. N. Duong, L. Reslan, C. A. Gerdes, T. Friess, U. Baer, H. Burtscher, M. Weidner, C. Dumontet, P. Umana, G. Niederfellner, M. Bacac, and C. Klein. 2013. "Preclinical activity of the type II CD20

antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models." *Mol Cancer Ther* 12 (10):2031-42. doi: 10.1158/1535-7163.MCT-12-1182.

Herter, S., F. Herting, G. Muth, E. van Puijenbroek, T. Schlothauer, C. Ferrara, K. Brady, S. Lang, M. Bacac, E. Mössner, P. Umana, and C. Klein. 2018. "GA101 P329GLALA, a variant of obinutuzumab with abolished ADCC, ADCP and CDC function but retained cell death induction, is as efficient as rituximab in B-cell depletion and antitumor activity." *Haematologica* 103 (2):e78-e81. doi: 10.3324/haematol.2017.178996.

Herting, F., T. Friess, S. Bader, G. Muth, G. Hölzlwimmer, N. Rieder, P. Umana, and C. Klein. 2014. "Enhanced anti-tumor activity of the glycoengineered type II CD20 antibody obinutuzumab (GA101) in combination with chemotherapy in xenograft models of human lymphoma." *Leuk Lymphoma* 55 (9):2151-5160. doi: 10.3109/10428194.2013.856008.

Hervé, M., I. Isnardi, Y. S. Ng, J. B. Bussel, H. D. Ochs, C. Cunningham-Rundles, and E. Meffre. 2007. "CD40 ligand and MHC class II expression are essential for human peripheral B cell tolerance." *J Exp Med* 204 (7):1583-93. doi: 10.1084/jem.20062287.

Hickey, W. F. 1999. "Leukocyte traffic in the central nervous system: the participants and their roles." *Semin Immunol* 11 (2):125-37. doi: 10.1006/smim.1999.0168.

Hickey, W. F., B. L. Hsu, and H. Kimura. 1991. "T-lymphocyte entry into the central nervous system." *J Neurosci Res* 28 (2):254-60. doi: 10.1002/jnr.490280213.

Hintiryan, H., L. Gou, B. Zingg, S. Yamashita, H. M. Lyden, M. Y. Song, A. K. Grewal, X. Zhang, A. W. Toga, and H. W. Dong. 2012. "Comprehensive connectivity of the mouse main olfactory bulb: analysis and online digital atlas." *Front Neuroanat* 6:30. doi: 10.3389/fnana.2012.00030.

Hiremath, M. M., Y. Saito, G. W. Knapp, J. P. Ting, K. Suzuki, and G. K. Matsushima. 1998. "Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice." *J Neuroimmunol* 92 (1-2):38-49.

Hjelmström, P., A. E. Juedes, J. Fjell, and N. H. Ruddle. 1998. "B-cell-deficient mice develop experimental allergic encephalomyelitis with demyelination after myelin oligodendrocyte glycoprotein sensitization." *J Immunol* 161 (9):4480-3.

Hochmeister, S., R. Grundtner, J. Bauer, B. Engelhardt, R. Lyck, G. Gordon, T. Korosec, A. Kutzelnigg, J. J. Berger, M. Bradl, R. E. Bittner, and H. Lassmann. 2006. "Dysferlin is a new marker for leaky brain blood vessels in multiple sclerosis." *J Neuropathol Exp Neurol* 65 (9):855-65. doi: 10.1097/01.jnen.0000235119.52311.16.

Holick, M. F. 2007. "Vitamin D deficiency." *N Engl J Med* 357 (3):266-81. doi: 10.1056/NEJMra070553.

Honeychurch, J., W. Alduaij, M. Azizyan, E. J. Cheadle, H. Pelicano, A. Ivanov, P. Huang, M. S. Cragg, and T. M. Illidge. 2012. "Antibody-induced nonapoptotic cell death in human lymphoma and leukemia cells is mediated through a novel reactive oxygen species-dependent pathway." *Blood* 119 (15):3523-33. doi: 10.1182/blood-2011-12-395541.

Horng, S., A. Therattil, S. Moyon, A. Gordon, K. Kim, A. T. Argaw, Y. Hara, J. N. Mariani, S. Sawai, P. Flodby, E. D. Crandall, Z. Borok, M. V. Sofroniew, C. Chapouly, and G. R. John. 2017. "Astrocytic tight junctions control inflammatory CNS lesion pathogenesis." *J Clin Invest* 127 (8):3136-3151. doi: 10.1172/JCI91301.

Hotchkiss, R. S., K. C. Chang, P. E. Swanson, K. W. Tinsley, J. J. Hui, P. Klender, S. Xanthoudakis, S. Roy, C. Black, E. Grimm, R. Aspiotis, Y. Han, D. W. Nicholson, and I. E. Karl. 2000. "Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte." *Nat Immunol* 1 (6):496-501. doi: 10.1038/82741.

Hotchkiss, R. S., P. E. Swanson, C. M. Knudson, K. C. Chang, J. P. Cobb, D. F. Osborne, K. M. Zollner, T. G. Buchman, S. J. Korsmeyer, and I. E. Karl. 1999. "Overexpression of Bcl-2 in transgenic mice decreases apoptosis and improves survival in sepsis." *J Immunol* 162 (7):4148-56.

Howell, O. W., C. A. Reeves, R. Nicholas, D. Carassiti, B. Radotra, S. M. Gentleman, B. Serafini, F. Aloisi, F. Roncaroli, R. Magliozzi, and R. Reynolds. 2011. "Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis." *Brain* 134 (Pt 9):2755-71. doi: 10.1093/brain/awr182.

Howell, O. W., E. K. Schulz-Trieglaff, D. Carassiti, S. M. Gentleman, R. Nicholas, F. Roncaroli, and R. Reynolds. 2015. "Extensive grey matter pathology in the cerebellum in multiple sclerosis is linked to inflammation in the subarachnoid space." *Neuropathol Appl Neurobiol* 41 (6):798-813. doi: 10.1111/nan.12199.

Humby, F., M. Bombardieri, A. Manzo, S. Kelly, M. C. Blades, B. Kirkham, J. Spencer, and C. Pitzalis. 2009. "Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium." *PLoS Med* 6 (1):e1. doi: 10.1371/journal.pmed.0060001.

Hurwitz, A. A., T. J. Sullivan, R. A. Sobel, and J. P. Allison. 2002. "Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits the expansion of encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice." *Proc Natl Acad Sci U S A* 99 (5):3013-7. doi: 10.1073/pnas.042684699.

Huse, M., E. J. Quann, and M. M. Davis. 2008. "Shouts, whispers and the kiss of death: directional secretion in T cells." *Nat Immunol* 9 (10):1105-11. doi: 10.1038/ni.f.215.

Häusler, D., S. Häusser-Kinzel, L. Feldmann, S. Torke, G. Lepennetier, C. C. A. Bernard, S. S. Zamvil, W. Brück, K. Lehmann-Horn, and M. S. Weber. 2018. "Functional characterization of reappearing B cells after anti-CD20 treatment of CNS autoimmune disease." *Proc Natl Acad Sci U S A* 115 (39):9773-9778. doi: 10.1073/pnas.1810470115.

Höftberger, R., M. Leisser, J. Bauer, and H. Lassmann. 2015. "Autoimmune encephalitis in humans: how closely does it reflect multiple sclerosis ?" *Acta Neuropathol Commun* 3:80. doi: 10.1186/s40478-015-0260-9.

Jang, E., W. S. Cho, M. L. Cho, H. J. Park, H. J. Oh, S. M. Kang, D. J. Paik, and J. Youn. 2011. "Foxp3+ regulatory T cells control humoral autoimmunity by suppressing the development of long-lived plasma cells." *J Immunol* 186 (3):1546-53. doi: 10.4049/jimmunol.1002942.

Jurynczyk, M., F. Probert, T. Yeo, G. Tackley, T. D. W. Claridge, A. Cavey, M. R. Woodhall, S. Arora, T. Winkler, E. Schiffer, A. Vincent, G. DeLuca, N. R. Sibson, M. Isabel Leite, P. Waters, D. C. Anthony, and J. Palace. 2017. "Metabolomics reveals distinct, antibody-independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease." *Acta Neuropathol Commun* 5 (1):95. doi: 10.1186/s40478-017-0495-8.

Juryńczyk, M., G. Tackley, Y. Kong, R. Geraldes, L. Matthews, M. Woodhall, P. Waters, W. Kuker, M. Craner, A. Weir, G. C. DeLuca, S. Kremer, M. I. Leite, A. Vincent, A. Jacob, J. de Sèze, and J. Palace. 2017. "Brain lesion distribution criteria distinguish MS from AQP4-antibody NMOSD and MOG-antibody disease." *J Neurol Neurosurg Psychiatry* 88 (2):132-136. doi: 10.1136/jnnp-2016-314005.

Juto, A., K. Fink, F. Al Nimer, and F. Piehl. 2019. "Interrupting rituximab treatment in relapsing-remitting multiple sclerosis; no evidence of rebound disease activity." *Mult Scler Relat Disord* 37:101468. doi: 10.1016/j.msard.2019.101468.

Juurlink, B. H. 1997. "Response of glial cells to ischemia: roles of reactive oxygen species and glutathione." *Neurosci Biobehav Rev* 21 (2):151-66.

Kantarci, O. H., C. Lebrun, A. Siva, M. B. Keegan, C. J. Azevedo, M. Inglese, M. Tintoré, B. D. Newton, F. Durand-Dubief, M. P. Amato, N. De Stefano, M. P. Sormani, D. Pelletier, and D. T. Okuda. 2016. "Primary Progressive Multiple Sclerosis Evolving From Radiologically Isolated Syndrome." *Ann Neurol* 79 (2):288-94. doi: 10.1002/ana.24564.

Kap, Y. S., N. van Driel, E. Blezer, P. W. Parren, W. K. Bleeker, J. D. Laman, J. L. Craigen, and B. A. 't Hart. 2010. "Late B cell depletion with a human anti-human CD20 IgG1κ monoclonal antibody halts the development of experimental autoimmune encephalomyelitis in marmosets." *J Immunol* 185 (7):3990-4003. doi: 10.4049/jimmunol.1001393.

Kappos, L., J. Antel, G. Comi, X. Montalban, P. O'Connor, C. H. Polman, T. Haas, A. A. Korn, G. Karlsson, E. W. Radue, and FTY720 D2201 Study Group. 2006. "Oral fingolimod (FTY720) for relapsing multiple sclerosis." *N Engl J Med* 355 (11):1124-40. doi: 10.1056/NEJMoa052643.

Kappos, L., A. Bar-Or, B. A. C. Cree, R. J. Fox, G. Giovannoni, R. Gold, P. Vermersch, D. L. Arnold, S. Arnould, T. Scherz, C. Wolf, E. Wallström, F. Dahlke, and EXPAND Clinical Investigators. 2018. "Siponimod versus placebo in secondary progressive multiple sclerosis (EXPAND): a double-blind, randomised, phase 3 study." *Lancet* 391 (10127):1263-1273. doi: 10.1016/S0140-6736(18)30475-6.

Kappos, L., H. P. Hartung, M. S. Freedman, A. Boyko, E. W. Radü, D. D. Mikol, M. Lamarine, Y. Hyvert, U. Freudensprung, T. Plitz, J. van Beek, and ATAMS Study Group. 2014. "Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial." *Lancet Neurol* 13 (4):353-63. doi: 10.1016/S1474-4422(14)70028-6.

Kappos, L., D. Li, P. A. Calabresi, P. O'Connor, A. Bar-Or, F. Barkhof, M. Yin, D. Leppert, R. Glanzman, J. Tinbergen, and S. L. Hauser. 2011. "Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial." *Lancet* 378 (9805):1779-87. doi: 10.1016/S0140-6736(11)61649-8.

Kappos, L., E. W. Radue, P. O'Connor, C. Polman, R. Hohlfeld, P. Calabresi, K. Selmaj, C. Agoropoulou, M. Leyk, L. Zhang-Auberson, P. Burtin, and FREEDOMS Study Group. 2010. "A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis." *N Engl J Med* 362 (5):387-401. doi: 10.1056/NEJMoa0909494.

Kaskow, B. J., and C. Baecher-Allan. 2018. "Effector T Cells in Multiple Sclerosis." *Cold Spring Harb Perspect Med* 8 (4). doi: 10.1101/cshperspect.a029025.

Kawachi, I., and H. Lassmann. 2017. "Neurodegeneration in multiple sclerosis and neuromyelitis optica." *J Neurol Neurosurg Psychiatry* 88 (2):137-145. doi: 10.1136/jnnp-2016-313300.

Kay, L. M., and W. J. Freeman. 1998. "Bidirectional processing in the olfactory-limbic axis during olfactory behavior." *Behav Neurosci* 112 (3):541-53.

