Regulatory and Effector Immune Cells in a Spontaneous Multiple Sclerosis Model

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by

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For my grandparents

1 Declaration of Originality

I, Daniel Elliot Lowther, hereby declare that this thesis is my own work. Furthermore, that information derived from the published or unpublished work of others has been clearly acknowledged in the text by suitable citation and referenced in the bibliography.

2 Acknowledgements

I would like to thank all those people who helped in the development of this thesis and supported me through the process of compiling it. The work described would not have been possible without the vision and support of my supervisor Professor Danny Altmann whose endless experimental ideas and guidance through the collection of results and writing process have been invaluable.

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3 Abstract

Multiple sclerosis (MS) affects around 2.5 million people worldwide, making it one of the most common neurological conditions in young adults. Experimental autoimmune encephalomyelitis (EAE) has been the standard animal model for MS where antigens are introduced alongside powerful T cell polarising adjuvants to induce disease. This general protocol has been both helpful and sometimes limiting in modelling T cell events as the immune adjuvants make interpretation of mechanism more difficult. There is a need for more relevant mouse models lacking the inherent biases of immune adjuvants and providing a human context for disease initiation and progression. For this thesis, I have characterized the disease and cellular dynamics in a humanized, T cell receptor (TCR) transgenic mouse model, termed Line7. The mice develop spontaneous, ascending paralysis. T cell responses defined in these transgenics have direct relevance to the human setting since they are by definition HLA-DR15 restricted and are allowed to progress without interference from modifying adjuvants. I have focused on the dynamics of Th1 and Th17 cells in both the initiation and progressive phase of disease and the role that regulatory T cells play in affecting this balance. Initial observation of naturally progressing disease showed that in this model IFNγ^+ cells dominate through disease initiation and IL-17$^+$ cells are only observed in the affected tissue once disease is established. The cytokine milieu favours Treg differentiation over Th17 cells and regulatory T cells accumulate in the central nervous system (CNS) but are ineffective at halting progression or initiating recovery. Through removal of the Tregs, severe acceleration of symptoms developed with uncontrolled infiltration of lymphocytes into

the CNS. IFNγ production by CD4 and CD8 cells is upregulated at the expense of IL-17 but reconstitution of the Treg population halts disease progression, illustrating the roles of Tregs in regulating autoreactive T cell activity and controlling cellular infiltration across immunological barriers. Therefore the use of this model, without the introduced biases of immune adjuvants, has highlighted the importance of the Th1/Treg balance in disease initiation over Th17 cells whilst underlining the critical role for Tregs in preventing autoimmune conditions or halting ongoing disease even in highly susceptible individuals.

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7 Abbreviations

AHR	Aryl Hydrocarbon Receptor
APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BFA	Brefeldin A
B2M	β-2 microglobulin
BP	Base Pairs
cDNA	Complementary DNA
CFA	Complete Freund's Antigen
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CPG ODN	CpG Oligodeoxynucleotide
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DT	Diphtheria Toxin
DTR	Diphtheria Toxin Receptor
EAE	Experimental Autoimmune Encephalomyelitis

EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FSL1	Fibroblast Stimulating Ligand-1
FOXP3	Forkhead Box P3
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
GFP	Green Fluorescent Protein
GWAS	Genome Wide Association Studies
HA	Haemagglutinin
HBSS	Hank's Balanced Salt Solution
HEL	Hen Egg Lysozyme
HKLM	Heat Killed *Listeria monocytogenes*
HLA	Human Leucocyte Antigen
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSP60	Heat Shock Protein 60
ICCS	Intracellular Cytokine Staining
iE-DAP	γ-D-glutamyl-*meso*-diaminopimelic acid
IFN	Interferon
IgG/M	Immunoglobulin G or M
iNOS	Inducible Nitric Oxide Synthase

iNKT	Invariant Natural Killer T Cell
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome
IVC	Individually Ventilated Cage
KO	Knockout
LM-MS	Lipomannan from *Mycobacterium smegmatis*
LPS	Lipopolysaccharide
LPS *E.coli* K12	Lipopolysaccharide from *Escherichia coli* K12
LTA	Lipoteichoic acid
MBP	Myelin Basic Protein
MCP1	Monocyte Chemotactic Protein 1
MDP	Muramyl Dipeptide
MHC	Major Histocompatibility Complex
MIP1α	Macrophage Inflammatory Protein 1 alpha
Mlsa	Minor-lymphocyte Stimulatory Antigen
MMTV	Mouse Mammary Tumour Virus
MOBP	Myelin-Associated Oligodendrocytic Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation Primary Response Gene 88

NAA	N-acetyl aspartate
NCBI	National Centre for Biotechnology Infomation
NK	Natural Killer Cell
NKT	Natural Killer T Cell
NOD	Non-Obese Diabetic Mice
NBOD	Nucleotide-Binding Oligomerization Domain
OCT	Optimal Cutting Temperature Compound
OT-1	MHC class I-restricted OVA specific TCR transgenic mice
OVA	Ovalbumin
Pam3CSK4	Synthetic Lipopeptide Pam_3Cys-Ser-$(Lys)_4$
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Triton
PFA	Paraformaldehyde
PG	Proteoglycan
PGN	Peptidoglycan
PGN-ECndi	Insoluble Peptidoglycan from *E.coli* K12
PHA	Phytohaemagglutinin
PLP	Proteolipid Protein
PMA	Phorbol 12-myristate 13-acetate
PML	Progressive Multifocal Leukoencephalopathy
PolyI:C	Polyinosinic:polycytidylic acid
PP	Primary Progressive
Ptx	Pertussis Toxin

PVE	Post-Vaccinal Encephalomyelitis
RA	Rheumatoid Arthritis
REST	Relative Expression Software Tool
RIP	Rat Insulin Promoter
RNA	Ribonucleic Acid
RORα	RAR-related Orphan Receptor Alpha
RORγt	RAR-related Orphan Receptor Gamma Second Isoform
RPL-13A	Ribosomal Protein Large Subunit 13A
RR	Relapsing Remitting
RT	Reverse Transcription
SDHA	Succinate Dehydrogenase Complex, Subunit A
SNP	Single Nucleotide Polymorphism
SP	Secondary Progressive
ssRNA40	20-mer GU-rich Single-Stranded RNA Oligonucleotide
STAT	Signal Transducers and Activators of Transcription
TBE	Tris/Borate/EDTA
T-BET	T-box Encoded Transcription Factor
TBP	TATA-Binding Protein
TCR	T Cell Receptor
Teff	Effector T cell
TFRC	Transferrin Receptor Protein Subunit C
TGFβ	Transforming Growth Factor β
TLR	Toll-Like Receptor

TMEV	Theiler's Murine Encephalomyelitis Virus
TNFα	Tumor Necrosis Factor α
Treg	Regulatory T Cell
TSP-1	Thrombospondin 1
UK	United Kingdom
USA	United States of America
VCAM-1	Vascular Cell Adhesion Molecule 1 (CD106)
VLA-4	Very Late Antigen 4 (CD49d)
WT	Wild Type
YWHAZ	14-3-3 Zelta/Delta Protein

8 Introduction

8.1 Multiple sclerosis

8.1.1 Clinical Presentation

Multiple sclerosis (MS) is the most common neurological condition in young adults and one of the many diseases lacking a clear causative agent or mechanism. At present, it is thought to be an autoimmune disease triggered in susceptible individuals by some form of environmental interaction which leads to an autoimmune attack on the myelin sheath and consequently, damage to central nervous system (CNS) axons (Compston 2002). Many factors have been thought to contribute to disease initiation and progression but none have been shown to be solely responsible. There is even debate as to whether MS is one condition or rather a collective syndrome, as there is a great deal of clinical heterogeneity (Lucchinetti 2000). This would impact greatly on selection and assessment of therapies as some may be effective in a subset of patients but not in others.

The characteristic pathology of MS is the presence of many large demarcated lesions or plaques throughout the CNS white matter and these tend to be located within the periventricular regions, optic nerve, brain stem and spinal cord. Around 10 years ago an attempt was made to classify the pathology of MS lesions into 4 categories based on the severity of tissue injury, extent of demyelination and inflammation (Lucchinetti 2000). Pattern I and II lesions were described as presenting with perivascular myelin loss, infiltration of lymphoid cells but relative sparing of oligodendrocytes. Patterns III and IV involve oligodendrocyte cell death either as a result of apoptosis (III) or necrosis (IV) within areas separate from blood vessels. Initially it was thought that lesions would be

formed though only one mechanism in each patient. However this has been shown to be inaccurate and rather a homogeneous pattern of demyelination occurs in all patients with established disease. This demyelination forms pattern II lesions and the formation of other lesion patterns is very rarely seen (Breij 2008).

While inter-individual variation in clinical presentation is substantial, there are three general, clinical forms often used to classify MS, varying in the pattern of symptoms and time course. The most severe is primary progressive (PP) where disease severity increases unabated and results in extensive neuronal disability without clear attacks. The most common in around 85% of cases is relapsing-remitting (RR) where periodic episodes of disease are broken by periods of recovery. This can develop into secondary progressive (SP) disease where there is further neuronal degeneration and no period of remission between relapses. Symptoms of MS are wide ranging, representing the integral role the CNS plays in controlling all aspects of the human body. Muscular weakness and/or control of movement, visual and speech problems, acute or chronic pain syndromes and other cognitive impairment are some potential symptoms but diagnosis is very difficult, particularly during the early stages of disease. RR disease is especially hard to diagnose as MS given the period of time between relapses and early attacks are often diagnosed in hindsight (McFarland 2007).

A major factor in determining the long-term disability of a patient is the degree of axonal loss. The use of magnetic resonance imaging (MRI) as a diagnostic tool has greatly improved our understanding and recognition of this disease. MRI has allowed longitudinal studies of RR-MS patients and has highlighted the importance of permanent CNS tissue damage and CNS atrophy as early signs of the disease (Brex 2001, Chard 2002)

and as a positive correlate for subsequent disability (Rudick 1999, Fisher 2002). MRI studies on progressive patients have not been able to place demyelination as primary before neuronal damage (Rudick 1999, Miller 2002). The improvement of MRI to image metabolites (MRSI) has highlighted some early checkpoints in neuronal loss such as the reduction of the glutamate metabolite N-actetyl aspartate (NAA). This is indicative of neuronal damage and it seems that a non-reversible NAA reduction in the white matter lesions occurs (De Stefano 1999) highlighting axonal loss and which could act as an early diagnostic tool. As it happens early in disease, and before overt lesions can be observed, it indicates that axonal loss is not necessarily a consequence of demyelination but could be a result of a separate immune attack. Indeed axonal pathology is seen in both normal appearing white matter and demyelinated lesions (Arnold 1992, Narayanan 1997, De Stefano 1998). Observation of precursor proteins characteristic of axonal loss in active inflammatory lesions of early biopsy material from MS patients (Kuhlmann 2002) support the use of metabolites as early checkpoints and may pave the way for more accurate early diagnostic tools before lesions can be seen on MRI.

The different types of MS seem to also present different CNS pathology. In early disease there are low degrees of cortical demyelination, this only becoming prominent at later stages of disease (Kutzelnigg 2005). The progressive stages of the disease, both primary and secondary show few classic active plaque white-matter lesions and a dominance for diffuse inflammation and extensive cortical demyelination (Koyanagi 1993, Bo 2003, Kutzelnigg 2005). Increased numbers of Na^+ channels and reduced ATP production along demyelinated axons in MS lesions (Craner 2004, Mahad 2009) indicate that the loss of myelin renders the axon more susceptible to physiological stresses. To support these

findings, it has also been shown that inhibition of remyelination in an animal model leads to increased axonal degeneration (Irvine 2008). The degree of axon and neuronal degeneration has been shown in MS patients to be linked to the numbers of activated T cells and macrophages/microglia (Ferguson 1997, Kuhlmann 2002) which both are thought to be directly involved in mediating neuronal damage. The roles of these and other immune cells in the pathogenesis of MS will be introduced in a later section.

8.1.2 Genetic and Environmental Influence

The mean age of onset is approximately 30 years of age and there is a clear gender bias of the condition, with females being more frequently affected than males (Compston 2002). There is a clear genetic link of MS as the concordance rate in monozygotic twins is around 30% compared to less than 5% in dizygotic twins (Ebers 1986, Dyment 2004a) and the incidence of disease in first-degree relatives is reported to be 20 times higher than the general population (Dyment 2004b). An association with the major histocompatibility complex (MHC) region of the genome was first shown almost 30 years ago (Jersild 1972) and has been since focused by genome wide association studies (GWAS) to show a link between the HLA-DRB1*15:01-B5*01:01 (originally called DR2) and HLA-DQA1*01:02-B1*06:02 haplotype and MS (Dyment 2004a, Hafler 2007). Several GWAS have been carried out to identify susceptibility loci for many complex diseases, including MS. They utilise chip-based technology to efficiently genotype several hundred thousand single nucleotide polymorphisms (SNPs) throughout the genome simultaneously. All of these studies have confirmed the association with HLA-

DRB1*15:01 but have also identified 16 non-HLA loci with genome wide significance as MS risk factors (Kemppinen 2011).

The first published MS related GWAS used trios (affected individual and both parents) from the UK and USA, and reported an association with a non-synonymous coding SNP in the *IL7RA* gene and 2 SNPs in intron 2 of the *IL2RA* (CD25) gene (Dyment 2004a, Hafler 2007). Both of these associations have been confirmed by candidate gene studies (Gregory 2007, Lundmark 2007, Rubio 2008, Weber 2008, Akkad 2009, Perera 2009) and due to their important roles in the life-cycle of a T cell and function, their association re-affirms the importance of the T cell in MS disease. As an extension, the proximal promoter of the *IL7RA* gene has been shown to include interferon beta (IFN-β) response elements and so is thought to have a role in patients' responses to therapy as well as individual immune responses (Cunningham 2005).

The original GWAS also found associations for a SNP in intron 19 of *CLEC16A*, a C-type lectin, and a SNP in *CD58* (Hafler 2007). The association of *CLEC16A* was confirmed through a replication cohort (IMSGC 2009), within Sardinian populations (Zoledziewska 2009) showing the same SNP association, from a meta-analysis (Hoppenbrouwers 2009) and in an African-American population (Johnson 2010). CD58 has shown both protective (De Jager 2009b) and susceptibility (Hafler 2007) associations with different SNPs. CD58 is involved in co-stimulation of T cells and CD58 mRNA is elevated in MS patients during remission (De Jager 2009a) suggesting a protective role although an association from an Australian GWAS and in a recent meta-analysis reaffirmed its role as a risk gene for MS (ANZgene 2009, Hoppenbrouwers 2009). However, for *CD58* unlike *CLEC16A*, no association was found in the African-American cohort (Johnson 2010).

Other non-HLA genes implicated in MS from GWAS include *TYK2* (Ban 2009, Mero 2010), *MMEL1* (ANZgene 2009, Ban 2010), *CD6*, *IRF8* and *TNFRSF1A* (De Jager 2009b) and *CD40* (ANZgene 2009) whilst follow-up studies on the original GWAS identified the *TMEM39A* and *KIF21B* genes as having risk variants for MS (IMSGC 2010). Interestingly, associations with *IL12A*, *MPHOSPH9*, *RGS1* and *STAT3* have also been confirmed recently (IMSGC 2010, Jakkula 2010) providing a direct link to T helper cell involvement in MS.

These genes and all others found in this manner confer very small increased risk of MS as measured by odds ratios (<1.4) (Kemppinen 2011) suggesting that the genetic model of MS could be polygenic, as suggested originally by Fisher (Fisher 1918). In this, a combination of risk allele variants will all exert weak effects and their interaction with the environment could provide an additional tipping point for development of MS. Further genetic and functional analysis should be carried out in co-ordination with GWAS to help provide a much more detailed picture of genetic pre-disposition and risk and understand the relevance of the highlighted pathways in disease pathology.

In conjunction with the genetic risk, environmental factors impact on both susceptibility and progression of MS. Latitude is the factor most strongly associated with MS risk represented by higher frequencies of MS in countries at higher latitudes and in patients with west European ancestry (Ebers 1993). In New Zealand the first admission to hospital for MS on the south island was much higher than on the north island (Skegg 1987, Fawcett 1988), and a similar pattern was seen in Europe and North America (Ebers 1993) representing the association of MS with latitude. The Viking theory has been suggested as an explanation for this, connecting the disease with the spread of

Scandinavian genes initially by the Vikings and then by further migration (Poser 1995) but twin concordance rates also differ by latitude of birth suggesting important environmental effects also determine MS risk (Islam 2006). Levels of sunlight follow a pattern similar to MS and so have implicated vitamin D levels to be a key environmental factor (Goldberg 1986). High serum levels of the Vitamin D precursor 2,5-hydroxyvitamin D have been shown to be protective against MS (Munger 2006) and Vitamin D levels during pregnancy are thought to affect likelihood of MS as babies born in May in the Northern Hemisphere are more susceptible than those born in November, reflecting the exposure to sunlight during pregnancy (Willer 2005).

Vitamin D levels throughout life, both in childhood and adulthood may be important in mediating the risk of MS but the difficulty in excluding reverse causation and correcting for recall bias in studies makes any conclusions very difficult to prove. Equally, Vitamin D is not solely derived from sunlight and dietary intake will influence serum levels. Both provide similar immunomodulatory effects (Munger 2006, Smolders 2008), increasing the difficulty of control within studies. Modern advances in sun cream and increased ability to travel throughout the world could average out regional differences, masking the more subtle regional differences but the identification of a vitamin D binding element in the promoter of the HLA-DRB1*15:01 gene adds support to the hypothesis that vitamin D levels affect MS susceptibility and progression (Ramagopalan 2009a).

The intrauterine environment has also been implicated from both the month of birth observations and also from concordance rates between full siblings and dizygotic twins in determining MS risk. Despite being genetically comparable, there is a higher

concordance in dizygotic twins (5.4% compared to 2.9%) and concordant dizygotic twins tend to develop MS at the same age (Bulman 1991, Willer 2003).

These results add weight to the importance of environmental factors over genetics and indeed migration of individuals from high-risk to low-risk areas has been shown to provide some protection from acquiring MS (Kurtzke 1993), although the critical age for this migration however is unknown. Studies have ranged from suggesting that migration must be before the age of 15 (Dean 1997) to those that suggest it may extend into adulthood, as far as the third decade of life (Hammond 2000). More general studies have shown that the MS rate among genetically comparable UK migrants to Tasmania or the south island of New Zealand resemble those remaining in the UK whereas they are five to six-fold lower in Queensland or the Northern Territory (Pugliatti 2002), further emphasizing the importance of environmental factors.

Alongside latitude, Epstein-Barr Virus (EBV) seropositivity is also strongly associated with MS risk. Almost all MS patients are EBV seropositive compared to a rate of 90% of healthy people (Ascherio 2007), both implicating EBV as important to MS progression but also demonstrating that it is not solely responsible. Individuals developing EBV-induced infectious mononucleosis have a higher risk of MS (Goldacre 2004, Sundstrom 2004, Ramagopalan 2009b), and those developing adult-onset MS have elevated levels of EBV antibodies is early adult life (Levin 2005, DeLorenze 2006) suggesting a pre-disposing role of infection. Viral infections, more specifically of the respiratory tract, have been associated with triggering relapses and new-onset MS in adulthood (Sibley 1985, Edwards 1998, Marrie 2000, Kriesel 2005), and high EBV antibody titres can act synergistically with HLA-DRB1*15:01 alleles to increase MS risk (De Jager 2008). It would

be however too far to say that EBV infection is a step in MS development, despite its candidacy as a risk factor.

Other environmental risk factors considered important include smoking. Many studies have shown an association between smoking and MS, independent of latitude and ancestry, with preliminary studies suggesting causality although not fully confirmed (De Jager 2008). After clinically isolated syndrome (CIS), the risk of developing definite MS is increased if the patient is a smoker and the likelihood of developing secondary progressive MS from relapsing-remitting also increases (Hernan 2005, Di Pauli 2008, Healy 2009).

8.1.3 Therapies

The lack of a clear mechanism of MS pathology, the differing clinical varieties and the challenge in targeting the drug to the correct location, has made the development of effective therapeutics very difficult. Immunosuppressants were first used in MS over twenty years ago and have showed minor successes but not sufficient to outweigh the risk of side effects including serious infections (Yudkin 1991). The advance from immunosuppressants was made through the use of disease modifying therapies, aiming to prevent relapses and disability progression without the immunosuppressive effects. At present, therapeutic intervention for MS is limited in most centres to three classes of compounds that act as disease modifying therapies. These are interferon beta (1a and 1b), glatiramer acetate (GA) and natalizumab although mitoxantrone is licensed for active MS patients in the USA and some parts of Europe.

IFN-β1a and 1b are both type one interferons and have antiviral and anti-inflammatory properties although they differ in their pharmacodynamics and pharmacokinetics (Alam 1997). On average, in RR-MS, relapse rates are reduced by approximately a third and severe attacks are cut by almost half through use of IFNβ and it has been shown to slow the rate of disease progression by around 1 year in the short term (Jacobs 1996). The mechanism of IFNβ action appears to be wide ranging. T cell activation is downregulated through a reduction in expression of MHC class II antigens and co-stimulatory molecules, impairing antigen recognition (Jiang 1995, Teleshova 2000). At the same time, an upregulation of cytotoxic T lymphocyte antigen 4 (CTLA-4) and Fas promotes the apoptosis of autoreactive cells (Hallal-Longo 2007). IFNβ administration causes a shift from pro-inflammatory to anti-inflammatory cytokine production and has been shown to decrease the responsiveness of both human peripheral blood mononuclear cells (PBMCs) and myelin specific T cells to IL-12 (Wang 2000). The permeability of the blood brain barrier (BBB) is reduced through the downregulation of very large antigen 4 (VLA-4) and upregulation of soluble vascular cell adhesion molecules (VCAMs) to block adhesion of cells to the BBB (Muraro 2000, Graber 2005) and CD73 to increase endothelial barrier function (Airas 2007). The final mechanism that IFNβ is thought to aid neuroprotection is by impairing macrophage and dendritic cell (DC) uptake of antigen. This would act to slow or prevent epitope spread, a mechanism implicated in progression of RR-MS (Axtell 2008).