Keaney, J., and M. Campbell. 2015. "The dynamic blood-brain barrier." *FEBS J* 282 (21):4067-79. doi: 10.1111/febs.13412.

Kebir, H., K. Kreymborg, I. Ifergan, A. Dodelet-Devillers, R. Cayrol, M. Bernard, F. Giuliani, N. Arbour, B. Becher, and A. Prat. 2007. "Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation." *Nat Med* 13 (10):1173-5. doi: 10.1038/nm1651.

Kelly-Scumpia, K. M., P. O. Scumpia, J. S. Weinstein, M. J. Delano, A. G. Cuenca, D. C. Nacionales, J. L. Wynn, P. Y. Lee, Y. Kumagai, P. A. Efron, S. Akira, C. Wasserfall, M. A. Atkinson, and L. L. Moldawer. 2011. "B cells enhance early innate immune responses during bacterial sepsis." *J Exp Med* 208 (8):1673-82. doi: 10.1084/jem.20101715.

Kerjaschki, D., H. M. Regele, I. Moosberger, K. Nagy-Bojarski, B. Watschinger, A. Soleiman, P. Birner, S. Krieger, A. Hovorka, G. Silberhumer, P. Laakkonen, T. Petrova, B. Langer, and I. Raab. 2004. "Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates." *J Am Soc Nephrol* 15 (3):603-12.

Kerschensteiner, M., C. Stadelmann, B. S. Buddeberg, D. Merkler, F. M. Bareyre, D. C. Anthony, C. Linington, W. Brück, and M. E. Schwab. 2004. "Targeting experimental autoimmune encephalomyelitis lesions to a predetermined axonal tract system allows for refined behavioral testing in an animal model of multiple sclerosis." *Am J Pathol* 164 (4):1455-69. doi: 10.1016/S0002-9440(10)63232-4.

Khakh, B. S., and M. V. Sofroniew. 2015. "Diversity of astrocyte functions and phenotypes in neural circuits." *Nat Neurosci* 18 (7):942-52. doi: 10.1038/nn.4043.

Kidd, D., F. Barkhof, R. McConnell, P. R. Algra, I. V. Allen, and T. Revesz. 1999. "Cortical lesions in multiple sclerosis." *Brain* 122 (Pt 1):17-26.

Kilsdonk, I. D., M. Schoonheim, and M. P. Wattjes. 2017. "In-vivo imaging of meningeal inflammation in multiple sclerosis: Presence of evidence or evidence of presence?" *Mult Scler* 23 (8):1169-1171. doi: 10.1177/1352458517704924.

Kinnunen, T., N. Chamberlain, H. Morbach, T. Cantaert, M. Lynch, P. Preston-Hurlburt, K. C. Herold, D. A. Hafler, K. C. O'Connor, and E. Meffre. 2013. "Specific peripheral B cell tolerance defects in patients with multiple sclerosis." *J Clin Invest* 123 (6):2737-41. doi: 10.1172/JCI68775.

Kinnunen, T., N. Chamberlain, H. Morbach, J. Choi, S. Kim, J. Craft, L. Mayer, C. Cancrini, L. Passerini, R. Bacchetta, H. D. Ochs, T. R. Torgerson, and E. Meffre. 2013. "Accumulation of peripheral autoreactive B

cells in the absence of functional human regulatory T cells." *Blood* 121 (9):1595-603. doi: 10.1182/blood-2012-09-457465.

Kipp, M., S. Nyamoya, T. Hochstrasser, and S. Amor. 2016. "Multiple sclerosis animal models: A clinical and histopathological perspective." *Brain Pathol.* doi: 10.1111/bpa.12454.

Kirk, J., J. Plumb, M. Mirakhur, and S. McQuaid. 2003. "Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination." *J Pathol* 201 (2):319-27. doi: 10.1002/path.1434.

Kitley, J., M. Woodhall, P. Waters, M. I. Leite, E. Devenney, J. Craig, J. Palace, and A. Vincent. 2012. "Myelin-oligodendrocyte glycoprotein antibodies in adults with a neuromyelitis optica phenotype." *Neurology* 79 (12):1273-7. doi: 10.1212/WNL.0b013e31826aac4e.

Klaus, G. G., J. H. Humphrey, A. Kunkl, and D. W. Dongworth. 1980. "The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory." *Immunol Rev* 53:3-28.

Klein, C., A. Lammens, W. Schäfer, G. Georges, M. Schwaiger, E. Mössner, K. P. Hopfner, P. Umaña, and G. Niederfellner. 2013. "Epitope interactions of monoclonal antibodies targeting CD20 and their relationship to functional properties." *MAbs* 5 (1):22-33. doi: 10.4161/mabs.22771.

Kleiter, I., K. Hellwig, A. Berthele, T. Kümpfel, R. A. Linker, H. P. Hartung, F. Paul, O. Aktas, and Neuromyelitis Optica Study Group. 2012. "Failure of natalizumab to prevent relapses in neuromyelitis optica." *Arch Neurol* 69 (2):239-45. doi: 10.1001/archneurol.2011.216.

Koch-Henriksen, N., and P. S. Sørensen. 2010. "The changing demographic pattern of multiple sclerosis epidemiology." *Lancet Neurol* 9 (5):520-32. doi: 10.1016/S1474-4422(10)70064-8.

Krumbholz, M., D. Theil, T. Derfuss, A. Rosenwald, F. Schrader, C. M. Monoranu, S. L. Kalled, D. M. Hess, B. Serafini, F. Aloisi, H. Wekerle, R. Hohlfeld, and E. Meinl. 2005. "BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma." *J Exp Med* 201 (2):195-200. doi: 10.1084/jem.20041674.

Kurtzke, J. F. 1983. "Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS)." *Neurology* 33 (11):1444-52.

Kurtzke, J. F. 2013. "Epidemiology in multiple sclerosis: a pilgrim's progress." *Brain* 136 (Pt 9):2904-17. doi: 10.1093/brain/awt220.

Kurtzke, John F. 1955. "A New Scale for Evaluating Disability in Multiple Sclerosis." *Neurology* 5 (8):580-580. doi: 10.1212/wnl.5.8.580.

Kushner, I. 1993. "Regulation of the acute phase response by cytokines." *Perspect Biol Med* 36 (4):611-22.

Kutzelnigg, A., J. C. Faber-Rod, J. Bauer, C. F. Lucchinetti, P. S. Sorensen, H. Laursen, C. Stadelmann, W. Brück, H. Rauschka, M. Schmidbauer, and H. Lassmann. 2007. "Widespread demyelination in the cerebellar cortex in multiple sclerosis." *Brain Pathol* 17 (1):38-44. doi: 10.1111/j.1750-3639.2006.00041.x.

Kutzelnigg, A., and H. Lassmann. 2005. "Cortical lesions and brain atrophy in MS." *J Neurol Sci* 233 (1-2):55-9. doi: 10.1016/j.jns.2005.03.027.

Kutzelnigg, A., and H. Lassmann. 2014. "Pathology of multiple sclerosis and related inflammatory demyelinating diseases." *Handb Clin Neurol* 122:15-58. doi: 10.1016/B978-0-444-52001-2.00002-9.

Kutzelnigg, A., C. F. Lucchinetti, C. Stadelmann, W. Brück, H. Rauschka, M. Bergmann, M. Schmidbauer, J. E. Parisi, and H. Lassmann. 2005. "Cortical demyelination and diffuse white matter injury in multiple sclerosis." *Brain* 128 (Pt 11):2705-12. doi: 10.1093/brain/awh641.

Ladányi, A., J. Kiss, B. Somlai, K. Gilde, Z. Fejos, A. Mohos, I. Gaudi, and J. Tímár. 2007. "Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor." *Cancer Immunol Immunother* 56 (9):1459-69. doi: 10.1007/s00262-007-0286-3.

Lanzavecchia, A. 1985. "Antigen-specific interaction between T and B cells." *Nature* 314 (6011):537-9.

Lassmann, H. 2014a. "Mechanisms of white matter damage in multiple sclerosis." *Glia* 62 (11):1816-30. doi: 10.1002/glia.22597.

Lassmann, H. 2014b. "Multiple sclerosis: lessons from molecular neuropathology." *Exp Neurol* 262 Pt A:2-7. doi: 10.1016/j.expneurol.2013.12.003.

Lassmann, H. 2018. "Multiple Sclerosis Pathology." *Cold Spring Harb Perspect Med* 8 (3). doi: 10.1101/cshperspect.a028936.

Lassmann, H., and M. Bradl. 2017. "Multiple sclerosis: experimental models and reality." *Acta Neuropathol* 133 (2):223-244. doi: 10.1007/s00401-016-1631-4.

Lassmann, H., W. Brück, and C. F. Lucchinetti. 2007. "The immunopathology of multiple sclerosis: an overview." *Brain Pathol* 17 (2):210-8. doi: 10.1111/j.1750-3639.2007.00064.x.

Lawson, L. J., V. H. Perry, P. Dri, and S. Gordon. 1990. "Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain." *Neuroscience* 39 (1):151-70.

Leary, S. M., D. H. Miller, V. L. Stevenson, P. A. Brex, D. T. Chard, and A. J. Thompson. 2003. "Interferon beta-1a in primary progressive MS: an exploratory, randomized, controlled trial." *Neurology* 60 (1):44-51.

Lee, Y., B. M. Morrison, Y. Li, S. Lengacher, M. H. Farah, P. N. Hoffman, Y. Liu, A. Tsingalia, L. Jin, P. W. Zhang, L. Pellerin, P. J. Magistretti, and J. D. Rothstein. 2012. "Oligodendroglia metabolically support axons and contribute to neurodegeneration." *Nature* 487 (7408):443-8. doi: 10.1038/nature11314.

Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. "Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen." *Nature* 358 (6382):155-7. doi: 10.1038/358155a0.

Lennon, V. A., T. J. Kryzer, S. J. Pittock, A. S. Verkman, and S. R. Hinson. 2005. "IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel." *J Exp Med* 202 (4):473-7. doi: 10.1084/jem.20050304.

Li, Q., and B. A. Barres. 2018. "Microglia and macrophages in brain homeostasis and disease." *Nat Rev Immunol* 18 (4):225-242. doi: 10.1038/nri.2017.125.

Li, R., A. Rezk, L. M. Healy, G. Muirhead, A. Prat, J. L. Gommerman, A. Bar-Or, and MSSRF Canadian B cells in MS Team. 2015. "Cytokine-Defined B Cell Responses as Therapeutic Targets in Multiple Sclerosis." *Front Immunol* 6:626. doi: 10.3389/fimmu.2015.00626.

Li, R., A. Rezk, Y. Miyazaki, E. Hilgenberg, H. Touil, P. Shen, C. S. Moore, L. Michel, F. Althekair, S. Rajasekharan, J. L. Gommerman, A. Prat, S. Fillatreau, A. Bar-Or, and Canadian B cells in MS Team. 2015. "Proinflammatory GM-CSF-producing B cells in multiple sclerosis and B cell depletion therapy." *Sci Transl Med* 7 (310):310ra166. doi: 10.1126/scitranslmed.aab4176.

Li, Z., Y. He, S. Fan, and B. Sun. 2015. "Clemastine rescues behavioral changes and enhances remyelination in the cuprizone mouse model of demyelination." *Neurosci Bull* 31 (5):617-25. doi: 10.1007/s12264-015-1555-3.

Liddelow, S. A., and B. A. Barres. 2017. "Reactive Astrocytes: Production, Function, and Therapeutic Potential." *Immunity* 46 (6):957-967. doi: 10.1016/j.immuni.2017.06.006.

Linington, C., M. Bradl, H. Lassmann, C. Brunner, and K. Vass. 1988. "Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein." *Am J Pathol* 130 (3):443-54.

Lisak, R. P., J. A. Benjamins, L. Nedelkoska, J. L. Barger, S. Ragheb, B. Fan, N. Ouamara, T. A. Johnson, S. Rajasekharan, and A. Bar-Or. 2012. "Secretory products of multiple sclerosis B cells are cytotoxic to oligodendroglia in vitro." *J Neuroimmunol* 246 (1-2):85-95. doi: 10.1016/j.jneuroim.2012.02.015.

Lovato, L., S. N. Willis, S. J. Rodig, T. Caron, S. E. Almendinger, O. W. Howell, R. Reynolds, K. C. O'Connor, and D. A. Hafler. 2011. "Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis." *Brain* 134 (Pt 2):534-41. doi: 10.1093/brain/awq350.

Lublin, F. D., and S. C. Reingold. 1996. "Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis." *Neurology* 46 (4):907-11.

Lucchinetti, C., W. Brück, J. Parisi, B. Scheithauer, M. Rodriguez, and H. Lassmann. 2000. "Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination." *Ann Neurol* 47 (6):707-17.