Glatiramer acetate is a synthetic co-polymer composed of four amino acids found in MBP. It was designed to simulate MBP and identify immune response gene effects but showed a suppressive effect on EAE development (Teitelbaum 1971). It achieves similar

efficacies as IFNβ in human disease (Mikol 2008) and promotes similar mechanisms to mediate neuroprotection. A shift in cytokine production from a pro-inflammatory to anti-inflammatory signature has been observed after GA administration (Miller 1998) as well as an increase in forkhead box P3 (Foxp3) expression that would act to increase Treg function (Hong 2005). GA also increased production of neuroprotective factors, which directly act to promote recovery from autoimmune damage in the CNS (Ziemssen 2005).

Natalizumab is a humanized monoclonal antibody directed to the α4 subunit of α4β1 integrin (VLA-4) and prevents the trafficking of lymphocytes across the blood brain barrier by blocking the interaction between VLA-4 and VCAM-1 required for this transmigration (Steinman 2005a). It is generally recommended for patients with more aggressive RR-MS and those who failed to respond to other disease modifying therapies, and in trials showed a 68% reduction in relapse rate and 42% reduction in disability progression over 2 years (Polman 2006). Although it was briefly withdrawn over safety fears linked to the development of progressive multifocal leucoencephalopathy (PML), it was reapproved as a therapy for MS in 2006. The risk of PML developing was originally estimated to be 1:1000, and is now thought to be even lower than that, with the risk-benefit analysis supporting its use (Thompson 2008).

The final approved therapy is mitoxantrone, along with IFNβ, licensed for use in secondary progressive MS cases. It belongs to the anthracenedione group of aromatic organic compounds and is also used to treat some forms of cancer (Shenkenberg 1986). In EAE, mitoxantrone suppresses disease activity (Lublin 1987, Watson 1991) and in patients has been shown to reduce treated relapses by 69% and lower a composite score

made up of expanded disability status scale (EDSS), relapse rates, ambulation and neurological status (Hartung 2002). However, less significant changes were seen from MRI observation in the same trials (Krapf 2005). Its mechanism of action is thought to be wide-ranging, suppressing proliferation of T cells, B cells and macrophages, inhibiting activation of T cells and production of pro-inflammatory cytokines whilst also reducing antigen presentation and macrophage-mediated demyelination (Fidler 1986a, Fidler 1986b, Fox 2004).

Other depleting antibodies have also been used, including the humanized anti-CD52 monoclonal antibody, alemtuzumab which depletes both CD4 and CD8 T lymphocytes, anti-CD20 monoclonal antibody, rituximab which depletes B lymphocytes (Reff 1994), and anti-IL-2R (CD25) humanized monoclonal antibody, daclizumab which limits T cell expansion (Waldmann 2007). Alemtuzumab has been shown to significantly reduce relapses and new lesion formation in the short term (Coles 2008) although side effects of CAMPATH-1H are idiopathic thrombocytopaenia purpura, thyroiditis and Goodpasture's syndrome (Moreau 1996, Coles 1999). The effectiveness of alemtuzumab was highest if given early in the disease course, leading to the inference by the authors that secondary progressive neurodegeneration occurred independently of inflammation (Coles 2004).

Rituximab therapy led to a reduction in both clinical disease and MRI markers in a phase II trial (Hauser 2008) and has been trialled in neuromyelitis optica, an autoimmune condition similar but distinct from MS, mediated by autoimmune B cells where it showed beneficial effects and an improvement in relapse rate and recovery (Cree 2005, Jacob 2008). The contribution of B lymphocytes in MS however is not clear but these trials have reaffirmed the conclusion that they do play an important role. The administration

of daclizumab leads to improved disease conditions through measurement of MRI and disability scores (Bielekova 2004, Rose 2004, Rose 2007, Bielekova 2009). There is an expansion of $CD56^{bright}$ natural killer (NK) cells and these are thought to bring about a reduction in CNS inflammation through inhibition of peripheral T cell survival (Bielekova 2006). $CD56^{bright}$ NK cells appear to be able to maintain IL-2 signalling despite the administration of daclizumab, and it appears that the reduced levels of IL-2 act to increase the cytotoxic potential of the NK cells (Martin 2010). In addition, recent work has shown a potential explanation for the increased efficacy of daclizumab at reducing T cell proliferation with higher doses, despite achieving saturation of CD25 in blood lymphocytes. The identification of a mechanism whereby myeloid DCs can present IL-2 in trans to T cells and 'lend' their CD25 to T cells to facilitate expansion offers an explanation for how lymphoproliferation can be inhibited by daclizumab whilst also diminishing the Treg population (Wuest 2011).

Oral immunomodulators have also shown promise as therapeutics in MS patients. FTY720 (fingolimod), a sphingosine-1-phosphate receptor modulator, inhibits T cell migration from lymphoid to peripheral organs thereby limiting inflammation without affecting function (Brinkmann 2004) and protects against experimental autoimmune encephalomyelitis (EAE) and showed protection in a phase III trial (Cohen 2010a). In oligodendrocyte progenitor cells treated *in vitro*, it also promoted survival but inhibited differentiation, having potentially both positive and negative effects on the remyelination process (Miron 2008). It was licensed for use in Russia and the USA in 2010, and by the EU in 2011, for use in patients with highly active RR-MS who had already received IFNβ treatment or those with highly evolving RR-MS. Other oral

immunomodulators have demonstrated encouraging results from animal studies or in clinical trials. The only other licensed oral therapy is Cladribine, approved in 2010 for use in the USA and Russia. Cladribine leads to the depletion of T cells (Carson 1983), teriflunomide works by inhibiting of lymphocyte expansion and differentiation (Tallantyre 2008) and laquinimod prevents lymphocyte migration (Brunmark 2002). Dimethyl fumarate activates neuronal protection and the maintenance of CNS myelin (Calabrese 2005, Hubbs 2007) whilst also inhibiting pro-inflammatory cytokines and cell adhesion molecules (de Jong 1996, Vandermeeren 1997) and firategrast, like natalizumab, acts on α4 integrins to block cell migration (Jackson 2002). This class of therapeutic approach have the advantage of an easier mode of delivery and reduced costs and so may end up being the front line treatment regime for long-term disease if they succeed in clinical trials.

8.1.4 One Disease?

The idea that multiple sclerosis is a spectrum of diseases based upon clinical considerations has been put forward (Weinshenker 1999). Schilder's disease, Marburg's disease, Baló's disease, Devic's disease and disseminated encephalomyelitis (DEM) are all described forms of demyelinated lesions in the CNS. However, rather than being considered forms of MS, they differ from MS in both presentation and location of lesions. In the case of Devic's disease, a different immune mechanism has been identified, with demyelination mediated by autoantibodies to aquaporin-4 (Lennon 2004). Therefore there is a clear separation of these conditions from MS, rather than classifying them within the boundaries and clinical spectrum of MS. Suggestions of genetic pre-

determination to the different demyelinating conditions have been put forward based on ethnicity of patients (Poser 2004b) and splitting the inflammatory demyelinating diseases into two groups, those of MS and DEM has been proposed as the simplest means of characterising the CNS demyelinating conditions (Poser 2004a). As more research is done, the individual similarities and differences between conditions will be fully described which should help to clarify the relationships between these conditions. There is an obvious requirement for the correct determination of which syndrome is affecting the patient. Although different mechanisms can lead to a similar phenotype characterized by demyelination in the CNS, each will display differential responses to therapies and proper identification of condition can lead to improved treatment regimes.

8.2 Animal Models of MS

8.2.1 EAE

A major difficulty with studying MS is the limited access to early stage and active tissue samples and the inability to causally relate autoimmune responses to attacks. Therefore, animal models have been used to try and fill this void and allow researchers to dissect immunological mechanisms that may be active in human MS. There are three major types of animal model for MS: EAE, virally induced chronic demyelinating disease, and toxin-induced demyelination. Of these, EAE has been the most widely used and has, despite limitations, been very informative and has directly led to the development of approved treatments for MS namely glatiramer acetate, mitoxantrone, natalizumab and fingolimod. However, these successes are tempered by therapies that showed initial

success in EAE but have proved to be ineffective in treating human disease e.g. administration of IFNγ and blockade of tumor necrosis factor alpha (TNFα) (Feldmann 2005). This mixed success is due to the limitations of EAE as a representative model of MS, unable to reproduce all facets of the human condition (Sriram 2005, Steinman 2005b, 2006). Individual models of EAE can be made to model all clinical forms of MS but therefore aspects of each of these models are unrepresentative. Toxicity of new drugs is rarely tested in EAE models and EAE experiments generally run for too short a time, and under conditions designed to limit pathogen interference, to detect infectious complications. Most therapies for the human condition would be used once disease is established rather than administering alongside or shortly after the initial attack. The use of knockout mice to study autoimmunity also creates problems of translation into therapies as cytokines etc often have diverse biological activities that may be replaced by other proteins, as there is large redundancy in most physiological processes. Many knockout mice show altered lymphoid architecture, leading to conclusions being drawn from unrealistic physiological conditions (Steinman 1997). EAE is induced by the introduction of an autoantigen alongside powerful immune adjuvants to initiate an immune response that overcomes self-tolerance to a self-peptide. Similar to MS, EAE is heterogeneous, influenced by the selected autoantigen, species, and genetic background used. It was discovered in the 1930s by Rivers et al (Rivers 1933) investigating neurological conditions observed after rabies vaccinations. Work had been carried out over the previous 38 years investigating an observation by Pasteur in attempting to vaccinate against rabies virus, that patients injected with dried spinal cord of rabies-infected rabbits developed neuropathological incidents, called post-vaccinal

encephalomyelitis (PVE) (Balaguer 1888, Baxter 2007), rather than immunity to the rabies virus. The initial version of the model involved immunizing rhesus monkeys with homogenised normal rabbit brain but this was cumbersome, involving many injections per animal over long time spans (Rivers 1935).

The model was improved by Elvin Kabat, by combining the brain extracts with the adjuvant recently developed by Jules Freund (Kabat 1946) originally designed to increase antibody production against horse serum in guinea pigs (Freund 1942). This method allowed induction of disease with only one injection, saving a great deal of time but arguably masking some of the more subtle initiating events. The similarities of EAE to human demyelinating diseases was noted at this same time (Wolf 1947) and since then it has been induced in a wide range of mammals and now, the mouse and rat are the main species used.

The importance of T cells in driving the disease was highlighted initially by the ability of adoptively transferred spleen cells from an EAE donor to induce EAE in naïve recipients. This observation was further specialised to myelin-specific $CD4^+$ T cells through similar adoptive transfer experiments of these specific cells inducing EAE in all animals (Ben-Nun 1981, Pettinelli 1981).

The usefulness of EAE as a model of MS has been questioned with some groups sighting a CD4-mediated mechanism as evidence for their scepticism. Whilst EAE is certainly a $CD4^+$ T cell mediated disease with the predominant cell type present in CNS lesions being $CD4^+$, there is not such clear evidence for the same importance in the human condition, where $CD8^+$ T cells and macrophages are more prevalent (Babbe 2000, Neumann 2002).