Lucchinetti, C. F., W. Brück, M. Rodriguez, and H. Lassmann. 1996. "Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis." *Brain Pathol* 6 (3):259-74.

Lucchinetti, C. F., B. F. Popescu, R. F. Bunyan, N. M. Moll, S. F. Roemer, H. Lassmann, W. Brück, J. E. Parisi, B. W. Scheithauer, C. Giannini, S. D. Weigand, J. Mandrekar, and R. M. Ransohoff. 2011. "Inflammatory cortical demyelination in early multiple sclerosis." *N Engl J Med* 365 (23):2188-97. doi: 10.1056/NEJMoa1100648.

Ludwin, S. K., V. T.s Rao, C. S. Moore, and J. P. Antel. 2016. "Astrocytes in multiple sclerosis." *Mult Scler* 22 (9):1114-24. doi: 10.1177/1352458516643396.

Lund, H., M. Krakauer, A. Skimminge, F. Sellebjerg, E. Garde, H. R. Siebner, O. B. Paulson, D. Hesse, and L. G. Hanson. 2013. "Blood-brain barrier permeability of normal appearing white matter in relapsing-remitting multiple sclerosis." *PLoS One* 8 (2):e56375. doi: 10.1371/journal.pone.0056375.

Lyons, J. A., M. San, M. P. Happ, and A. H. Cross. 1999. "B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide." *Eur J Immunol* 29 (11):3432-9. doi: 10.1002/(SICI)1521-4141(199911)29:11<3432::AID-IMMU3432>3.0.CO;2-2.

Machado-Santos, J., E. Saji, A. R. Tröscher, M. Paunovic, R. Liblau, G. Gabriely, C. G. Bien, J. Bauer, and H. Lassmann. 2018. "The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells." *Brain* 141 (7):2066-2082. doi: 10.1093/brain/awy151.

MacLennan, I. C. 1994. "Germinal centers." *Annu Rev Immunol* 12:117-39. doi: 10.1146/annurev.iy.12.040194.001001.

Magliozzi, R., S. Columba-Cabezas, B. Serafini, and F. Aloisi. 2004. "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." *J Neuroimmunol* 148 (1-2):11-23. doi: 10.1016/j.jneuroim.2003.10.056.

Magliozzi, R., O. Howell, A. Vora, B. Serafini, R. Nicholas, M. Puopolo, R. Reynolds, and F. Aloisi. 2007. "Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology." *Brain* 130 (Pt 4):1089-104. doi: 10.1093/brain/awm038.

Magliozzi, R., O. W. Howell, R. Nicholas, C. Cruciani, M. Castellaro, C. Romualdi, S. Rossi, M. Pitteri, M. D. Benedetti, A. Gajofatto, F. B. Pizzini, S. Montemezzi, S. Rasia, R. Capra, A. Bertoldo, F. Facchiano, S. Monaco, R. Reynolds, and M. Calabrese. 2018. "Inflammatory intrathecal profiles and cortical damage in multiple sclerosis." *Ann Neurol* 83 (4):739-755. doi: 10.1002/ana.25197.

Magliozzi, R., O. W. Howell, C. Reeves, F. Roncaroli, R. Nicholas, B. Serafini, F. Aloisi, and R. Reynolds. 2010. "A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis." *Ann Neurol* 68 (4):477-93. doi: 10.1002/ana.22230.

Mahad, D. H., B. D. Trapp, and H. Lassmann. 2015. "Pathological mechanisms in progressive multiple sclerosis." *Lancet Neurol* 14 (2):183-93. doi: 10.1016/S1474-4422(14)70256-X.

Mahad, D. J., I. Ziabreva, G. Campbell, N. Lax, K. White, P. S. Hanson, H. Lassmann, and D. M. Turnbull. 2009. "Mitochondrial changes within axons in multiple sclerosis." *Brain* 132 (Pt 5):1161-74. doi: 10.1093/brain/awp046.

Maier, S. F., and L. R. Watkins. 1998. "Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition." *Psychol Rev* 105 (1):83-107.

Makshakov, G., E. Magonov, N. Totolyan, V. Nazarov, S. Lapin, A. Mazing, E. Verbitskaya, T. Trofimova, V. Krasnov, M. Shumilina, A. Skoromets, and E. Evdoshenko. 2017. "Leptomeningeal Contrast Enhancement Is Associated with Disability Progression and Grey Matter Atrophy in Multiple Sclerosis." *Neurol Res Int* 2017:8652463. doi: 10.1155/2017/8652463.

Mancardi, G. L., R. Saccardi, M. Filippi, F. Gualandi, A. Murialdo, M. Inglese, M. G. Marrosu, G. Meucci, L. Massacesi, A. Lugaresi, F. Pagliai, M. P. Sormani, F. Sardanelli, A. Marmont, and Italian GITMO-NEURO Intergroup on Autologous Hematopoietic Stem Cell Transplantation for Multiple Sclerosis. 2001. "Autologous hematopoietic stem cell transplantation suppresses Gd-enhanced MRI activity in MS." *Neurology* 57 (1):62-8.

Mandel, T. E., R. P. Phipps, A. Abbot, and J. G. Tew. 1980. "The follicular dendritic cell: long term antigen retention during immunity." *Immunol Rev* 53:29-59.

Marcus, R., A. Davies, K. Ando, W. Klapper, S. Opat, C. Owen, E. Phillips, R. Sangha, R. Schlag, J. F. Seymour, W. Townsend, M. Trněný, M. Wenger, G. Fingerle-Rowson, K. Rufibach, T. Moore, M. Herold, and W. Hiddemann. 2017. "Obinutuzumab for the First-Line Treatment of Follicular Lymphoma." *N Engl J Med* 377 (14):1331-1344. doi: 10.1056/NEJMoa1614598.

Mardiguian, S., E. Ladds, R. Turner, H. Shepherd, S. J. Campbell, and D. C. Anthony. 2017. "The contribution of the acute phase response to the pathogenesis of relapse in chronic-relapsing experimental autoimmune encephalitis models of multiple sclerosis." *J Neuroinflammation* 14 (1):196. doi: 10.1186/s12974-017-0969-4.

Markoullis, K., I. Sargiannidou, N. Schiza, F. Roncaroli, R. Reynolds, and K. A. Kleopa. 2014. "Oligodendrocyte gap junction loss and disconnection from reactive astrocytes in multiple sclerosis gray matter." *J Neuropathol Exp Neurol* 73 (9):865-79. doi: 10.1097/NEN.0000000000000106.

Martin, M.el P, P. D. Cravens, R. Winger, B. C. Kieseier, S. Cepok, T. N. Eagar, S. S. Zamvil, M. S. Weber, E. M. Frohman, B. K. Kleinschmidt-Demasters, T. J. Montine, B. Hemmer, C. M. Marra, and O. Stüve. 2009. "Depletion of B lymphocytes from cerebral perivascular spaces by rituximab." *Arch Neurol* 66 (8):1016-20. doi: 10.1001/archneurol.2009.157.

Mathias, A., G. Perriard, M. Canales, C. Soneson, M. Delorenzi, M. Schluep, and R. A. Du Pasquier. 2017. "Increased ex vivo antigen presentation profile of B cells in multiple sclerosis." *Mult Scler* 23 (6):802-809. doi: 10.1177/1352458516664210.

Matsushima, G. K., and P. Morell. 2001. "The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system." *Brain Pathol* 11 (1):107-16.

Matyszak, M. K., and V. H. Perry. 1995. "Demyelination in the central nervous system following a delayed-type hypersensitivity response to bacillus Calmette-Guérin." *Neuroscience* 64 (4):967-77.

Matyszak, M. K., and V. H. Perry. 1996. "Delayed-type hypersensitivity lesions in the central nervous system are prevented by inhibitors of matrix metalloproteinases." *J Neuroimmunol* 69 (1-2):141-9.

Mayo, L., S. A. Trauger, M. Blain, M. Nadeau, B. Patel, J. I. Alvarez, I. D. Mascanfroni, A. Yeste, P. Kivisäkk, K. Kallas, B. Ellezam, R. Bakshi, A. Prat, J. P. Antel, H. L. Weiner, and F. J. Quintana. 2014. "Regulation of astrocyte activation by glycolipids drives chronic CNS inflammation." *Nat Med* 20 (10):1147-56. doi: 10.1038/nm.3681.

McGinley, M. P., B. P. Moss, and J. A. Cohen. 2017. "Safety of monoclonal antibodies for the treatment of multiple sclerosis." *Expert Opin Drug Saf* 16 (1):89-100. doi: 10.1080/14740338.2017.1250881.

McNicholas, N., M. Hutchinson, C. McGuigan, and J. Chataway. 2018. "2017 McDonald diagnostic criteria: A review of the evidence." *Mult Scler Relat Disord* 24:48-54. doi: 10.1016/j.msard.2018.05.011.

McWilliam, O., F. Sellebjerg, H. V. Marquart, and M. R. von Essen. 2018. "B cells from patients with multiple sclerosis have a pathogenic phenotype and increased LTα and TGFβ1 response." *J Neuroimmunol* 324:157-164. doi: 10.1016/j.jneuroim.2018.09.001.

Mei, F., S. P. J. Fancy, Y. A. Shen, J. Niu, C. Zhao, B. Presley, E. Miao, S. Lee, S. R. Mayoral, S. A. Redmond, A. Etxeberria, L. Xiao, R. J. M. Franklin, A. Green, S. L. Hauser, and J. R. Chan. 2014. "Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis." *Nat Med* 20 (8):954-960. doi: 10.1038/nm.3618.

Mei, F., K. Lehmann-Horn, Y. A. Shen, K. A. Rankin, K. J. Stebbins, D. S. Lorrain, K. Pekarek, S. A Sagan, L. Xiao, C. Teuscher, H. C. von Büdingen, J. Wess, J. J. Lawrence, A. J. Green, S. P. Fancy, S. S. Zamvil, and J. R. Chan. 2016. "Accelerated remyelination during inflammatory demyelination prevents axonal loss and improves functional recovery." *Elife* 5. doi: 10.7554/eLife.18246.

Merkler, D., T. Ernsting, M. Kerschensteiner, W. Brück, and C. Stadelmann. 2006. "A new focal EAE model of cortical demyelination: multiple sclerosis-like lesions with rapid resolution of inflammation and extensive remyelination." *Brain* 129 (Pt 8):1972-83. doi: 10.1093/brain/awl135.

Meuth, S. G., A. M. Herrmann, O. J. Simon, V. Siffrin, N. Melzer, S. Bittner, P. Meuth, H. F. Langer, S. Hallermann, N. Boldakowa, J. Herz, T. Munsch, P. Landgraf, O. Aktas, M. Heckmann, V. Lessmann, T. Budde, B. C. Kieseier, F. Zipp, and H. Wiendl. 2009. "Cytotoxic CD8+ T cell-neuron interactions: perforin-dependent electrical silencing precedes but is not causally linked to neuronal cell death." *J Neurosci* 29 (49):15397-409. doi: 10.1523/JNEUROSCI.4339-09.2009.

Mi, S., B. Hu, K. Hahm, Y. Luo, E. S. Kam Hui, Q. Yuan, W. M. Wong, L. Wang, H. Su, T. H. Chu, J. Guo, W. Zhang, K. F. So, B. Pepinsky, Z. Shao, C. Graff, E. Garber, V. Jung, E. X. Wu, and W. Wu. 2007. "LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis." *Nat Med* 13 (10):1228-33. doi: 10.1038/nm1664.

Mi, S., X. Lee, Z. Shao, G. Thill, B. Ji, J. Relton, M. Levesque, N. Allaire, S. Perrin, B. Sands, T. Crowell, R. L. Cate, J. M. McCoy, and R. B. Pepinsky. 2004. "LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex." *Nat Neurosci* 7 (3):221-8. doi: 10.1038/nn1188.

Mi, S., R. H. Miller, X. Lee, M. L. Scott, S. Shulag-Morskaya, Z. Shao, J. Chang, G. Thill, M. Levesque, M. Zhang, C. Hession, D. Sah, B. Trapp, Z. He, V. Jung, J. M. McCoy, and R. B. Pepinsky. 2005. "LINGO-1 negatively regulates myelination by oligodendrocytes." *Nat Neurosci* 8 (6):745-51. doi: 10.1038/nn1460.

Mi, S., R. H. Miller, W. Tang, X. Lee, B. Hu, W. Wu, Y. Zhang, C. B. Shields, S. Miklasz, D. Shea, J. Mason, R. J. Franklin, B. Ji, Z. Shao, A. Chédotal, F. Bernard, A. Roulois, J. Xu, V. Jung, and B. Pepinsky. 2009. "Promotion of central nervous system remyelination by induced differentiation of oligodendrocyte precursor cells." *Ann Neurol* 65 (3):304-15. doi: 10.1002/ana.21581.

Miller, A. E., J. S. Wolinsky, L. Kappos, G. Comi, M. S. Freedman, T. P. Olsson, D. Bauer, M. Benamor, P. Truffinet, P. W. O'Connor, and TOPIC Study Group. 2014. "Oral teriflunomide for patients with a first clinical episode suggestive of multiple sclerosis (TOPIC): a randomised, double-blind, placebo-controlled, phase 3 trial." *Lancet Neurol* 13 (10):977-86. doi: 10.1016/S1474-4422(14)70191-7.

Misu, T., R. Höftberger, K. Fujihara, I. Wimmer, Y. Takai, S. Nishiyama, I. Nakashima, H. Konno, M. Bradl, F. Garzuly, Y. Itoyama, M. Aoki, and H. Lassmann. 2013. "Presence of six different lesion types suggests diverse mechanisms of tissue injury in neuromyelitis optica." *Acta Neuropathol* 125 (6):815-27. doi: 10.1007/s00401-013-1116-7.

Mittapalli, R. K., V. K. Manda, C. E. Adkins, W. J. Geldenhuys, and P. R. Lockman. 2010. "Exploiting nutrient transporters at the blood-brain barrier to improve brain distribution of small molecules." *Ther Deliv* 1 (6):775-84.

Mizuno, T., G. Zhang, H. Takeuchi, J. Kawanokuchi, J. Wang, Y. Sonobe, S. Jin, N. Takada, Y. Komatsu, and A. Suzumura. 2008. "Interferon-gamma directly induces neurotoxicity through a neuron specific, calcium-permeable complex of IFN-gamma receptor and AMPA GluR1 receptor." *FASEB J* 22 (6):1797-806. doi: 10.1096/fj.07-099499.

Moll, N. M., A. M. Rietsch, A. J. Ransohoff, M. B. Cossoy, D. Huang, F. S. Eichler, B. D. Trapp, and R. M. Ransohoff. 2008. "Cortical demyelination in PML and MS: Similarities and differences." *Neurology* 70 (5):336-43. doi: 10.1212/01.WNL.0000284601.54436.e4.

Molnarfi, N., U. Schulze-Topphoff, M. S. Weber, J. C. Patarroyo, T. Prod'homme, M. Varrin-Doyer, A. Shetty, C. Linington, A. J. Slavin, J. Hidalgo, D. E. Jenne, H. Wekerle, R. A. Sobel, C. C. Bernard, M. J. Shlomchik, and S. S. Zamvil. 2013. "MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies." *J Exp Med* 210 (13):2921-37. doi: 10.1084/jem.20130699.

Monson, N. L., P. D. Cravens, E. M. Frohman, K. Hawker, and M. K. Racke. 2005. "Effect of rituximab on the peripheral blood and cerebrospinal fluid B cells in patients with primary progressive multiple sclerosis." *Arch Neurol* 62 (2):258-64. doi: 10.1001/archneur.62.2.258.

Monson, N. L., P. Cravens, R. Hussain, C. T. Harp, M. Cummings, M. de Pilar Martin, L. H. Ben, J. Do, J. A. Lyons, A. Lovette-Racke, A. H. Cross, M. K. Racke, O. Stüve, M. Shlomchik, and T. N. Eagar. 2011. "Rituximab therapy reduces organ-specific T cell responses and ameliorates experimental autoimmune encephalomyelitis." *PLoS One* 6 (2):e17103. doi: 10.1371/journal.pone.0017103.

Montalban, X., S. L. Hauser, L. Kappos, D. L. Arnold, A. Bar-Or, G. Comi, J. de Seze, G. Giovannoni, H. P. Hartung, B. Hemmer, F. Lublin, K. W. Rammohan, K. Selmaj, A. Traboulsee, A. Sauter, D. Masterman, P. Fontoura, S. Belachew, H. Garren, N. Mairon, P. Chin, J. S. Wolinsky, and ORATORIO Clinical Investigators. 2016. "Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis." *N Engl J Med*. doi: 10.1056/NEJMoa1606468.

Montalban, X., S. L. Hauser, L. Kappos, D. L. Arnold, A. Bar-Or, G. Comi, J. de Seze, G. Giovannoni, H. P. Hartung, B. Hemmer, F. Lublin, K. W. Rammohan, K. Selmaj, A. Traboulsee, A. Sauter, D. Masterman, P. Fontoura, S. Belachew, H. Garren, N. Mairon, P. Chin, J. S. Wolinsky, and ORATORIO Clinical Investigators. 2017. "Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis." *N Engl J Med* 376 (3):209-220. doi: 10.1056/NEJMoa1606468.

Moore, C. S., S. L. Abdullah, A. Brown, A. Arulpragasam, and S. J. Crocker. 2011. "How factors secreted from astrocytes impact myelin repair." *J Neurosci Res* 89 (1):13-21. doi: 10.1002/jnr.22482.

Moore, C. S., R. Milner, A. Nishiyama, R. F. Frausto, D. R. Serwanski, R. R. Pagarigan, J. L. Whitton, R. H. Miller, and S. J. Crocker. 2011. "Astrocytic tissue inhibitor of metalloproteinase-1 (TIMP-1) promotes oligodendrocyte differentiation and enhances CNS myelination." *J Neurosci* 31 (16):6247-54. doi: 10.1523/JNEUROSCI.5474-10.2011.

Morrison, S. L., M. J. Johnson, L. A. Herzenberg, and V. T. Oi. 1984. "Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains." *Proc Natl Acad Sci U S A* 81 (21):6851-5. doi: 10.1073/pnas.81.21.6851.

Moshage, H. 1997. "Cytokines and the hepatic acute phase response." *J Pathol* 181 (3):257-66. doi: 10.1002/(SICI)1096-9896(199703)181:3<257::AID-PATH756>3.0.CO;2-U.

Mouly, A. M., A. Fort, N. Ben-Boutayab, and R. Gervais. 2001. "Olfactory learning induces differential long-lasting changes in rat central olfactory pathways." *Neuroscience* 102 (1):11-21.

Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund, and T. D. Randall. 2004. "Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity." *Nat Med* 10 (9):927-34. doi: 10.1038/nm1091.

Murakami, J., Y. Shimizu, Y. Kashii, T. Kato, M. Minemura, K. Okada, S. Nambu, T. Takahara, K. Higuchi, Y. Maeda, T. Kumada, and A. Watanabe. 1999. "Functional B-cell response in intrahepatic lymphoid follicles in chronic hepatitis C." *Hepatology* 30 (1):143-50. doi: 10.1002/hep.510300107.

Murray, T. J. 2005. *Multiple sclerosis : the history of a disease*. New York: Demos.

Mössner, E., P. Brünker, S. Moser, U. Püntener, C. Schmidt, S. Herter, R. Grau, C. Gerdes, A. Nopora, E. van Puijenbroek, C. Ferrara, P. Sondermann, C. Jäger, P. Strein, G. Fertig, T. Friess, C. Schüll, S. Bauer, J. Dal Porto, C. Del Nagro, K. Dabbagh, M. J. Dyer, S. Poppema, C. Klein, and P. Umaña. 2010. "Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity." *Blood* 115 (22):4393-402. doi: 10.1182/blood-2009-06-225979.

Nagarajan, S., K. Venkiteswaran, M. Anderson, U. Sayed, C. Zhu, and P. Selvaraj. 2000. "Cell-specific, activation-dependent regulation of neutrophil CD32A ligand-binding function." *Blood* 95 (3):1069-77.

Nakamura, K., A. Kitani, and W. Strober. 2001. "Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta." *J Exp Med* 194 (5):629-44.

Nakamura, M., T. Matsuoka, N. Chihara, S. Miyake, W. Sato, M. Araki, T. Okamoto, Y. Lin, M. Ogawa, M. Murata, T. Aranami, and T. Yamamura. 2014. "Differential effects of fingolimod on B-cell populations in multiple sclerosis." *Mult Scler* 20 (10):1371-80. doi: 10.1177/1352458514523496.

Nandoskar, A., J. Raffel, A. S. Scalfari, T. Friede, and R. S. Nicholas. 2017. "Pharmacological Approaches to the Management of Secondary Progressive Multiple Sclerosis." *Drugs* 77 (8):885-910. doi: 10.1007/s40265-017-0726-0.

Nedergaard, M., B. Ransom, and S. A. Goldman. 2003. "New roles for astrocytes: redefining the functional architecture of the brain." *Trends Neurosci* 26 (10):523-30. doi: 10.1016/j.tins.2003.08.008.

Nemazee, D. 2017. "Mechanisms of central tolerance for B cells." *Nat Rev Immunol* 17 (5):281-294. doi: 10.1038/nri.2017.19.

Newman, T. A., S. T. Woolley, P. M. Hughes, N. R. Sibson, D. C. Anthony, and V. H. Perry. 2001. "T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases." *Brain* 124 (Pt 11):2203-14. doi: 10.1093/brain/124.11.2203.

Niewoehner, J., B. Bohrmann, L. Collin, E. Urich, H. Sade, P. Maier, P. Rueger, J. O. Stracke, W. Lau, A. C. Tissot, H. Loetscher, A. Ghosh, and P. O. Freskgård. 2014. "Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle." *Neuron* 81 (1):49-60. doi: 10.1016/j.neuron.2013.10.061.

Nikić, I., D. Merkler, C. Sorbara, M. Brinkoetter, M. Kreutzfeldt, F. M. Bareyre, W. Brück, D. Bishop, T. Misgeld, and M. Kerschensteiner. 2011. "A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis." *Nat Med* 17 (4):495-9. doi: 10.1038/nm.2324.

Nimmerjahn, A., F. Kirchhoff, and F. Helmchen. 2005. "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo." *Science* 308 (5726):1314-8. doi: 10.1126/science.1110647.

Njenga, M. K., M. J. Coenen, N. DeCuir, H. Y. Yeh, and M. Rodriguez. 2000. "Short-term treatment with interferon-alpha/beta promotes remyelination, whereas long-term treatment aggravates demyelination in a murine model of multiple sclerosis." *J Neurosci Res* 59 (5):661-70. doi: 10.1002/(SICI)1097-4547(20000301)59:5<661::AID-JNR9>3.0.CO;2-E.

Noseworthy, J. H., C. Lucchinetti, M. Rodriguez, and B. G. Weinshenker. 2000. "Multiple sclerosis." *N Engl J Med* 343 (13):938-52. doi: 10.1056/NEJM200009283431307.

Nylander, A., and D. A. Hafler. 2012. "Multiple sclerosis." *J Clin Invest* 122 (4):1180-8. doi: 10.1172/JCI58649.

O'Loughlin, E., C. Madore, H. Lassmann, and O. Butovsky. 2018. "Microglial Phenotypes and Functions in Multiple Sclerosis." *Cold Spring Harb Perspect Med* 8 (2). doi: 10.1101/cshperspect.a028993.

Obermeier, B., L. Lovato, R. Mentele, W. Brück, I. Forne, A. Imhof, F. Lottspeich, K. W. Turk, S. N. Willis, H. Wekerle, R. Hohlfeld, D. A. Hafler, K. C. O'Connor, and K. Dornmair. 2011. "Related B cell clones that populate the CSF and CNS of patients with multiple sclerosis produce CSF immunoglobulin." *J Neuroimmunol* 233 (1-2):245-8. doi: 10.1016/j.jneuroim.2011.01.010.

Ohira, H., K. Abe, J. Yokokawa, J. Takiguchi, T. Rai, S. Shishido, and Y. Sato. 2003. "Adhesion molecules and CXC chemokines in endotoxin-induced liver injury." *Fukushima J Med Sci* 49 (1):1-13.

Ohira, H., T. Ueno, S. Shakado, M. Sakamoto, T. Torimura, S. Inuzuka, M. Sata, and K. Tanikawa. 1994. "Cultured rat hepatic sinusoidal endothelial cells express intercellular adhesion molecule-1 (ICAM-1) by tumor necrosis factor-alpha or interleukin-1 alpha stimulation." *J Hepatol* 20 (6):729-34.

Okuda, D. T., E. M. Mowry, A. Beheshtian, E. Waubant, S. E. Baranzini, D. S. Goodin, S. L. Hauser, and D. Pelletier. 2009. "Incidental MRI anomalies suggestive of multiple sclerosis: the radiologically isolated syndrome." *Neurology* 72 (9):800-5. doi: 10.1212/01.wnl.0000335764.14513.1a.

Opie, E. L., and J. Freund. 1937. "AN EXPERIMENTAL STUDY OF PROTECTIVE INOCULATION WITH HEAT KILLED TUBERCLE BACILLI." *J Exp Med* 66 (6):761-88.