However, its usefulness in leading to disease modifying therapies is without doubt (Steinman 2006).

EAE is not a complete model of human MS, but rather replicates distinct characteristics of the human condition with each individual model reproducing a different clinical manifestation allowing isolated investigation. Relapsing-remitting, chronic and acute disease can be modelled by using a different combination of mouse strain and myelin peptide, immunized alongside Complete Freund's Adjuvant (CFA) and *Bordetella pertussis* toxin. SJL (H-2^{s}) and Biozzi ABH (H-2^{dq1}) (Colombani 1979, Baker 1990) mice typically develop a relapsing-remitting clinical profile, B10.PL or PL/J (H-2^{u}) an acute monophasic disease (Waxman 1980), and C57BL/6 (H-2^{b}) mice develop chronic paralysis although they may also show acute attacks followed by resolution (McRae 1992, Tompkins 2002). These different profiles are due largely but not exclusively to the MHC class II antigens of the strains used, determining which myelin peptides the mouse strains are susceptible to.

SJL, PL/J and B10.PL mice can be induced to develop EAE with MBP (Zamvil 1986), PLP (Tuohy 1989, Greer 1992, McRae 1992) and MOBP (Kaye 2000) peptides. PL/J and B10.PL mice are however resistant to EAE induced by MOG peptides whereas SJL, Biozzi ABH and C57BL/6 mice are susceptible (Amor 1994, Mendel 1995). C57BL/6 and Biozzi ABH mice are also susceptible to PLP induced EAE (Amor 1993, Tompkins 2002) but only show limited susceptibility to MBP. It is a minor encephalitogen in Biozzi mice (Amor 1996) and only acts as a successful antigen in C57BL/6 mice when bovine MBP is used rather than MBP derived from any other species (Faunce 2004).

As relapsing-remitting MS is the most common form of the human condition, the SJL model appears the most relevant for direct comparisons with human disease. It shows histopathological similarities to MS, with areas of cellular infiltration linked to regions of demyelination (Brown 1982, Paterson 1988). Biozzi ABH mice share these similarities (Baker 1990) and along with SJL mice show reactivity to a wide range of myelin-derived peptides, likely to be the source of the remitting disease. It is thought that the scavenging and subsequent re-presentation of myelin peptides from an initial demyelinating event can lead to epitope spread and new inflammatory attacks (McRae 1995). The Biozzi ABH mice can also be used to study neurological inflammation in a variety of models including virally-induced (Amor 2005), and EAE induced with CNS-derived non-myelin peptides e.g. αBcrystallin and GFAP (Thoua 2000), which have been shown to elicit T cell reactivity in MS patients (van Noort 1995). They have also been used to model secondary progressive MS (Hampton 2008), a clinical form neglected in most EAE studies. Despite the strengths of these models, both are still primarily $CD4^+$-mediated conditions, at a scale much higher than MS and so do not provide a comprehensive representation of human disease.

The recent development of a MBP-PLP fusion protein (MP4) to induce EAE in C57BL/6 mice (Kuerten 2006) provides an improvement in diversity of C57BL/6 models but the most commonly used EAE model is a MOG-induced disease in mouse strains on the C57BL/6 background (Mendel 1995). This chronic progressive EAE model does not represent the largest clinical form of human MS, rather models primary progressive MS, but still provides important information regarding CNS inflammation. Tissue damage is characterised by T cell and macrophage mediated demyelination but occurs in the

absence of a demyelinating autoantibody response, which is blocked by the H-2^b MHC haplotype, when rat or mouse MOG is used (Bourquin 2003). Therefore human MOG must be used to induce an autoantibody response (Oliver 2003), to elicit axonal and neuronal damage (Bourquin 2003). Lesions in MOG-specific models form in the forebrain and optic nerve, in a similar way to MS, suggesting that MOG may be involved in the development of optic neuritis but as the clinical score is measure by paralysis, the degree of CNS damage does not always directly correlate with achieved score (Berger 1997). Despite these limitations, the C57BL/6 strain is widely used because of the ease of generating transgenic lines. There is a large availability of knockout mice on the C57BL/6 background and so using this model allows the comparison of congenic strains where they differ in only one locus, necessary for mechanistic studies (Owens 2001). The speed of disease induction allows rapid pre-clinical testing of potential therapeutic compounds increasing its popularity and this further accelerates its use but to the potential detriment of the human condition as the variety of models is neglected over ease of use when it is clear how large an impact genetic diversity can have on disease outcome.

8.2.2 Other Animal Models

There are two other animal models of MS, namely virally induced demyelination by Theiler's murine encephalomyelitis virus (TMEV) and toxin-induced demyelination. TMEV is a single-stranded RNA picornavirus and was initially described to cause flaccid paralysis in mice but not monkeys (Theiler 1934). Intracereberal injection of the virus, mice develop transient meningo-encephalomyelitis peaking at around day 7 after infection and clearing after 3 weeks. In some cases, the infection may become bi-phasic

and demyelinating lesions appear in the spinal cord (Dal Canto 1996). A less virulent strain of the virus is used to model MS as the more virulent strains cause fatal encephalitis (Zoecklein 2003). TMEV infection is an attractive model for MS as the pathology resembles MS (Lipton 1975, Dal Canto 1977) and it is a model for a viral CNS infection, thought to be a potential cause of MS (Rodriguez 1987). It has been claimed that TMEV may be a more accurate predictor of therapeutic potential than EAE (Nelson 2004) but until all potential therapies for MS and EAE have been tried, then the claim cannot be validated.

Toxin–induced demyelination is a model for investigating mechanisms of focal demyelination and remyelination and those cells involved (Blakemore 2008). Injection of lysolecithin into the spinal cord radidly induces areas of non-immune mediated demyelination with complete relmyelination within 5-6 weeks (Shields 1999, Bieber 2003). Cuprizone added to the diet of the animal however leads to the development of spongiform encephalopathy (Carlton 1969, Suzuki 1969) and pathology develops due to a depletion of oligodendrocytes, induced to undergo apoptosis. Removal of cuprizone from the diet allows remyelination as homeostasis returns (Matsushima 2001).

There is no one mouse model that can recapitulate all the immune events involved in the human disease, especially given the heterogeneity of the condition. However, mouse models should be made as relevant and faithful to the human condition as possible whilst understanding their limitations to better interpret the results generated.

8.3 Immune Mechanisms in MS

8.3.1 CD4 T cell subset divisions

8.3.1.1 Historical – Th1 and Th2

It would seem logical that in such a complex immune system such as the one evolved in mammals to react to a wide variety of pathogens should be formed of many interconnected but yet distinct subsets of cells. Each has their own specificity and armoury to protect the host from a group of pathogens or threats, whether these be from outside or from the host itself. It has been this rationale that has lead people to try and classify cellular subsets, assuming that through classification, our knowledge of the system and diseases will simplify, and aid advances in therapy.

The first division of the $CD4^+$ T cell lineage was made by Mossmann and Coffman in 1986 based initially upon functional characteristics. They proposed that two mutually exclusive subsets could be described called Th1 and Th2, and this paradigm survived for almost twenty years. Th1 cells were described as producing large amounts of IFNγ enabling them to kill intracellular pathogens whereas Th2 cells produced IL-4, IL-5, IL-13 and IL-25 to control extracellular pathogens mainly by promoting antibody class switching (Mosmann 1986, Mosmann 1989, Fort 2001). Cytokine release however is not sufficient to classify cell fate determination and it was ten years later that transcriptional signatures were identified for Th1 and Th2 cells. T-bet (Tbx21) a TATA box binding protein and GATA3, a member of the GATA binding family were shown to be lineage specific for Th1 and Th2 $CD4^+$ T cells respectively (Zhang 1997b, Zheng 1997, Szabo 2000). Alongside these master transcription factors, signal transducer and activator of

transcription (STAT) proteins also act in a lineage specific fashion. They are signaling molecules of cytokine receptors; STAT4 is activated after IL-12R ligation and STAT6 activated after IL-4R ligation (Kaplan 1996, Thierfelder 1996). The activity of these proteins is determined not just by expression as in the case of the master transcription factors but by post-transcriptional modification in response to cytokine release. Some STAT proteins are directly responsible for controlling the expression of master regulator transcription factors whilst others act directly on cytokine genes and therefore work alongside the master transcription factors to polarise T cells. It was shown that STAT4 works with T-bet in Th1 cells and STAT6 is active in Th2 cells.

From this simple division, many human conditions could be put in the perspective of aberrant T cell activity. Uncontrolled Th1 cell activity was assumed to lead to autoimmune inflammatory conditions (e.g. MS, RA) and over-reactive Th2 responses were thought to be responsible for allergic reactions.

Support for Th1 cells being important in autoimmune conditions came from observations in multiple sclerosis and its animal model EAE. Myelin specific Th1 cells producing IFNγ upon *in vitro* restimulation were identified at higher frequencies in blood and cerebrospinal fluid (CSF) of MS patients (Olsson 1990, Link 1992) and accumulate in CNS lesions of MS patients (Traugott 1988) and mice (Merrill 1992). Th1 cells were shown to require the presence of α4 integrins on their surface to enter the brain parenchyma, and despite maintaining their response to antigen and cytokine production, those cells with low levels of α4 integrins were non-encephalitogenic (Baron 1993). Treatment of MS patients with IFNγ exacerbated disease (Panitch 1987) and mice lacking p40, a subunit of IL-12 which is required for Th1 differentiation, were resistant to EAE (Becher 2002).

However, several observations including more severe EAE in IFNγ$^{-/-}$ and STAT1$^{-/-}$ mice that lack Th1 cells (Ferber 1996, Bettelli 2004) and the ability of MBP specific Th2 cells that lack IFNγ production to cause EAE in immunodeficient animals, probably through TNFα (Lafaille 1997) confused the issue and suggested that the situation was more complicated than just Th1 and Th2.

8.3.1.2 Paradigm Revision

The discovery of IL-23 partially resolved these confused results. IL-23 shares a p40 subunit with IL-12 but has a specific p19 subunit as compared to the p35 subunit of IL-12. p19$^{-/-}$ mice were resistant to EAE whereas p35$^{-/-}$ mice remained susceptible (Gran 2002) illustrating that it was not IL-12 that was important for EAE induction but IL-23 (Cua 2003). IL-23R knockout mice are also resistant to EAE induction (Awasthi 2009, McGeachy 2009). IL-23 was shown to be necessary for the induction or expansion of CD4^{+} cells expressing IL-17, which were called Th17 cells.

Since then there has been a great deal of focus on Th17 cells and they have been shown to be a distinct subset of pro-inflammatory cells alongside classical Th1 and Th2 cells, releasing IL-17A, IL-17F, IL-21, IL-9 and TNFα and being independent of T-bet or GATA3 transcription factors (Harrington 2005, Park 2005). Retinoid-related orphan receptor gamma second isoform (RORγt) and alpha (RORα) were shown to be the master transcription factors related to this subset and initially differentiating conditions of transforming growth factor beta (TGFβ) and IL-6 reinforced that idea (Ivanov 2006, Yang 2008b) with IL-23 considered vital for their expansion (Mangan 2006) and maintaining the Th17 phenotype (Stritesky 2008, Ciric 2009, Lee 2009). However, since the initial

discovery, TGFβ has been shown to be dispensable for Th17 differentiation and is more likely to function by suppressing Th1 and Th2 cells, and therefore their products which inhibit Th17 differentiation (Das 2009, Santarlasci 2009). IL-1 has been shown to be required for Th17 differentiation as IL-1RI$^{-/-}$ mice are resistant to EAE and show defective Th17 responses (Sutton 2006), and a role for IL-21 in differentiation of human Th17 cells has been suggested although this has only been shown *in vitro* (Yang 2008a).