Oshima, T., F. S. Laroux, L. L. Coe, Z. Morise, S. Kawachi, P. Bauer, M. B. Grisham, R. D. Specian, P. Carter, S. Jennings, D. N. Granger, T. Joh, and J. S. Alexander. 2001. "Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function." *Microvasc Res* 61 (1):130-43. doi: 10.1006/mvre.2000.2288.

Owens, G. P., J. L. Bennett, D. H. Gilden, and M. P. Burgoon. 2006. "The B cell response in multiple sclerosis." *Neurol Res* 28 (3):236-44. doi: 10.1179/016164106X98099.

Owens, G. P., A. M. Ritchie, D. H. Gilden, M. P. Burgoon, D. Becker, and J. L. Bennett. 2007. "Measles virus-specific plasma cells are prominent in subacute sclerosing panencephalitis CSF." *Neurology* 68 (21):1815-9. doi: 10.1212/01.wnl.0000262036.56594.7c.

Pachner, A. R., L. Li, and D. Lagunoff. 2011. "Plasma cells in the central nervous system in the Theiler's virus model of multiple sclerosis." *J Neuroimmunol* 232 (1-2):35-40. doi: 10.1016/j.jneuroim.2010.09.026.

Palandri, A., V. R. Salvador, J. Wojnacki, A. L. Vivinetto, R. L. Schnaar, and P. H. Lopez. 2015. "Myelin-associated glycoprotein modulates apoptosis of motoneurons during early postnatal development via NgR/p75(NTR) receptor-mediated activation of RhoA signaling pathways." *Cell Death Dis* 6:e1876. doi: 10.1038/cddis.2015.228.

Palanichamy, A., L. Apeltsin, T. C. Kuo, M. Sirota, S. Wang, S. J. Pitts, P. D. Sundar, D. Telman, L. Z. Zhao, M. Derstine, A. Abounasr, S. L. Hauser, and H. C. von Büdingen. 2014. "Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis." *Sci Transl Med* 6 (248):248ra106. doi: 10.1126/scitranslmed.3008930.

Panitch, H., A. Miller, D. Paty, B. Weinshenker, and North American Study Group on Interferon beta-1b in Secondary Progressive MS. 2004. "Interferon beta-1b in secondary progressive MS: results from a 3-year controlled study." *Neurology* 63 (10):1788-95.

Panitch, H. S. 1994. "Influence of infection on exacerbations of multiple sclerosis." *Ann Neurol* 36 Suppl:S25-8.

Park, J. B., G. Yiu, S. Kaneko, J. Wang, J. Chang, X. L. He, K. C. Garcia, and Z. He. 2005. "A TNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors." *Neuron* 45 (3):345-51. doi: 10.1016/j.neuron.2004.12.040.

Parodis, I., F. Söder, F. Faustini, Z. Kasza, I. Samuelsson, A. Zickert, E. Svenungsson, R. F. van Vollenhoven, V. Malmström, F. Wermeling, and I. Gunnarsson. 2018. "Rituximab-mediated late-onset neutropenia in systemic lupus erythematosus - distinct roles of BAFF and APRIL." *Lupus*:961203318777116. doi: 10.1177/0961203318777116.

Patrikios, P., C. Stadelmann, A. Kutzelnigg, H. Rauschka, M. Schmidbauer, H. Laursen, P. S. Sorensen, W. Brück, C. Lucchinetti, and H. Lassmann. 2006. "Remyelination is extensive in a subset of multiple sclerosis patients." *Brain* 129 (Pt 12):3165-72. doi: 10.1093/brain/awl217.

Paty, D. W., and D. K. Li. 1993. "Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group." *Neurology* 43 (4):662-7.

Pedersen, A. E., M. B. Jungersen, and C. D. Pedersen. 2011. "Monocytes mediate shaving of B-cell-bound anti-CD20 antibodies." *Immunology* 133 (2):239-45. doi: 10.1111/j.1365-2567.2011.03434.x.

Peferoen, L. A., D. Y. Vogel, K. Ummenthum, M. Breur, P. D. Heijnen, W. H. Gerritsen, R. M. Peferoen-Baert, P. van der Valk, C. D. Dijkstra, and S. Amor. 2015. "Activation status of human microglia is dependent on lesion formation stage and remyelination in multiple sclerosis." *J Neuropathol Exp Neurol* 74 (1):48-63. doi: 10.1097/NEN.0000000000000149.

Pelletier, D., and D. A. Hafler. 2012. "Fingolimod for multiple sclerosis." *N Engl J Med* 366 (4):339-47. doi: 10.1056/NEJMct1101691.

Peterson, J. W., L. Bö, S. Mörk, A. Chang, and B. D. Trapp. 2001. "Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions." *Ann Neurol* 50 (3):389-400.

Petrillo, Jennifer, Stanley Cohan, Carmen Castrillo-Viguera , Ih Chang , Yi Chai , Shih-Yin Chen , and Bing Zhu. 2018. "Effects of Opicinumab on Patient-Reported Outcomes in Participants with Relapsing Multiple Sclerosis: A Post-Hoc Analysis of the Phase 2 SYNERGY Study (P6.345)." *Neurology* 90 (15 supplement):P6.345.

Piehl, F. 2014. "A changing treatment landscape for multiple sclerosis: challenges and opportunities." *J Intern Med* 275 (4):364-81. doi: 10.1111/joim.12204.

Pikor, N. B., J. L. Astarita, L. Summers-Deluca, G. Galicia, J. Qu, L. A. Ward, S. Armstrong, C. X. Dominguez, D. Malhotra, B. Heiden, R. Kay, V. Castanov, H. Touil, L. Boon, P. O'Connor, A. Bar-Or, A. Prat, V. Ramaglia, S. Ludwin, S. J. Turley, and J. L. Gommerman. 2015. "Integration of Th17- and Lymphotoxin-Derived Signals Initiates Meningeal-Resident Stromal Cell Remodeling to Propagate Neuroinflammation." *Immunity* 43 (6):1160-73. doi: 10.1016/j.immuni.2015.11.010.

Pikor, N. B., A. Prat, A. Bar-Or, and J. L. Gommerman. 2015. "Meningeal Tertiary Lymphoid Tissues and Multiple Sclerosis: A Gathering Place for Diverse Types of Immune Cells during CNS Autoimmunity." *Front Immunol* 6:657. doi: 10.3389/fimmu.2015.00657.

Pipi, E., S. Nayar, D. H. Gardner, S. Colafrancesco, C. Smith, and F. Barone. 2018. "Tertiary Lymphoid Structures: Autoimmunity Goes Local." *Front Immunol* 9:1952. doi: 10.3389/fimmu.2018.01952.

Pirich, T., E. Zwickl-Traxler, M. Pecherstorfer, and J. Singer. 2018. "Tolerability of obinutuzumab therapy in patients with rituximab-relapsed/refractory B-cell malignancies - a retrospective single center cohort study." *Oncotarget* 9 (52):29944-29956. doi: 10.18632/oncotarget.25714.

Pitzalis, C., G. W. Jones, M. Bombardieri, and S. A. Jones. 2014. "Ectopic lymphoid-like structures in infection, cancer and autoimmunity." *Nat Rev Immunol* 14 (7):447-62. doi: 10.1038/nri3700.

Plemel, J. R., W. Q. Liu, and V. W. Yong. 2017. "Remyelination therapies: a new direction and challenge in multiple sclerosis." *Nat Rev Drug Discov* 16 (9):617-634. doi: 10.1038/nrd.2017.115.

Plemel, J. R., S. B. Manesh, J. S. Sparling, and W. Tetzlaff. 2013. "Myelin inhibits oligodendroglial maturation and regulates oligodendrocytic transcription factor expression." *Glia* 61 (9):1471-87. doi: 10.1002/glia.22535.

Pollak, Y., H. Ovadia, I. Goshen, R. Gurevich, K. Monsa, R. Avitsur, and R. Yirmiya. 2000. "Behavioral aspects of experimental autoimmune encephalomyelitis." *J Neuroimmunol* 104 (1):31-6.

Polman, C. H., P. W. O'Connor, E. Havrdova, M. Hutchinson, L. Kappos, D. H. Miller, J. T. Phillips, F. D. Lublin, G. Giovannoni, A. Wajgt, M. Toal, F. Lynn, M. A. Panzara, A. W. Sandrock, and AFFIRM Investigators. 2006. "A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." *N Engl J Med* 354 (9):899-910. doi: 10.1056/NEJMoa044397.

Ponath, G., S. Ramanan, M. Mubarak, W. Housley, S. Lee, F. R. Sahinkaya, A. Vortmeyer, C. S. Raine, and D. Pitt. 2017. "Myelin phagocytosis by astrocytes after myelin damage promotes lesion pathology." *Brain* 140 (2):399-413. doi: 10.1093/brain/aww298.

Popescu, B. F., and C. F. Lucchinetti. 2012. "Pathology of demyelinating diseases." *Annu Rev Pathol* 7:185-217. doi: 10.1146/annurev-pathol-011811-132443.

Popescu, V., R. Klaver, P. Voorn, Y. Galis-de Graaf, D. L. Knol, J. W. Twisk, A. Versteeg, G. J. Schenk, P. Van der Valk, F. Barkhof, H. E. De Vries, H. Vrenken, and J. J. Geurts. 2015. "What drives MRI-measured cortical atrophy in multiple sclerosis?" *Mult Scler* 21 (10):1280-90. doi: 10.1177/1352458514562440.

Poser, C. M., and V. V. Brinar. 2004. "Diagnostic criteria for multiple sclerosis: an historical review." *Clin Neurol Neurosurg* 106 (3):147-58. doi: 10.1016/j.clineuro.2004.02.004.

Poser, C. M., D. W. Paty, L. Scheinberg, W. I. McDonald, F. A. Davis, G. C. Ebers, K. P. Johnson, W. A. Sibley, D. H. Silberberg, and W. W. Tourtellotte. 1983. "New diagnostic criteria for multiple sclerosis: guidelines for research protocols." *Ann Neurol* 13 (3):227-31. doi: 10.1002/ana.410130302.

Praet, J., C. Guglielmetti, Z. Berneman, A. Van der Linden, and P. Ponsaerts. 2014. "Cellular and molecular neuropathology of the cuprizone mouse model: clinical relevance for multiple sclerosis." *Neurosci Biobehav Rev* 47:485-505. doi: 10.1016/j.neubiorev.2014.10.004.

Prineas, J. W., R. O. Barnard, E. E. Kwon, L. R. Sharer, and E. S. Cho. 1993. "Multiple sclerosis: remyelination of nascent lesions." *Ann Neurol* 33 (2):137-51. doi: 10.1002/ana.410330203.

Prineas, J. W., and F. Connell. 1979. "Remyelination in multiple sclerosis." *Ann Neurol* 5 (1):22-31. doi: 10.1002/ana.410050105.

Prineas, J. W., E. E. Kwon, E. S. Cho, L. R. Sharer, M. H. Barnett, E. L. Oleszak, B. Hoffman, and B. P. Morgan. 2001. "Immunopathology of secondary-progressive multiple sclerosis." *Ann Neurol* 50 (5):646-57.

Prinz, M., D. Erny, and N. Hagemeyer. 2017. "Ontogeny and homeostasis of CNS myeloid cells." *Nat Immunol* 18 (4):385-392. doi: 10.1038/ni.3703.

Putzki, N., M. K. Baranwal, B. Tettenborn, V. Limmroth, and E. Kreuzfelder. 2010. "Effects of natalizumab on circulating B cells, T regulatory cells and natural killer cells." *Eur Neurol* 63 (5):311-7. doi: 10.1159/000302687.

Périer, O., and A. Grégoire. 1965. "Electron microscopic features of multiple sclerosis lesions." *Brain* 88 (5):937-52.

Qin, D., J. Wu, K. A. Vora, J. V. Ravetch, A. K. Szakal, T. Manser, and J. G. Tew. 2000. "Fc gamma receptor IIB on follicular dendritic cells regulates the B cell recall response." *J Immunol* 164 (12):6268-75.

Quartuccio, L., M. Fabris, M. Moretti, F. Barone, M. Bombardieri, M. Rupolo, S. Lombardi, C. Pitzalis, C. A. Beltrami, F. Curcio, and S. De Vita. 2008. "Resistance to rituximab therapy and local BAFF overexpression in Sjögren's syndrome-related myoepithelial sialadenitis and low-grade parotid B-cell lymphoma." *Open Rheumatol J* 2:38-43. doi: 10.2174/1874312900802010038.

Racke, M. K., A. E. Lovett-Racke, and N. J. Karandikar. 2010. "The mechanism of action of glatiramer acetate treatment in multiple sclerosis." *Neurology* 74 Suppl 1:S25-30. doi: 10.1212/WNL.0b013e3181c97e39.

Ramagopalan, S. V., N. J. Maugeri, L. Handunnetthi, M. R. Lincoln, S. M. Orton, D. A. Dyment, G. C. Deluca, B. M. Herrera, M. J. Chao, A. D. Sadovnick, G. C. Ebers, and J. C. Knight. 2009. "Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D." *PLoS Genet* 5 (2):e1000369. doi: 10.1371/journal.pgen.1000369.

Ranger, A., S. Ray, S. Szak, A. Dearth, N. Allaire, R. Murray, R. Gardner, D. Cadavid, and S. Mi. 2018. "Anti-LINGO-1 has no detectable immunomodulatory effects in preclinical and phase 1 studies." *Neurol Neuroimmunol Neuroinflamm* 5 (1):e417. doi: 10.1212/NXI.0000000000000417.

Ransohoff, R. M. 2007. "Natalizumab for multiple sclerosis." *N Engl J Med* 356 (25):2622-9. doi: 10.1056/NEJMct071462.

Ransohoff, R. M., D. A. Hafler, and C. F. Lucchinetti. 2015. "Multiple sclerosis-a quiet revolution." *Nat Rev Neurol* 11 (3):134-42. doi: 10.1038/nrneurol.2015.14.

Raposo, G., and W. Stoorvogel. 2013. "Extracellular vesicles: exosomes, microvesicles, and friends." *J Cell Biol* 200 (4):373-83. doi: 10.1083/jcb.201211138.

Rauch, P. J., A. Chudnovskiy, C. S. Robbins, G. F. Weber, M. Etzrodt, I. Hilgendorf, E. Tiglao, J. L. Figueiredo, Y. Iwamoto, I. Theurl, R. Gorbatov, M. T. Waring, A. T. Chicoine, M. Mouded, M. J. Pittet, M. Nahrendorf, R. Weissleder, and F. K. Swirski. 2012. "Innate response activator B cells protect against microbial sepsis." *Science* 335 (6068):597-601. doi: 10.1126/science.1215173.

Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. "C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS

through the choroid plexus is required for the initiation of EAE." *Nat Immunol* 10 (5):514-23. doi: 10.1038/ni.1716.

Regard, L., C. Martin, L. Zemoura, V. Geolier, E. Sage, and P. R. Burgel. 2018. "Peribronchial tertiary lymphoid structures persist after rituximab therapy in patients with cystic fibrosis." *J Clin Pathol* 71 (8):752-753. doi: 10.1136/jclinpath-2018-205160.

Reich, D. S., C. F. Lucchinetti, and P. A. Calabresi. 2018. "Multiple Sclerosis." *N Engl J Med* 378 (2):169-180. doi: 10.1056/NEJMra1401483.

Rivas-Fuentes, S., E. García-García, G. Nieto-Castañeda, and C. Rosales. 2010. "Fcgamma receptors exhibit different phagocytosis potential in human neutrophils." *Cell Immunol* 263 (1):114-21. doi: 10.1016/j.cellimm.2010.03.006.

Rivers, T. M., and F. F. Schwentker. 1935. "ENCEPHALOMYELITIS ACCOMPANIED BY MYELIN DESTRUCTION EXPERIMENTALLY PRODUCED IN MONKEYS." *J Exp Med* 61 (5):689-702.

Rivers, T. M., D. H. Sprunt, and G. P. Berry. 1933. "OBSERVATIONS ON ATTEMPTS TO PRODUCE ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MONKEYS." *J Exp Med* 58 (1):39-53.

Robertson, N. P., D. Clayton, M. Fraser, J. Deans, and D. A. Compston. 1996. "Clinical concordance in sibling pairs with multiple sclerosis." *Neurology* 47 (2):347-52.

Robinson, M. W., C. Harmon, and C. O'Farrelly. 2016. "Liver immunology and its role in inflammation and homeostasis." *Cell Mol Immunol* 13 (3):267-76. doi: 10.1038/cmi.2016.3.

Rodda, L. B., O. Bannard, B. Ludewig, T. Nagasawa, and J. G. Cyster. 2015. "Phenotypic and Morphological Properties of Germinal Center Dark Zone Cxcl12-Expressing Reticular Cells." *J Immunol* 195 (10):4781-91. doi: 10.4049/jimmunol.1501191.

Rodríguez-Pinto, D. 2005. "B cells as antigen presenting cells." *Cell Immunol* 238 (2):67-75. doi: 10.1016/j.cellimm.2006.02.005.

Rommer, P. S., R. Patejdl, A. Winkelmann, R. Benecke, and U. K. Zettl. 2011. "Rituximab for secondary progressive multiple sclerosis: a case series." *CNS Drugs* 25 (7):607-13. doi: 10.2165/11589390-000000000-00000.

Rosati, G. 2001. "The prevalence of multiple sclerosis in the world: an update." *Neurol Sci* 22 (2):117-39.

Rothhammer, V., and F. J. Quintana. 2015. "Control of autoimmune CNS inflammation by astrocytes." *Semin Immunopathol* 37 (6):625-38. doi: 10.1007/s00281-015-0515-3.

Rovaris, M., D. Barnes, N. Woodrofe, G. H. du Boulay, J. W. Thorpe, A. J. Thompson, W. I. McDonald, and D. H. Miller. 1996. "Patterns of disease activity in multiple sclerosis patients: a study with quantitative gadolinium-enhanced brain MRI and cytokine measurement in different clinical subgroups." *J Neurol* 243 (7):536-42.

Ruddle, N. H. 2014. "Lymphatic vessels and tertiary lymphoid organs." *J Clin Invest* 124 (3):953-9. doi: 10.1172/JCI71611.

Saade, C., R. Bou-Fakhredin, D. M. Yousem, K. Asmar, L. Naffaa, and F. El-Merhi. 2018. "Gadolinium and Multiple Sclerosis: Vessels, Barriers of the Brain, and Glymphatics." *AJNR Am J Neuroradiol.* doi: 10.3174/ajnr.A5773.

Sadovnick, A. D., H. Armstrong, G. P. Rice, D. Bulman, L. Hashimoto, D. W. Paty, S. A. Hashimoto, S. Warren, W. Hader, and T. J. Murray. 1993. "A population-based study of multiple sclerosis in twins: update." *Ann Neurol* 33 (3):281-5. doi: 10.1002/ana.410330309.

Saiz, A., and F. Graus. 2004. "Neurological complications of hematopoietic cell transplantation." *Semin Neurol* 24 (4):427-34. doi: 10.1055/s-2004-861537.

Sakamoto, S., T. Okanoue, Y. Itoh, K. Sakamoto, K. Nishioji, Y. Nakagawa, N. Yoshida, T. Yoshikawa, and K. Kashima. 1997. "Intercellular adhesion molecule-1 and CD18 are involved in neutrophil adhesion and its

cytotoxicity to cultured sinusoidal endothelial cells in rats." *Hepatology* 26 (3):658-63. doi: 10.1053/jhep.1997.v26.pm0009303496.

Salles, G. A., F. Morschhauser, P. Solal-Céligny, C. Thieblemont, T. Lamy, H. Tilly, E. Gyan, G. Lei, M. Wenger, E. Wassner-Fritsch, and G. Cartron. 2013. "Obinutuzumab (GA101) in patients with relapsed/refractory indolent non-Hodgkin lymphoma: results from the phase II GAUGUIN study." *J Clin Oncol* 31 (23):2920-6. doi: 10.1200/JCO.2012.46.9718.

Salzer, J., R. Svenningsson, P. Alping, L. Novakova, A. Björck, K. Fink, P. Islam-Jakobsson, C. Malmeström, M. Axelsson, M. Vågberg, P. Sundström, J. Lycke, F. Piehl, and A. Svenningsson. 2016. "Rituximab in multiple sclerosis: A retrospective observational study on safety and efficacy." *Neurology* 87 (20):2074-2081. doi: 10.1212/WNL.0000000000003331.

Sansonno, D., G. Lauletta, V. De Re, F. A. Tucci, P. Gatti, V. Racanelli, M. Boiocchi, and F. Dammacco. 2004. "Intrahepatic B cell clonal expansions and extrahepatic manifestations of chronic HCV infection." *Eur J Immunol* 34 (1):126-36. doi: 10.1002/eji.200324328.

Satoh, J., H. Tabunoki, T. Yamamura, K. Arima, and H. Konno. 2007. "TROY and LINGO-1 expression in astrocytes and macrophages/microglia in multiple sclerosis lesions." *Neuropathol Appl Neurobiol* 33 (1):99-107. doi: 10.1111/j.1365-2990.2006.00787.x.

Sawcer, S., G. Hellenthal, M. Pirinen, C. C. Spencer, N. A. Patsopoulos, L. Moutsianas, A. Dilthey, Z. Su, C. Freeman, S. E. Hunt, S. Edkins, E. Gray, D. R. Booth, S. C. Potter, A. Goris, G. Band, A. B. Oturai, A. Strange, J. Saarela, C. Bellenguez, B. Fontaine, M. Gillman, B. Hemmer, R. Gwilliam, F. Zipp, A. Jayakumar, R. Martin, S. Leslie, S. Hawkins, E. Giannoulatou, S. D'alfonso, H. Blackburn, F. Martinelli Boneschi, J. Liddle, H. F. Harbo, M. L. Perez, A. Spurkland, M. J. Waller, M. P. Mycko, M. Ricketts, M. Comabella, N. Hammond, I. Kockum, O. T. McCann, M. Ban, P. Whittaker, A. Kemppinen, P. Weston, C. Hawkins, S. Widaa, J. Zajicek, S. Dronov, N. Robertson, S. J. Bumpstead, L. F. Barcellos, R. Ravindrarajah, R. Abraham, L. Alfredsson, K. Ardlie, C. Aubin, A. Baker, K. Baker, S. E. Baranzini, L. Bergamaschi, R. Bergamaschi, A. Bernstein, A. Berthele, M. Boggild, J. P. Bradfield, D. Brassat, S. A. Broadley, D. Buck, H. Butzkueven, R. Capra, W. M. Carroll, P. Cavalla, E. G. Celius, S. Cepok, R. Chiavacci, F. Clerget-Darpoux, K. Clysters, G. Comi, M. Cossburn, I. Cournu-Rebeix, M. B. Cox, W. Cozen, B. A. Cree, A. H. Cross, D. Cusi, M. J. Daly, E. Davis, P. I. de Bakker, M. Debouverie, M. B. D'hooghe, K. Dixon, R. Dobosi, B. Dubois, D. Ellinghaus, I. Elovaara, F. Esposito, C. Fontenille, S. Foote, A. Franke, D. Galimberti, A. Ghezzi, J. Glessner, R. Gomez, O. Gout, C. Graham, S. F. Grant, F. R. Guerini, H. Hakonarson, P. Hall, A. Hamsten, H. P. Hartung, R. N. Heard, S. Heath, J. Hobart, M. Hoshi, C. Infante-Duarte, G. Ingram, W. Ingram, T. Islam, M. Jagodic, M. Kabesch, A. G. Kermode, T. J. Kilpatrick, C. Kim, N. Klopp, K. Koivisto, M. Larsson, M. Lathrop, J. S. Lechner-Scott, M. A. Leone, V. Leppä, U. Liljedahl, I. L. Bomfim, R. R. Lincoln, J. Link, J. Liu, A. R. Lorentzen, S. Lupoli, F. Macciardi, T. Mack, M. Marriott, V. Martinelli, D. Mason, J. L. McCauley, F. Mentch, I. L. Mero, T. Mihalova, X. Montalban, J. Mottershead, K. M. Myhr, P. Naldi, W. Ollier, A. Page, A. Palotie, J. Pelletier, L. Piccio, T. Pickersgill, F. Piehl, S. Pobywajlo, H. L. Quach, P. P. Ramsay, M. Reunanen, R. Reynolds, J. D. Rioux, M. Rodegher, S. Roesner, J. P. Rubio, I. M. Rückert, M. Salvetti, E. Salvi, A. Santaniello, C. A. Schaefer, S. Schreiber, C. Schulze, R. J. Scott, F. Sellebjerg, K. W. Selmaj, D. Sexton, L. Shen, B. Simms-Acuna, S. Skidmore, P. M. Sleiman, C. Smestad, P. S. Sørensen, H. B. Søndergaard, J. Stankovich, R. C. Strange, A. M. Sulonen, E. Sundqvist, A. C. Syvänen, F. Taddeo, B. Taylor, J. M. Blackwell, P. Tienari, E. Bramon, A. Tourbah, M. A. Brown, E. Tronczynska, J. P. Casas, N. Tubridy, A. Corvin, J. Vickery, J. Jankowski, P. Villoslada, H. S. Markus, K. Wang, C. G. Mathew, J. Wason, C. N. Palmer, H. E. Wichmann, R. Plomin, E. Willoughby, A. Rautanen, J. Winkelmann, M. Wittig, R. C. Trembath, J. Yaouanq, A. C. Viswanathan, H. Zhang, N. W. Wood, R. Zuvich, P. Deloukas, C. Langford, A. Duncanson, J. R. Oksenberg, M. A. Pericak-Vance, J. L. Haines, T. Olsson, J. Hillert, A. J. Ivinson, P. L. De Jager, L. Peltonen, G. J. Stewart, D. A. Hafler, S. L. Hauser, G. McVean, P. Donnelly, A. Compston, International Multiple Sclerosis Genetics Consortium, and Wellcome Trust Case Control Consortium 2. 2011. "Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis." *Nature* 476 (7359):214-9. doi: 10.1038/nature10251.