Th17 cells were therefore thought to be the most important subset in mediating EAE. PLP-specific, IL-23 induced Th17 cells induced EAE in naïve SJL mice whereas IL-12 induced Th1 cells did not and neutralizing anti-IL-17 antibody reduced severity of EAE whereas anti-IFNγ exacerbated it (Langrish 2005). Studies inducing EAE in IL-17$^{-/-}$ mice resulted in very mild disease with delayed onset and early recovery (Komiyama 2006) and neutralizing IL-17 in a MOG-induced EAE model improved clinical signs (Hofstetter 2005). RORγt$^{-/-}$ mice also developed a much milder EAE later than wildtype mice (Ivanov 2006) and studies showing that adoptive transfer of MOG_{35-55} primed Th17 cells could initiate EAE (Jager 2009) have all provided further evidence for their importance.

Increased expression of IL-17 mRNA has been detected in the CSF and lesions of MS patients where there is also an increase in IL-6 mRNA (Lock 2002). CD4$^+$ T cells producing IL-17 at both the mRNA and protein level have been detected in active lesions alongside both IL-23 subunits (p19 and p40) (Li 2007) and the borders of chronically active lesions (Tzartos 2008).

The discovery of Th17 cells has lead to greater acceptance of the possibility of new CD4 subsets. In the last few years, new CD4$^+$ T cell subsets have been touted, including Th9 and Th22 (Veldhoen 2008b, Duhen 2009, Trifari 2009). Th9 cells are characterised based

upon their production of IL-9, a cytokine initially thought to be derived from Th2 or even Th17 cells (Nowak 2009) but now shown to not be expressed with IL-4, IL-5, IL-13, IL-17 or IL-22. Th22 cells have been described in the human setting as skin-homing cells that do not produce IL-17 or RORγt, but as with Th9 cells, at present the evidence is only *in vitro* and without the identification of a master transcription factor, it is not clear whether they are a new subset or just an adaption of another.

This openness to new subsets should however be approached with caution. Just as the Th1 and Th2 paradigm simply provided simple guidelines, recent evidence is muddying the waters and confusing the issue of whether terminal differentiation of cells exists or if it is just a convenient method of classification.

8.3.1.3 Inconsistencies

With the discovery of Th17 cells and their importance to EAE brought an ignorance/dismissal of the role of Th1 cells in EAE and MS. However some observations opened the door to the fact that it may not be that simple. Purified Th17 populations devoid of IFNγ producing cells were reported to be unable to induce EAE by adoptive transfer whereas purified Th1 populations could, and Th17 cells seemed to require prior Th1 access to the CNS to infiltrate (O'Connor 2008). The plasticity between these subsets poses a major caveat, particularly the ease with which Th17 polarised cells can switch to Th1 cells (Abromson-Leeman 2009, Bending 2009, Nurieva 2009, Domingues 2010). Using Rosa26 fate reporter mice, it was shown that in the MOG/C57BL/6 EAE model, the majority of CNS-infiltrating CD4 cells are ex-Th17, Th1 cells (Hirota 2011). Conversely, it is argued by others that experiments that reported successful EAE induction by adoptive

transfer of Th1 cells contained a contaminating population of Th17 cell because the length of *in vitro* stimulation with IL-12 was not long enough to suppress Th17 cells (Jager 2009). It has been shown that EAE induced by Th1 and Th17 cells differ in both the clinical and pathological features and is determined by the Th1:Th17 ratio (Stromnes 2008). IL-12 polarized cells promoted expression of monocyte attracting chemokines and the infiltrating cell populations into the spinal cord were rich in macrophages but IL-23 polarized cells promoted neutrophil infiltration into the brain and their attracting chemokines (Kroenke 2008). In addition, T-bet^{+}RORγt^{+} cells have also been observed in the infiltrating population in EAE and Th17 cells can switch to produce IFNγ (Shi 2008, Abromson-Leeman 2009) unlike Th1 cell switching to a Th17 phenotype. There are repressive histone methylation marks in both the RORc and IL-17 loci in Th1 cells making it unlikely that Th1 cells can switch to becoming Th17 cells (Wei 2009). In fact it has been suggested that T-bet expression defines encephalitogenicity of T cells (Yang 2009b) backed up as inhibition of T-bet ameliorates EAE by inhibiting both Th1 and Th17 cells (Bettelli 2004, Nath 2006, Gocke 2007) raising the question of just how defined these subsets are. T cells expressing both IFNγ and IL-17 have been observed in the MS brain (Kebir 2009) and although IL-17 and IFNγ are elevated during very early disease, it is enhancement of IFNγ alone which is associated with relapses (Frisullo 2008). IFNγ responsiveness is also thought to be responsible for lesion localisation (Lees 2008a) and a large proportion of the IL-17 observed in brain lesions comes from host cells, mainly the γδ T cells (Lees 2008b).

It may be that the model itself is skewing these results however. Pertussis toxin, given alongside CFA and peptide to induce EAE, promotes secretion of IL-6 and inhibits IL-2

expression enhancing Th17 differentiation (Chen 2006) whist the mycobacterium within the CFA can stimulate IL-17 production (Veldhoen 2006a, Veldhoen 2006b). This would suggest that the induction of EAE increases the likelihood of observing Th17 responses and that they may not be the direct cause to the pathology seen. Additionally, the mouse model used should be incorporated into the interpretation of results. In C57BL/6 mice Th1 cells predominate at the peak of disease, whereas in the SJL model, more Th17 cells are observed (Langrish 2005, Korn 2007a).

8.3.1.4 Tregs

Th1, Th2 and Th17 cells protect the host from diverse pathogens through their wide repertoire of T cell receptor (TCR) expression. However, a number of them will be generated with autoreactive TCRs and any that are not negatively selected in the thymus must be inhibited in the periphery or the host will develop autoimmunity. Tregs appear to be a critical mechanism by which this peripheral tolerance is maintained, while retaining the correct scaling of immune responses to foreign antigens (Cobbold 2006, Belkaid 2007). They were originally discovered after experiments involving the inoculation of normal T cells into thymectomised syngeneic mice were able to suppress the development of autoimmunity that would normally develop after thymectomy (Sakaguchi 1982). Therefore animals possessed cells capable to protecting themselves from autoimmunity as well as those which are potentially pathogenic. The cell population responsible was initially identified by adoptive transfer experiments. $CD4^+$ spleen cell populations depleted *ex vivo* of $CD5^{high}$ and $CD45RC^{low}$ were transferred into athymic nude mice or rats and autoimmunity developed (Sakaguchi 1985, Powrie 1990).

These initial experiments identifying a phenotypic marker and the further discopvery of a more specific marker, CD25, affirmed these cells as an individual population responsible of protection from autoimmunity (Sakaguchi 1995).

Tregs include both natural and inducible Treg cells, the natural subset classically defined as $CD4^{+}CD25^{+}$ and positive for their characteristic transcription factor Foxp3 (Fontenot 2003, Hori 2004). Inducible Tregs (Tr-1, Th3) are derived from naïve T cells in the periphery. They are stimulated by antigen in the presence of anti-inflammatory cytokines e.g. IL-10 and TGFβ and act to increase the levels of these cytokines in the local milieu. nTregs on the other hand have been shown to utilise multiple mechanisms of action including cell-cell contact, local competition for growth factors and local secretion of inhibitory cytokines (Sojka 2008).

The importance of Tregs is most apparent when mutations occur that prevent normal function of these cells. Mutations in the Foxp3 gene lead to a severe and rapidly fatal condition called Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans and scurfy in mice (Wildin 2002, Khattri 2003). Both lead to systemic autoimmunity from birth and human patients usually die within a year of birth. Using an *in vivo* ablation model, it has also been shown that Tregs are required not just in neonates but throughout adult life to prevent the onset of large-scale autoimmunity (Kim 2007).

Therefore Tregs were assumed to play a role in MS. An initial study reporting a loss of suppressive ability of Tregs derived from patients with MS suggested that it was a disease of aberrant regulation (Viglietta 2004), but since there have been mixed reports on the importance of Tregs. Some studies have found a reduced frequency of nTregs in the

blood of MS patients (Haas 2007, Venken 2008b), especially those that have recently left the thymus but most have found no difference between patients and controls (Hellings 2001, Haas 2005, Feger 2007). However, and slightly paradoxically, an increased frequency of nTregs was found in the CSF but not the blood of MS patients (Venken 2008b). This would suggest an impairment in the function of Tregs rather than their population size or migration potential and using *in vitro* suppression assays, a number of groups have found impairments in the functionality of Tregs in MS patients (Tsaknaridis 2003, Viglietta 2004, Haas 2005, Kumar 2006, Feger 2007, Venken 2008a, Frisullo 2009) but this may be reversed after therapy (Hong 2005, de Andres 2007, Korporal 2008). The complication in these results was the limitation in being able to sort Tregs based upon $CD25^{hi}$ expression. CD25 is also upregulated on activated T cells and so some effector cells may also have been sorted and this led to the inconsistent results. However in two of the studies, the functional impairments of the Tregs was associated with lower Foxp3 expression at both the mRNA and protein level (Huan 2005, Venken 2008b) but at later stages of the disease, such as in secondary progressive forms, the Tregs showed a normal suppressive function and Foxp3 levels compared to RR-MS patients (Venken 2006). By observation of $Foxp3^{+}$ by immunostaining, one group has also noted an absence of Tregs in active MS lesions (Tzartos 2008) throwing doubt on the ability of Tregs to migrate into the inflamed CNS.

In mice, more direct evidence has been generated that Tregs have an important role in EAE. An early study showed that the presence of non-transgenic $CD4^{+}$ T cells prevents spontaneous autoimmunity in MBP TCR transgenic mice, an observation subsequently interpreted in support of the role of Tregs (Lafaille 1994, Olivares-Villagomez 1998), and

transfer of $CD4^+CD25^+$ T cells reduced disease severity in MOG-induced EAE (Kohm 2002). The frequency of antigen-specific Tregs has also been shown to correlate with disease susceptibility across a range of mouse strains in the PLP-induced model (Reddy 2004). Depletion of $CD25^+$ cells decreased the threshold of antigen required to induce EAE and prevented efficient recovery (Stephens 2005, Korn 2007a). Resistant B10 mice can be made susceptible to EAE through administration of anti-CD25 (Reddy 2004), a component of this dependent on gender as the susceptibility increases to a greater extent in the male mice compared to the female (Reddy 2005). Disease severity and mortality were increased in a $PLP_{139-151}$ induced EAE model following Treg depletion with anti-CD25 (Zhang 2004b).

As in humans, in mice it has been seen that Tregs accumulate in the CNS through EAE although they were mainly seen during the recovery phase of the disease (McGeachy 2005, Liu 2006b, Korn 2007b). Depletion of the Treg population inhibits recovery of the animals (McGeachy 2005) but the suppressive ability is not clear: Tregs taken from one model at peak of disease were unable to suppress CNS derived effector T cells (Korn 2007b) but from another CNS –derived Tregs taken from the CNS during the recovery phase of EAE could suppress MOG-specific IFNγ but not IL-17 *in vitro* (O'Connor 2007). The Tregs from the CNS appear to be derived from proliferation of host Tregs rather than conversion of non-Tregs (Korn 2007b, O'Connor 2007) and the inflamed CNS has been shown to specifically drive the proliferation of Treg cells (O'Connor 2007) in opposition to a study that suggested that Tregs could be converted from effector cells in the CNS (Liu 2006b).

Adoptive transfer of Tregs has been shown to provide protection against MOG_{35-55} induced EAE (Kohm 2002) and in this instance there was a decreased infiltration of cells into the CNS. As a final complication, recent observations are that $Foxp3^+$ Tregs can produce IL-17 and IFNγ which may explain the difficulties in showing suppressive effects, but also opens the question of how terminal is the differentiation into a Treg phenotype (Koenen 2008, Ayyoub 2009, Beriou 2009, Komatsu 2009).

It was thought that Tregs and Th17 cells may be reciprocally differentiated, and come from the same progenitor cells (Bettelli 2006b). Support from this came from the need for TGFβ to differentiate both (Oukka 2007), with Th17 cells favoured in inflammatory conditions (IL-1, IL-6) or when other Th17 cells are able to produce these cytokines (Veldhoen 2006a). *In vitro* it was shown that Tregs could substitute for TGFβ in a Th17 generating cell culture (Veldhoen 2006a) and in a model of systemic autoimmunity, the transfer of Tregs enhanced the Th17 population (Lohr 2006). IL-2 is required for the maintenance of the Treg population (Yu 2006) but inhibits the differentiation of Th17 cells (and Th1, Th2) (Laurence 2007) so it could be, as with the other subsets that the cytokine milieu in fact determines differentiation above all else. Other factors such as the aryl hydrocarbon receptor provide a more specific developmental switch incorporating environmental stimuli in the form of ligands driving one lineage at the expense of the other (Quintana 2008a, Veldhoen 2008a). Such a reciprocal relationship may have evolved to allow a targeted cellular function in response to the environment, both internal and external.

A great deal of research has centred on the role immune cells play in mediating the pathology observed in MS. Studies in humans and using animal models have all thrown

their contribution into what is a very complicated set of events. Each model or study has been focused on a different aspect but without the complete model it is hard to tie together observations from disparate settings and try to reach a consensus about how the immune pathology is mediated and which cell types are the most important or limiting. In this section I will summarise what is known about each of the other (non-CD4) major cell types and their hypothesised role in MS, coming from experiments with human samples and animal models.

8.3.2 Roles of Other Immune Cells

8.3.2.1 Mast cells

Mast cells are present in the parenchyma of the normal brain and at the blood brain barrier (Silver 1996). They have been seen to cluster in CNS lesions of MS patients in both MS plaques and normal appearing white matter (Olsson 1974, Toms 1990, Kruger 2001) and mice lacking mast cells develop a milder form of EAE (Secor 2000). However, the role of mast cells is not clear as a recent studies in mast cell deficient mice have described more severe EAE (Bennett 2009) despite their usual trafficking to the CNS in EAE (Bennett 2009) and a significant reduction in mast cells has been observed in brains of rats with EAE (Brenner 1994). In the latter case, a higher proportion of mast cells appear to be degranulated (Brenner 1994) and this corresponds with other data showing that mast cells can be activated by myelin (Medic 2008) and mast cell derived proteases could contribute to MBP degradation (Johnson 1988). Mast cells are also thought to have a role in the breakdown of the blood brain barrier as molecules such as histamine and tryptase released by mast cells can promote the infiltration of leucocytes into the

CNS (Lock 2002, Lapilla 2010). Roles such as these highlight the importance of the interplay between the adaptive and innate immune systems in the pathogenesis of MS and whilst not directly investigated in this study, certainly contribute to the variety of clinical forms observed, beyond the directly T cell mediated component (Pedotti 2003).

8.3.2.2 γδ T Cells

The exact role that TCR γδ T cells play in MS is unclear. They have been observed in MS lesions (Selmaj 1991a) and clonally expanded cells were found in CSF taken from patients with recent-onset disease (Shimonkevitz 1993) along with increases in their number in peripheral blood (Stinissen 1995). Studies in EAE have shown γδ T cells to be protective (Kobayashi 1997, Ponomarev 2004, Ponomarev 2005), promote EAE (Olive 1995, Rajan 1996, Rajan 1998, Spahn 1999, Odyniec 2004) or have no effect (Matsumoto 1998). γδ T cells are found in the CNS of mice with EAE (Sobel 1992) and they may lyse oligodendrocytes (Freedman 1991) and prevent remyelination (Selmaj 1991a) or contribute to recovery through Fas-FasL induced apoptosis of encephalitogenic T cells (Ponomarev 2004, Ponomarev 2005).

8.3.2.3 Dendritic Cells

Dendritic cells are implicated in the pathogenesis of MS as they present antigen to T cells and so can therefore drive an autoimmune response by presenting self peptides as well as shaping polarisation of the T cell response. DCs are reported to be found in elevated levels in the blood and CSF of MS patients as well in white matter lesions (Zozulya 2010),

some of which are seen to have taken up myelin (Serafini 2006). DCs have also been shown to accumulate in the CNS and either initiate EAE (Dittel 1999, Weir 2002, Bailey 2007) or mediate tolerance (Khoury 1995, Xiao 2004) in studies involving the adoptive transfer of activated myelin antigen pulsed DCs.

Different subsets of DCs appear to behave differently in MS patients. DCs from peripheral blood of MS patients are activated, with expression of activation markers and increased production of inflammatory cytokines whereas plasmacytoid DCs show decreased or delayed expression of activation markers when isolated from MS patient peripheral blood (Stasiolek 2006). DCs are also likely to be the cells driving epitope spreading as observed in MS patients. CNS-invading myeloid DCs have been reported to drive this spread in both a relapsing EAE and TMEV model of demyelination (McMahon 2005).

Numerous attempts have also been made to manipulate DCs in order to treat EAE. Severity of disease could be reduced after injection of mature DCs as they promoted high levels of IL-10 production at the expense of IL-12, inhibiting the pro-inflammatory response (Zhang 2002). IFNγ treated DCs have also been shown to reduce EAE severity somewhat paradoxically, through prevention of the upregulation of co-stimulatory molecules in the presence of pro-inflammatory cytokines (Xiao 2004), and DCs matured in the presence of TNFα with an autoantigenic peptide lead to a similar phenotype but by an opposite mechanism, through an up-regulation of co-stimulatory molecules but weak pro-inflammatory cytokine response (Menges 2002). Other groups promoted IL-10 production and inhibited IL-12, IFNγ and IL-23 production by transferring DCs transduced

with SOCS-3 (suppressor of cytokine signalling inhibitor) (Li 2006), or loaded mitomycin C treated DCs with MBP to inhibit disease induction and severity (Terness 2008).

8.3.2.4 Macrophages/Microglia

Microglia are considered to be CNS resident macrophages, reflected by their roles in antigen presentation, phagocytosis and cytokine production (Benveniste 1997). All known Toll-like receptors (TLRs) are expressed by microglial cells and their expression is increased in CNS lesions in both EAE and human disease (Bsibsi 2002). MS patients' T cells specific for myelin have been show to activate microglia and macrophages and as these cells have been shown to be involved in demyelination and phagocytosis of degraded myelin (Bauer 1994). This phagocytosis can lead to increased expression of myeloperoxidases (Gray 2008), causing neuronal damage.

Microglia are also involved in activating T cell populations and increasing infiltration into the CNS, while themselves mediating damage directly. The secretion of macrophage inflammatory protein 1 alpha (MIP1α) and monocyte chemotactic protein 1 (MCP1) (Hofmann 2002) and upregulation of their receptors CCR1 and CCR2 have been reported on microglia (Eltayeb 2007), leading to increased attraction for leucocytes to the CNS. In this way, MCP1 knock-out mice or those treated with anti-MIP1α antibodies developed EAE with lower leucocyte infiltration and therefore less severe signs of disease (Karpus 1995, Izikson 2000). CD40 expression on brain resident microglia is essential for CNS leucocyte infiltration and EAE induction (Becher 2001) and MHC class II molecules and associated transcription factors have been found to be upregulated on microglia in both MS and EAE (Konno 1989, Gobin 2001) as well as co-stimulatory molecules e.g. CD80 and

CD40 (Windhagen 1995, Gerritse 1996). The expression of MHC class II molecules could have both protective and pathogenic roles depending on the cells expressing it. MHC class II antigens expressed on microglia may activate Tregs (Almolda 2010) favouring remission, whereas expression on perivascular macrophages activates pathogenic helper T cells and thereby promotes EAE (Hickey 1988). In EAE, macrophages have been seen to upregulate CD80 and downregulate CD86, suggesting pathogenic and protective roles for these co-stimulatory molecules respectively. By blocking the CD80-CD28 interaction, EAE can be suppressed by deletion of peripheral antigen specific $CD4^+$ T cells (Srinivasan 2002) whereas blocking of CD86 or its ligand, CTLA-4, EAE could be made worse (Kuchroo 1995, Hurwitz 1997).

As well as directly activating T cells, microglia can produce factors that mediate CNS damage. Glutamate production by macrophages/microglia correlates with axonal damage in EAE (Werner 2001) and the main factors driving glutamate degradation, glutamate transporters and degrading enzymes were also downregulated (Hardin-Pouzet 1997, Ohgoh 2002). Blocking of the glutamate receptor reduces demyelination and stops disease progression (Pitt 2000). Macrophages/microglia are also potent producers of inducible nitric oxide synthase (iNOS) in EAE which can be mediated by MBP-primed T cells (Dasgupta 2002) and leads to the production of the free-radical NO. Inhibiting NOS suppressed the disease (Zhao 1996) but complete lack of NO exacerbated disease (Fenyk-Melody 1998) so there must be a minimal threshold necessary for homeostasis.

Microglia are reported also to secrete anti-inflammatory cytokines such as IL-10 in the appropriate cytokine milieu, and are capable of secreting factors that promote

neurogenesis, such as brain-derived neurotrophic factor, insulin-like growth factor and neurotropin-3 (Napoli 2010).

8.3.2.5 B cells

B cells have dual roles in mediating an immune response, by releasing antibodies and acting as antigen presenting cells for T cells. MS-like disease is not transferable by antibodies (Lassmann 1983) and plasmapharesis has not been a successful treatment for MS, so the role of the B cell response in this disease has been difficult to elucidate. One clue to the role of B cells came from characterization of EAE in Igμ-null mice (Kitamura 1991). In these mice the development of B cells is arrested at the pre-B cell stage as they are unable to produce the heavy chain of IgM (μ chain) and so mice are generated lacking mature B cells. Induction of EAE in these mice was comparable to controls but recovery was impaired leading to the conclusion that B cells influence the immune regulation in EAE (Wolf 1996). A key study by Genain and colleagues proposed a possible contributory role of antibody in a primate model of MOG-induced EAE (Genain 1999) in agreement with long established observations from rodent models (Linnington 1984). However, many of the myelin components targeted by the immune response in autoimmunity are not accessible to antibody on the surface of the myelin sheath.