Schlothauer, T., S. Herter, C. F. Koller, S. Grau-Richards, V. Steinhart, C. Spick, M. Kubbies, C. Klein, P. Umaña, and E. Mössner. 2016. "Novel human IgG1 and IgG4 Fc-engineered antibodies with completely abolished immune effector functions." *Protein Eng Des Sel* 29 (10):457-466. doi: 10.1093/protein/gzw040.

Schneider, R. 2015. "CD20+ T cells in multiple sclerosis." *Mult Scler Relat Disord* 4 (1):58-9. doi: 10.1016/j.msard.2014.09.213.

Schnell, L., S. Fearn, H. Klassen, M. E. Schwab, and V. H. Perry. 1999. "Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord." *Eur J Neurosci* 11 (10):3648-58.

Schnell, L., S. Fearn, M. E. Schwab, V. H. Perry, and D. C. Anthony. 1999. "Cytokine-induced acute inflammation in the brain and spinal cord." *J Neuropathol Exp Neurol* 58 (3):245-54.

Schuh, E., K. Berer, M. Mulazzani, K. Feil, I. Meinl, H. Lahm, M. Krane, R. Lange, K. Pfannes, M. Subklewe, R. Gürkov, M. Bradl, R. Hohlfeld, T. Kümpfel, E. Meinl, and M. Krumbholz. 2016. "Features of Human CD3+CD20+ T Cells." *J Immunol* 197 (4):1111-7. doi: 10.4049/jimmunol.1600089.

Schuhmann, M. K., F. Langhauser, P. Kraft, and C. Kleinschnitz. 2017. "B cells do not have a major pathophysiologic role in acute ischemic stroke in mice." *J Neuroinflammation* 14 (1):112. doi: 10.1186/s12974-017-0890-x.

Schumacher, George A., Gilbert Beebe, Robert F. Kibler, Leonard T. Kurland, John F. Kurtzke, Fletcher McDowell, Benedict Nagler, William A. Sibley, Wallace W. Tourtellotte, and Thomas L. Willmon. 1965. "PROBLEMS OF EXPERIMENTAL TRIALS OF THERAPY IN MULTIPLE SCLEROSIS: REPORT BY THE PANEL ON THE EVALUATION OF EXPERIMENTAL TRIALS OF THERAPY IN MULTIPLE SCLEROSIS." *Annals of the New York Academy of Sciences* 122 (1):552-568. doi: 10.1111/j.1749-6632.1965.tb20235.x.

Seda, V., and M. Mraz. 2015. "B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells." *Eur J Haematol* 94 (3):193-205. doi: 10.1111/ejh.12427.

Seewann, A., H. Vrenken, P. van der Valk, E. L. Blezer, D. L. Knol, J. A. Castelijns, C. H. Polman, P. J. Pouwels, F. Barkhof, and J. J. Geurts. 2009. "Diffusely abnormal white matter in chronic multiple sclerosis: imaging and histopathologic analysis." *Arch Neurol* 66 (5):601-9. doi: 10.1001/archneurol.2009.57.

Sefia, E., G. Pryce, U. C. Meier, G. Giovannoni, and D. Baker. 2017. "Depletion of CD20 B cells fails to inhibit relapsing mouse experimental autoimmune encephalomyelitis." *Mult Scler Relat Disord* 14:46-50. doi: 10.1016/j.msard.2017.03.013.

Segal, B. M., C. S. Constantinescu, A. Raychaudhuri, L. Kim, R. Fidelus-Gort, L. H. Kasper, and Ustekinumab MS Investigators. 2008. "Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study." *Lancet Neurol* 7 (9):796-804. doi: 10.1016/S1474-4422(08)70173-X.

Selmaj, K., C. S. Raine, B. Cannella, and C. F. Brosnan. 1991. "Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions." *J Clin Invest* 87 (3):949-54. doi: 10.1172/JCI115102.

Serafini, B., B. Rosicarelli, R. Magliozzi, E. Stigliano, and F. Aloisi. 2004. "Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis." *Brain Pathol* 14 (2):164-74.

Serres, S., D. C. Anthony, Y. Jiang, S. J. Campbell, K. A. Broom, A. Khrapitchev, and N. R. Sibson. 2009. "Comparison of MRI signatures in pattern I and II multiple sclerosis models." *NMR Biomed* 22 (10):1014-24. doi: 10.1002/nbm.1404.

Shah, S., C. G. Kavadichanda, P. Belani, R. N. Ganesh, and V. S. Negi. 2018. "Early onset neutropenia and thrombocytopenia following rituximab in lupus nephritis." *Int J Rheum Dis*. doi: 10.1111/1756-185X.13365.

Shao, Z., J. L. Browning, X. Lee, M. L. Scott, S. Shulga-Morskaya, N. Allaire, G. Thill, M. Levesque, D. Sah, J. M. McCoy, B. Murray, V. Jung, R. B. Pepinsky, and S. Mi. 2005. "TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration." *Neuron* 45 (3):353-9. doi: 10.1016/j.neuron.2004.12.050.

Shao, Z., X. Lee, G. Huang, G. Sheng, C. E. Henderson, D. Louvard, J. Sohn, B. Pepinsky, and S. Mi. 2017. "LINGO-1 Regulates Oligodendrocyte Differentiation through the Cytoplasmic Gelsolin Signaling Pathway." *J Neurosci* 37 (12):3127-3137. doi: 10.1523/JNEUROSCI.3722-16.2017.

Sharma, R., M. T. Fischer, J. Bauer, P. A. Felts, K. J. Smith, T. Misu, K. Fujihara, M. Bradl, and H. Lassmann. 2010. "Inflammation induced by innate immunity in the central nervous system leads to primary astrocyte dysfunction followed by demyelination." *Acta Neuropathol* 120 (2):223-36. doi: 10.1007/s00401-010-0704-z.

Shen, P., and S. Fillatreau. 2015. "Antibody-independent functions of B cells: a focus on cytokines." *Nat Rev Immunol* 15 (7):441-51. doi: 10.1038/nri3857.

Shepro, D., and N. M. Morel. 1993. "Pericyte physiology." *FASEB J* 7 (11):1031-8.

Silva, B. A., M. C. Leal, M. I. Farías, J. C. Avalos, C. H. Besada, F. J. Pitossi, and C. C. Ferrari. 2018. "A new focal model resembling features of cortical pathology of the progressive forms of multiple sclerosis: Influence of innate immunity." *Brain Behav Immun* 69:515-531. doi: 10.1016/j.bbi.2018.01.010.

Simpson, S., B. Taylor, L. Blizzard, A. L. Ponsonby, F. Pittas, H. Tremlett, T. Dwyer, P. Gies, and I. van der Mei. 2010. "Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis." *Ann Neurol* 68 (2):193-203. doi: 10.1002/ana.22043.

Singh, V., D. Gupta, R. Arora, R. P. Tripathi, A. Almasan, and R. M. Macklis. 2014. "Surface levels of CD20 determine anti-CD20 antibodies mediated cell death in vitro." *PLoS One* 9 (11):e111113. doi: 10.1371/journal.pone.0111113.

Skopelja-Gardner, S., J. D. Jones, B. J. Hamilton, A. V. Danilov, and W. F. C. Rigby. 2017. "Role for ZAP-70 Signaling in the Differential Effector Functions of Rituximab and Obinutuzumab (GA101) in Chronic Lymphocytic Leukemia B Cells." *J Immunol* 199 (4):1275-1282. doi: 10.4049/jimmunol.1602105.

Sobel, R. A., R. C. van der Veen, and M. B. Lees. 1986. "The immunopathology of chronic experimental allergic encephalomyelitis induced in rabbits with bovine proteolipid protein." *J Immunol* 136 (1):157-63.

Sofroniew, M. V. 2009. "Molecular dissection of reactive astrogliosis and glial scar formation." *Trends Neurosci* 32 (12):638-47. doi: 10.1016/j.tins.2009.08.002.

Sofroniew, M. V. 2014. "Astrogliosis." *Cold Spring Harb Perspect Biol* 7 (2):a020420. doi: 10.1101/cshperspect.a020420.

Sofroniew, M. V. 2015. "Astrocyte barriers to neurotoxic inflammation." *Nat Rev Neurosci* 16 (5):249-63. doi: 10.1038/nrn3898.

Sorensen, P. S., S. Lisby, R. Grove, F. Derosier, S. Shackelford, E. Havrdova, J. Drulovic, and M. Filippi. 2014. "Safety and efficacy of ofatumumab in relapsing-remitting multiple sclerosis: a phase 2 study." *Neurology* 82 (7):573-81. doi: 10.1212/WNL.0000000000000125.

Spain, R., K. Powers, C. Murchison, E. Heriza, K. Winges, V. Yadav, M. Cameron, E. Kim, F. Horak, J. Simon, and D. Bourdette. 2017. "Lipoic acid in secondary progressive MS: A randomized controlled pilot trial." *Neurol Neuroimmunol Neuroinflamm* 4 (5):e374. doi: 10.1212/NXI.0000000000000374.

Stern, J. N., G. Yaari, J. A. Vander Heiden, G. Church, W. F. Donahue, R. Q. Hintzen, A. J. Huttner, J. D. Laman, R. M. Nagra, A. Nylander, D. Pitt, S. Ramanan, B. A. Siddiqui, F. Vigneault, S. H. Kleinstein, D. A. Hafler, and K. C. O'Connor. 2014. "B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes." *Sci Transl Med* 6 (248):248ra107. doi: 10.1126/scitranslmed.3008879.

Stern, Y. 2012. "Cognitive reserve in ageing and Alzheimer's disease." *Lancet Neurol* 11 (11):1006-12. doi: 10.1016/S1474-4422(12)70191-6.

Stills, H. F. 2005. "Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants." *ILAR J* 46 (3):280-93.

Sun, J. J., Q. G. Ren, L. Xu, and Z. J. Zhang. 2015. "LINGO-1 antibody ameliorates myelin impairment and spatial memory deficits in experimental autoimmune encephalomyelitis mice." *Sci Rep* 5:14235. doi: 10.1038/srep14235.

Sá-Pereira, I., J. Roodselaar, Y. Couch, M. Consentino Kronka Sosthenes, M. C. Evans, D. C. Anthony, and H. B. Stolp. 2018. "Hepatic acute phase response protects the brain from focal inflammation during postnatal window of susceptibility." *Brain Behav Immun* 69:486-498. doi: 10.1016/j.bbi.2018.01.008.

Takemura, S., P. A. Klimiuk, A. Braun, J. J. Goronzy, and C. M. Weyand. 2001. "T cell activation in rheumatoid synovium is B cell dependent." *J Immunol* 167 (8):4710-8.

Tanaka, M., K. Park, and K. Tanaka. 2013. "Reduced fingolimod dosage treatment for patients with multiple sclerosis and lymphopenia or neutropenia." *Mult Scler* 19 (9):1244-5. doi: 10.1177/1352458512472750.

Tang, H., M. Zhu, J. Qiao, and Y. X. Fu. 2017. "Lymphotoxin signalling in tertiary lymphoid structures and immunotherapy." *Cell Mol Immunol* 14 (10):809-818. doi: 10.1038/cmi.2017.13.

Tanuma, N., H. Sakuma, A. Sasaki, and Y. Matsumoto. 2006. "Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis." *Acta Neuropathol* 112 (2):195-204. doi: 10.1007/s00401-006-0083-7.

Thaunat, O., A. C. Field, J. Dai, L. Louedec, N. Patey, M. F. Bloch, C. Mandet, M. F. Belair, P. Bruneval, O. Meilhac, B. Bellon, E. Joly, J. B. Michel, and A. Nicoletti. 2005. "Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response." *Proc Natl Acad Sci U S A* 102 (41):14723-8. doi: 10.1073/pnas.0507223102.

Thaunat, O., N. Patey, C. Gautreau, S. Lechaton, V. Fremeaux-Bacchi, M. C. Dieu-Nosjean, E. Cassuto-Viguier, C. Legendre, M. Delahousse, P. Lang, J. B. Michel, and A. Nicoletti. 2008. "B cell survival in intragraft tertiary lymphoid organs after rituximab therapy." *Transplantation* 85 (11):1648-53. doi: 10.1097/TP.0b013e3181735723.