An important role for B cells in EAE was highlighted as removal of them before immunization worsened the disease course. There was an increase in leucocyte infiltration into the CNS and more severe demyelination (Matsushita 2008) probably a result of removing a regulatory B cell subset as adoptive transfer of these cells prior to immunization regained control. This protection was only provided before immunization

as administration with rituximab (anti-CD20) during disease onset actually reduced leucocyte infiltration and demyelination. There were fewer and less active T cells probably as a result of lacking co-stimulation in disease initiation (Matsushita 2008). In the human setting, rituximab has been effective in reducing inflammatory CNS lesions and relapses over 48 weeks (Hauser 2008).

Aggregates of B cells in the meninges of MS patients were first described by Serafini, present in 40% of secondary progressive cases (Serafini 2004). The aggregates resembled germinal centres with B cells, plasma cells, T cells, macrophages and follicular dendritic cells. The white matter lesions did not differ but these patients showed more severe grey matter lesions (Magliozzi 2007). However, this secondary lymphoid structure has been observed in other autoimmune diseases and in other neurological conditions (Carragher 2008). These ectopic B cell structures have been reconstituted in mice to an extent as adoptive transfer of Th17 cells co-cultured with IL-23 could be seen to form B cell aggregates (Jager 2009).

When MOG-specific B cells are present to act as APC, MOG-specific T cells show enhanced proliferation and secretion of IFNγ in response to the recombinant protein (Bettelli 2006a). It has also been shown that transgenic mice expressing MOG-specific B and T cells develop spontaneous EAE and the B and T cells produced more MOG-specific IgG and IFNγ and IL-17 respectively than control mice carrying a MOG-specific B or T cell component (Bettelli 2006a).

Clonally expanded B cells have been found in the CSF and CNS of patients. Positive selection based on the matching antigen was suggested as the Ig heavy-chain variable regions were hypermutated (Baranzini 1999). A longstanding diagnostic criterion for MS

is the identification of oligoclonal IgG bands by isoelectric focusing in the CSF, and myelin specific antibodies have been detected in the CSF reactive to MBP, PLP (Warren 1994) and MOG where they often bind to disintegrating myelin (Genain 1999). More recently these oligoclonal Ig bands have been linked to the Ig transcriptomes of B cells derived from MS patients' CSF (Obermeier 2008). This work confirmed that the intrathecal Ig response is directly relevant to MS pathogenesis adding retrospective support to previous studies of CSF derived B cells and antibodies.

Resistance to EAE by early removal of B cells through anti-IgM could be overcome through administration of serum containing MBP or MOG specific antibodies although this required administration of the requisite myelin peptide as well as passive transfer of serum alone was not sufficient to induce EAE (Lassmann 1983, Willenborg 1986, Lyons 2002). An acceleration of EAE in a mouse overproducing MOG-specific antibodies irrespective of the myelin antigen used has been observed (Litzenburger 1998) and antibodies to other myelin antigens have been implicated in promotion of demyelination (Schluesener 1987, Lassmann 1988). Therefore autoantibodies may play a crucial role in driving MS pathogenesis and more recently, attempts have been made to identify a different antibody profile for MS patients with each various form of the disease to aid diagnosis (Reindl 2006, Quintana 2008b).

8.3.2.6 iNKT cells

Invariant Natural Killer T cells (iNKT) are considered part of the innate repertoire in the sense that they do not utilise clonotypic receptors and express immune memory, but have a classical (but invariant) TCR coupled to CD3 as well as CD56, a marker of natural

killer (NK) cells. They recognise glycolipid antigens presented by CD1d. They express TCRα and β chains but have a highly limited repertoire because they express only one α chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) that couple with only a few β chains (Vβ11 in humans, Vβ8.2, Vβ2 and Vβ7 in mice). The Vβ chains show a degree of CDR3 diversity, presumed to impact on antigen binding affinity (Matulis 2010). A consensus regarding their role in the immune system is still a matter for debate. After activation, iNKT cells have been shown to produce a wide array of cytokines, both pro- and anti-inflammatory including IL-4, IL-6, IL-10, IL-17A, IFNγ and TGFβ (Taniguchi 2003, Kronenberg 2005). Therefore iNKT cells are able to display both pathogenic (e.g. Chlamydia muridarum) and protective (e.g. *Streptococcus pneumoniae* and *Leishmania major*) roles in different microbial infections (Brigl 2003, Ronet 2005, Tupin 2007). This variety of cytokines secreted implicates iNKT cells in both protective and promotional roles for autoimmune conditions (Miyamoto 2001, Singh 2001, Wu 2009, Caielli 2011), whereby the usual Th2 bias would lead to the reduction of symptoms but the polarisation of iNKT cells to produce Th1 cytokines could exacerbate existing inflammation (Wilson 2003, Kent 2005).

Overexpression of invariant TCR in non-obese diabetic (NOD) mice was seen to lead to protection from EAE by reducing IFNγ release (Mars 2002) and lower numbers have been detected in MS patients (van der Vliet 2001), most specifically in the $CD4^-$ subpopulation (Araki 2003). Dysfunctions in NKT activity have also been implicated in susceptibility to EAE (Gombert 1996) potentially by inhibiting Th17 differentiation. EAE can also be prevented through the use of synthetic glycolipids to stimulate iNKT cells (Miyamoto 2001, Pal 2001). Therapeutic polarization of the iNKT response could act to augment

either a protective or pathogenic outcome, despite perhaps not playing a direct role in mediating damage.

8.3.2.7 NK cells

NK cells are another form of lymphocyte separate from the B and T cell lineage. They do not express TCRs and do not require prior stimulation to recognise and lyse cells (Trinchieri 1989). The major target for NK cells are tumour lines and virally infected cells which are directed towards apoptosis through the release of perforin and granzymes (Russell 2002). They are found in demyelinating MS lesions (Traugott 1985) and in autoimmune diseases have been thought to play both protective and pathogenic roles, as evidence has been accumulated for both. Initial division of NK cells on the basis of CD56 expression separates the $CD56^{dim}$ subset as those with cytotoxic function, and the $CD56^{bright}$ subset which secrete cytokines (Cooper 2001). This division has also been taken further, separating NK cells based upon their cytokine production. Those secreting IFNγ and IL-10 called NK1, and those secreting IL-5 and IL-13 called NK2 (Peritt 1998).

The first indication of an effect in autoimmune disease is any alteration in disease progression after depletion of the cell type of interest. Depletion of NK cells has been shown to exacerbate EAE (Zhang 1997a, Matsumoto 1998, Xu 2005) or reduce EAE severity if depletion was done before MOG immunization (Winkler-Pickett 2008) illustrating both protective and pathogenic phenotypes. NK cells have been shown to have cytotoxic activity directed at neurons (Backstrom 2003) oligodendrocytes, astrocytes and microglial cells (Antel 1998, Morse 2001, Saikali 2007) *in vitro* through interactions with NKp46 and NKG2D receptors and subsequent release of perforin (Lunemann 2008). The secretion of IFNγ from NK cells can also play a role in driving Th1

cell polarization (Martin-Fontecha 2004, Morandi 2006) and can restore susceptibility to EAE in IL-18$^{-/-}$ mice (Shi 2000) suggesting a pathogenic role for NK cells in autoimmunity. Supporting the studies noting an exacerbation of EAE in the absence of NK cells, decreased numbers of NK cells have been recorded in MS patients (Munschauer 1995) and the observed reduction in relapse rate in pregnancy has been associated with an increase in the CD56^{bright} and decrease in CD56^{dim} NK cell poplations (Airas 2008). An increase in NK2 NK cells during remission (Takahashi 2001) potentially negatively regulating autoreactive T cells (Takahashi 2004) and a decrease in the cytotoxic activity of circulating NK cells during relapses have been described (Kastrukoff 1998, Kastrukoff 2003). Also, an increase in NK cells number is seen through use of immunomodulatory and immunosuppressant therapies such as daclizumab (Bielekova 2006), and IFNβ (Saraste 2007). CNS infiltrating NK cells are also observed to produce factors favouring neuroprotection such as brain-derived neurotrophic growth factor and neurotropin-3 (Hammarberg 2000) during EAE, supporting their role in prevention of damage in the CNS.

8.3.2.8 CD8^{+} T Cells

The role of CD8 cells in MS has not until recently received as much attention as that of CD4 cells. This may be because EAE had been considered CD8-independent and the immunization protocol favours presentation through MHC class II antigens and thus, activation of CD4 cells. Unlike CD4 cells which typically reside in the meningeal and perivascular space, CD8 T cells have been found to migrate to the brain parenchyma in MS (Gay 1997) and CD8 T cells outnumber CD4 cells in acute and chronic lesions of MS

patients. Many of these are clonally expanded, which suggests an antigen-driven process (Babbe 2000, Junker 2007). There is an enrichment of $CD8^+$ T cells with an effector/memory phenotype in the CNS and CSF of MS patients (Jilek 2007) and these show an increased adhesiveness and ability to cross an *in vitro* BBB model (Battistini 2003). CD8 T cell responses to MBP and MOG were also shown to be increased in MS patients as compared to controls (Van der Aa 2003, Zang 2004) further implicating an important role for CD8 T cells in MS pathology.

In contrast to $CD4^+$ T cells, CD8 T cells recognize antigens presented by MHC class I molecules. In the CNS, MHC class I antigens are more widely expressed than MHC class II antigens, which is only seen on microglia. Most CNS resident cells, including oligodendrocytes and neurons can express MHC class antigens, and they upregulate it early in the MS disease course, before any demyelination (Hayashi 1988, Gobin 2001). The cerebral endothelium presents antigens on MHC class I, allowing CD8 T cells to adhere and cross the BBB (Galea 2007) and in addition, astrocytes, oligodendrocytes and neurons express upregulated MHC class I antigens in acute MS lesions (Hoftberger 2004). Contributions have been found for the MHC class I region in MS (Haines 1998, Sawcer 2005), although the strong influence through linkage disequilibrium of the neighbouring HLA-DRB1*15:01 has been suggested to influence these results (Chao 2008). The HLA class I antigen HLA-A3 (A*03:01) has been shown to provide a genetic risk factor in contrast to HLA-A2 (A*02:01) which reduces genetic risk (Fogdell-Hahn 2000, Brynedal 2007, Burfoot 2008) underlining the importance of HLA class I antigens in autoimmunity and therefore the likely involvement of CD8 T cells in the disease process. HLA-A*03:01 transgenic mice along with a human TCR for PLP_{45-53} developed an MS like disease after

immunization with PLP_{45-53} with CD8 T cells shown to mediate disease initiation. However, on introduction of the transgenic HLA-A*02:01 allele, mice were protected, as autoreactive T cells were deleted in the thymus (Friese 2008).