Theotokis, P., and N. Grigoriadis. 2018. "p75NTR and TROY: Uncharted Roles of Nogo Receptor Complex in Experimental Autoimmune Encephalomyelitis." *Mol Neurobiol* 55 (8):6329-6336. doi: 10.1007/s12035-017-0841-7.

Tobinai, K., C. Klein, N. Oya, and G. Fingerle-Rowson. 2017. "A Review of Obinutuzumab (GA101), a Novel Type II Anti-CD20 Monoclonal Antibody, for the Treatment of Patients with B-Cell Malignancies." *Adv Ther* 34 (2):324-356. doi: 10.1007/s12325-016-0451-1.

Torkildsen, Ø, K. M. Myhr, and L. Bø. 2016. "Disease-modifying treatments for multiple sclerosis - a review of approved medications." *Eur J Neurol* 23 Suppl 1:18-27. doi: 10.1111/ene.12883.

Tourbah, A., C. Lebrun-Frenay, G. Edan, M. Clanet, C. Papeix, S. Vukusic, J. De Sèze, M. Debouverie, O. Gout, P. Clavelou, G. Defer, D. A. Laplaud, T. Moreau, P. Labauge, B. Brochet, F. Sedel, J. Pelletier, and MS-SPI study group. 2016. "MD1003 (high-dose biotin) for the treatment of progressive multiple sclerosis: A randomised, double-blind, placebo-controlled study." *Mult Scler* 22 (13):1719-1731. doi: 10.1177/1352458516667568.

Tradtrantip, L., T. Duan, M. R. Yeaman, and A. S. Verkman. 2019. "CD55 upregulation in astrocytes by statins as potential therapy for AQP4-IgG seropositive neuromyelitis optica." *J Neuroinflammation* 16 (1):57. doi: 10.1186/s12974-019-1448-x.

Tran, J. Q., J. Rana, F. Barkhof, I. Melamed, H. Gevorkyan, M. P. Wattjes, R. de Jong, K. Brosofsky, S. Ray, L. Xu, J. Zhao, E. Parr, and D. Cadavid. 2014. "Randomized phase I trials of the safety/tolerability of anti-LINGO-1 monoclonal antibody BIIB033." *Neurol Neuroimmunol Neuroinflamm* 1 (2):e18. doi: 10.1212/NXI.0000000000000018.

Trapp, B. D., and P. K. Stys. 2009. "Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis." *Lancet Neurol* 8 (3):280-91. doi: 10.1016/S1474-4422(09)70043-2.

Tremblay, M., C. Lecours, L. Samson, V. Sánchez-Zafra, and A. Sierra. 2015. "From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia." *Front Neuroanat* 9:45. doi: 10.3389/fnana.2015.00045.

Tsenova, L., R. Harbacheuski, N. Sung, E. Ellison, D. Fallows, and G. Kaplan. 2007. "BCG vaccination confers poor protection against M. tuberculosis HN878-induced central nervous system disease." *Vaccine* 25 (28):5126-32. doi: 10.1016/j.vaccine.2006.11.024.

Tsuboi, N., K. Asano, M. Lauterbach, and T. N. Mayadas. 2008. "Human neutrophil Fcgamma receptors initiate and play specialized nonredundant roles in antibody-mediated inflammatory diseases." *Immunity* 28 (6):833-46. doi: 10.1016/j.immuni.2008.04.013.

Uhlar, C. M., and A. S. Whitehead. 1999. "Serum amyloid A, the major vertebrate acute-phase reactant." *Eur J Biochem* 265 (2):501-23.

Ukai, J., S. Imagama, T. Ohgomori, Z. Ito, K. Ando, N. Ishiguro, and K. Kadomatsu. 2016. "Nogo receptor 1 is expressed in both primary cultured glial cells and neurons." *Nagoya J Med Sci* 78 (3):303-11.

van Oosten, B. W., M. Lai, S. Hodgkinson, F. Barkhof, D. H. Miller, I. F. Moseley, A. J. Thompson, P. Rudge, A. McDougall, J. G. McLeod, H. J. Adèr, and C. H. Polman. 1997. "Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody cM-T412: results of a randomized, double-blind, placebo-controlled, MR-monitored phase II trial." *Neurology* 49 (2):351-7.

van Well, G. T., C. W. Wieland, S. Florquin, J. J. Roord, T. van der Poll, and A. M. van Furth. 2007. "A new murine model to study the pathogenesis of tuberculous meningitis." *J Infect Dis* 195 (5):694-7. doi: 10.1086/511273.

Vandvik, B., E. Norrby, H. J. Nordal, and M. Degré. 1976. "Oligoclonal measles virus-specific IgG antibodies isolated from cerebrospinal fluids, brain extracts, and sera from patients with subacute sclerosing panencephalitis and multiple sclerosis." *Scand J Immunol* 5 (8):979-92.

Vaughan, J. H., J. R. Valbracht, M. D. Nguyen, H. H. Handley, R. S. Smith, K. Patrick, and G. H. Rhodes. 1995. "Epstein-Barr virus-induced autoimmune responses. I. Immunoglobulin M autoantibodies to proteins mimicking and not mimicking Epstein-Barr virus nuclear antigen-1." *J Clin Invest* 95 (3):1306-15. doi: 10.1172/JCI117781.

Villapol, S., D. Kryndushkin, M. G. Balarezo, A. M. Campbell, J. M. Saavedra, F. P. Shewmaker, and A. J. Symes. 2015. "Hepatic expression of serum amyloid A1 is induced by traumatic brain injury and modulated by telmisartan." *Am J Pathol* 185 (10):2641-52. doi: 10.1016/j.ajpath.2015.06.016.

von Büdingen, H. C., T. C. Kuo, M. Sirota, C. J. van Belle, L. Apeltsin, J. Glanville, B. A. Cree, P. A. Gourraud, A. Schwartzburg, G. Huerta, D. Telman, P. D. Sundar, T. Casey, D. R. Cox, and S. L. Hauser. 2012. "B cell exchange across the blood-brain barrier in multiple sclerosis." *J Clin Invest* 122 (12):4533-43. doi: 10.1172/JCI63842.

von Essen, M. R., C. Ammitzbøll, R. H. Hansen, E. R. S. Petersen, O. McWilliam, H. V. Marquart, P. Damm, and F. Sellebjerg. 2019. "Proinflammatory CD20+ T cells in the pathogenesis of multiple sclerosis." *Brain* 142 (1):120-132. doi: 10.1093/brain/awy301.

Vos, K., R. M. Thurlings, C. A. Wijbrandts, D. van Schaardenburg, D. M. Gerlag, and P. P. Tak. 2007. "Early effects of rituximab on the synovial cell infiltrate in patients with rheumatoid arthritis." *Arthritis Rheum* 56 (3):772-8. doi: 10.1002/art.22400.

Wang, D., M. M. Ayers, D. V. Catmull, L. J. Hazelwood, C. C. Bernard, and J. M. Orian. 2005. "Astrocyte-associated axonal damage in pre-onset stages of experimental autoimmune encephalomyelitis." *Glia* 51 (3):235-40. doi: 10.1002/glia.20199.

Wang, S. Y., and G. Weiner. 2008. "Complement and cellular cytotoxicity in antibody therapy of cancer." *Expert Opin Biol Ther* 8 (6):759-68. doi: 10.1517/14712598.8.6.759.

Waters, P., M. Woodhall, K. C. O'Connor, M. Reindl, B. Lang, D. K. Sato, M. Juryńczyk, G. Tackley, J. Rocha, T. Takahashi, T. Misu, I. Nakashima, J. Palace, K. Fujihara, M. I. Leite, and A. Vincent. 2015. "MOG cell-based assay detects non-MS patients with inflammatory neurologic disease." *Neurol Neuroimmunol Neuroinflamm* 2 (3):e89. doi: 10.1212/NXI.0000000000000089.

Weber, M. S., T. Prod'homme, J. C. Patarroyo, N. Molnarfi, T. Karnezis, K. Lehmann-Horn, D. M. Danilenko, J. Eastham-Anderson, A. J. Slavin, C. Linington, C. C. Bernard, F. Martin, and S. S. Zamvil. 2010. "B-cell

activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity." *Ann Neurol* 68 (3):369-83. doi: 10.1002/ana.22081.

Wegner, C., M. M. Esiri, S. A. Chance, J. Palace, and P. M. Matthews. 2006. "Neocortical neuronal, synaptic, and glial loss in multiple sclerosis." *Neurology* 67 (6):960-7. doi: 10.1212/01.wnl.0000237551.26858.39.

Wilcockson, D. C., S. J. Campbell, D. C. Anthony, and V. H. Perry. 2002. "The systemic and local acute phase response following acute brain injury." *J Cereb Blood Flow Metab* 22 (3):318-26. doi: 10.1097/00004647-200203000-00009.

Wolf, S. D., B. N. Dittel, F. Hardardottir, and C. A. Janeway. 1996. "Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice." *J Exp Med* 184 (6):2271-8.

Wolswijk, G. 2000. "Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis." *Brain* 123 (Pt 1):105-15.

Wolswijk, G. 2002. "Oligodendrocyte precursor cells in the demyelinated multiple sclerosis spinal cord." *Brain* 125 (Pt 2):338-49.

Wu, E., and C. S. Raine. 1992. "Multiple sclerosis. Interactions between oligodendrocytes and hypertrophic astrocytes and their occurrence in other, nondemyelinating conditions." *Lab Invest* 67 (1):88-99.

Xiong, B., A. Li, Y. Lou, S. Chen, B. Long, J. Peng, Z. Yang, T. Xu, X. Yang, X. Li, T. Jiang, Q. Luo, and H. Gong. 2017. "Precise Cerebral Vascular Atlas in Stereotaxic Coordinates of Whole Mouse Brain." *Front Neuroanat* 11:128. doi: 10.3389/fnana.2017.00128.

Yoshida, H., A. Naito, J. Inoue, M. Satoh, S. M. Santee-Cooper, C. F. Ware, A. Togawa, and S. Nishikawa. 2002. "Different cytokines induce surface lymphotoxin-alphabeta on IL-7 receptor-alpha cells that differentially engender lymph nodes and Peyer's patches." *Immunity* 17 (6):823-33.

Young, I. R., A. S. Hall, C. A. Pallis, N. J. Legg, G. M. Bydder, and R. E. Steiner. 1981. "Nuclear magnetic resonance imaging of the brain in multiple sclerosis." *Lancet* 2 (8255):1063-6.

Young, K. M., K. Psachoulia, R. B. Tripathi, S. J. Dunn, L. Cossell, D. Attwell, K. Tohyama, and W. D. Richardson. 2013. "Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling." *Neuron* 77 (5):873-85. doi: 10.1016/j.neuron.2013.01.006.

Yu, Y. J., Y. Zhang, M. Kenrick, K. Hoyte, W. Luk, Y. Lu, J. Atwal, J. M. Elliott, S. Prabhu, R. J. Watts, and M. S. Dennis. 2011. "Boosting brain uptake of a therapeutic antibody by reducing its affinity for a transcytosis target." *Sci Transl Med* 3 (84):84ra44. doi: 10.1126/scitranslmed.3002230.

Zendedel, A., C. Beyer, and M. Kipp. 2013. "Cuprizone-induced demyelination as a tool to study remyelination and axonal protection." *J Mol Neurosci* 51 (2):567-72. doi: 10.1007/s12031-013-0026-4.

Zhang, B. 2009. "Ofatumumab." *MAbs* 1 (4):326-31. doi: 10.4161/mabs.1.4.8895.

Zhao, C., S. P. Fancy, M. R. Kotter, W. W. Li, and R. J. Franklin. 2005. "Mechanisms of CNS remyelination--the key to therapeutic advances." *J Neurol Sci* 233 (1-2):87-91. doi: 10.1016/j.jns.2005.03.008.

Zivadinov, R., M. Zorzon, L. Monti Bragadin, G. Pagliaro, and G. Cazzato. 1999. "Olfactory loss in multiple sclerosis." *J Neurol Sci* 168 (2):127-30.

Zrzavy, T., S. Hametner, I. Wimmer, O. Butovsky, H. L. Weiner, and H. Lassmann. 2017. "Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis." *Brain* 140 (7):1900-1913. doi: 10.1093/brain/awx113.

Zucchi, F. C., A. Pelegrini-da-Silva, L. Neder, C. L. Silva, A. M. Tsanaclis, and O. M. Takayanagui. 2012. "The contribution of a murine CNS-TB model for the understanding of the host-pathogen interactions in the formation of granulomas." *J Neurosci Methods* 206 (1):88-93. doi: 10.1016/j.jneumeth.2012.02.015.