The exact role that CD8 T cells play in autoimmunity is a matter for debate. Evidence has been compiled for both a protective and pathogenic role for these cells. Through studies with $CD8^{-/-}$ mice, a reduction in demyelination and CNS inflammation during EAE induction would support a pathogenic role for CD8s in MS (Abdul-Majid 2003). These results are supported by some adoptive transfer experiments in mice. MOG_{35-55} reactive CD8 T cells generated *in vivo* or *in vitro* and MBP-specific CD8 T cells have been shown to transfer EAE to recipient mice (Huseby 2001a, Sun 2001, Ford 2005). This EAE more closely resembled human MS than CD4 transferred EAE, with lesions found in the higher CNS rather than being limited to the spinal cord and the development of other neurological symptoms not constrained to ascending paralysis and cellular infiltration into the spinal cord.

CD8 cells may not be directly involved in demyelination, but their role may be in mediating an attack against the neurons. Axonal damage within lesions appears to correlate best with the numbers of $CD8^+$ T cells (Bitsch 2000, Kuhlmann 2002) and macrophages/microglia (Ferguson 1997). In MHC class I antigen deficient mice there was no axonal damage despite widespread demyelination (Rivera-Quinones 1998), compared to MHC class II antigen knock-out mice which developed both axonal damage and demyelination. In the TMEV model of MS, axonal function could be preserved through depletion of the immunodominant $CD8^+$ T cell response (Howe 2007).

CD8 T cells can however show direct action against oligodendrocytes. In two models utilising a neo-self antigen transgenic strategy, the specific expression of either ovalbumin (OVA) or haemagglutinin (HA) in oligodendrocytes led to their lysis by CD8 cells and pathology resembling MS lesions upon introduction of the cognate MHC class I antigen-restricted T cell receptor or of pre-activated cells (Na 2008, Saxena 2008).

Several studies have attempted to identify differences in frequency or activation of CD8 T cells between healthy controls and MS patients, although with limited success. The frequency of myelin-specific CD8 T cells has found to be increased in blood of MS patients compared to controls (Tsuchida 1994, Crawford 2004, Zang 2004) and these cells have been seen to be clonally expanded and carry effector or memory phenotypes in MS patients rather than the naïve phenotype seen when extracted from healthy control blood (Babbe 2000, Crawford 2004, Skulina 2004, Zang 2004). CD8 cells extracted from secondary progressive patient blood produced more lymphotoxin (Buckle 2003) which acts to induce apoptosis and can be toxic for oligodendrocytes *in vitro* (Selmaj 1991b). Other studies have reported no difference in frequency of myelin-specific effector CD8 T cells as measured by proliferative potential (Huizinga 2009) or IFNγ ELISPOT (Berthelot 2008). In response to IL-23, IL-17 production by $CD8^+$ cells rivals that produced by $CD4^+$ cells under the same conditions, is produced alongside IFNγ and IL-10 (Vanden Eijnden 2005) and in the CNS equal numbers of both CD4 and CD8 T cells have been detected producing IL-17 (Tzartos 2008). CD8 T cells producing IL-17 have been called Tc17 cells and appear to differentiate in a mode similar to Th17 cells (Huber 2009). They have been directly implicated in development of autoimmunity through an adoptive transfer model of diabetes but in EAE showed decreased cytotoxic function (Ciric 2009) and are different

to normal cytotoxic T lymphocytes (CTLs) (Tc1) as they produce less perforin and granzyme B, which may explain the results seen.

A regulatory mode of action for $CD8^+$ T cells is a hypothesis that has received a lot of support. Observations from EAE sighting an inverse correlation between disease severity and proportion of CNS infiltrating CD8 cells (Weiss 2007) and CD8-deficient mice showing increased relapses despite an initial reduction in mortality (Jiang 1992, Koh 1992) indicate a presence of a CD8 regulatory T cell subset. Indeed, CD8 T cells isolated from mice that had recovered from EAE have also been shown to be capable of eliminating MBP-specific CD4 T cells both *in vitro* and *in vivo* (Jiang 2003) and this suppressive ability of CD8 T cells is seen to be impaired in MS patients with chronic, progressive disease (Balashov 1995).

Two populations of regulatory CD8 subsets have been described, the first lacking CD28 expression with the other characterised by expression of CD122. The $CD28^-$ subset have been seen to exhibit a regulatory function in the MOG_{35-55} EAE model by inhibiting DCs from upregulating co-stimulatory molecules and forcing them into a tolerogenic phenotype, unable to activate autoreactive CD4 T cells (Najafian 2003). The $CD8^+CD122^+$ subset act differently, secreting IL-10 and suppressing proliferation and IFNγ production by $CD8^+$ T cells (Rifa'i 2004, Endharti 2005) such that in the MOG_{35-55} EAE model, depletion of these cells increased the length of time mice persisted with high clinical scores and reconstitution of the population by adoptive transfer of *in vitro* activated cells significantly reduced EAE severity (Lee 2008). Work in humans suggests that $CD8^+CXCR3^+$ T cells are the counterpart for mouse $CD8^+CD122^+$ regulatory cells (Suzuki 2008) and the

action of copaxone has been attributed to the induction of regulatory $CD8^+$ cells (Karandikar 2002).

8.3.3 Innate Immune Signalling

8.3.3.1 TLRs

TLR activation offers an attractive hypothesis for the bridge between innate/environmental and adaptive triggers leading to an autoimmune attack on the CNS. TLRs are found throughout the CNS, on macrophages and microglia, mast cells and dendritic cells. There are differences of expression patterns between mice and humans, for example, human astrocytes are limited to TLR3 expression only whereas mouse astrocytes also transcribe TLR2, TLR4, TLR5 and TL9 mRNA (Bowman 2003, Farina 2005). Microglia from both species however express all TLRs (Olson 2004, Jack 2005) illustrating their central role in CNS surveillance. The requirement for adjuvants to initiate EAE has implicated the TLRs in initiating the immune attack in animal models. Analysis of CSF and brain lesions of MS patients and in EAE brains, have shown an increase in TLR expression (Bsibsi 2002, Hanisch 2008) and through ligation with their cognate ligands, TLRs lead to production of inflammatory cytokines which could drive T cell polarization. Specifically, Th1 polarizing cytokines have been shown to be secreted as a result of TLR3 signalling, leading to the upregulation of MHC class II molecules on microglia and differentiation of T helper cells (Jack 2007). However stimulation of the same TLR can also have anti-inflammatory effects, as PolyI:C induces the release of IFNβ and led to the suppression of EAE (Touil 2006).

Specific TLR knockout mouse lines have been made and used to study their role in CNS autoimmunity. TLR4$^{-/-}$ mice demonstrate more severe EAE (Marta 2008) whereas TLR2$^{-/-}$ (Prinz 2006) and TLR6$^{-/-}$ (Marta 2008) mice showed a similar clinical course to C57BL/6 mice. Contrasting roles have been shown for TLR9 in EAE with groups showing protection of knockout mice from EAE (Prinz 2006) or increased susceptibility (Marta 2008).

Linking most TLRs is myeloid differentiation primary response gene 88 (MyD88), the main adapter protein used in TLR signalling. MyD88 knock-out mice have been shown to be resistant to both induced and passive EAE involving the adoptive transfer of autoreactive T cells (Marta 2008, Cohen 2010b) illustrating the central role of TLRs in autoimmunity. Tissue damage releases endogenous TLR ligands that have been shown to stimulate TLRs on microglia to promote neurotoxic inflammatory mediators, more specifically heat shock protein 60 (HSP60) release stimulates nitric oxide release through TLR4 signalling (Lehnardt 2010). TLR stimulation on B cells however has been shown to promote recovery from EAE through increased IL-10 production as mice with B cell-restricted deficiencies of MyD88, TLR2 or TLR4 develop chronic EAE with DC-dependent production of IL-6 blocked, but a deficiency of TLR9 does not affect normal recovery (Lampropoulou 2008).

The major role of TLRs to detect microbial pathogens and their products provides a direct link between TLRs and EAE as one of the major components of the immunization protocol is *Mycobacterium tuberculosis*, a potent TLR4 activating micro-organism. Other microbial products have been shown to be capable to inducing EAE along with myelin proteolipid protein including CpG oligodeoxynucelotide (CPG ODN) (TLR9) and

lipopolysaccharide (LPS) (TLR4), albeit at delayed onset (Waldner 2004) demonstrating how micro-organisms can prime T-cell dependent inflammation through TLR-mediated activation of antigen presenting cells (APCs). Another microbial product, pertussis toxin has even more severe impact on EAE as it not only promotes T helper cell differentiation but also promotes T cell transendothelial migration across the BBB (Hou 2003, Kügler 2007). Therefore stimulation of APCs by the innate immune system can overcome peripheral tolerance and lead to autoimmunity, with TLRs being central to mediating this fine balance.

8.4 TCR Transgenic Mice in Autoimmunity

8.4.1 Background

Animal models of diseases allow greater depth of investigation than simply human studies and provide one other large advantage, that the genetics of the animal can be manipulated and controlled to investigate specific aspects of a condition without having to control for the large genetic diversity of a human population. The creation of T cell receptor transgenic mice provides a cohort of T cells with defined restriction and specificity that can be monitored phenotypically throughout the course of normal development and in the context of a disease. It is usually hard to detect and follow the fate of antigen specific cells through an immune response, as there is low frequency of their precursors in the naïve animal. It has been estimated through limiting dilution and TCR sequencing experiments to be between 1 in 10^5 or 10^6 T cells (Casrouge 2000, Hou 2003) and studies using tetramers, which enable identification of individual T cell clones,

generated ranges of 1:200,000 to 1:2,000,000 for $CD4^+$ T cells (Moon 2007, Chu 2009) and 1:20,000 to 1:1,300,000 for $CD8^+$ T cells (Kotturi 2008, Obar 2008) in support of these figures. TCR transgenic mice increase this frequency, such that, when examined on a RAG-null background to obviate endogenous rearrangements and dual-TCR expression, mice can be considered functionally monoclonal. This allows studies of T cell development to be carried out at a clonotypic level, watching naïve precursors develop into memory and effector cell populations. The location of T cell activation in response to specific antigens can also be assessed and this may have very far-reaching consequences with regard to drug development and interventions in clinic. The choice of MHC class restriction of the TCR in the mice allows investigation of the CD4 or CD8 response to the antigen of choice and the extension of this selection is the creation of humanized mice where human HLA class I or II heterodimer is introduced, so that the immune response more closely resembles that which normally occurs in the human setting. The TCR itself can also be derived directly from a human patient's T cells, thereby providing even more relevant immune responses in the context of development and diseases.

8.4.2 Initial Development

The first αβ TCR transgenic mice were reported in 1988 from the lab of von Boehmer. They generated mice with a TCR that recognized the male HY antigen in the context of H-$2D^b$. They investigated the development of thymic selection, illustrating the deletion of self-reactive T cells in the thymus (Kisielow 1988a), that the interaction of the TCR with the MHC molecule drives selection of CD4 or CD8 lymphocytes from the double positive

stage (Teh 1988) and the interaction with the MHC class I molecule is necessary to generate antigen specific CD8 lymphocytes (Kisielow 1988b). Shortly after, the 2C TCR transgenic mouse was developed, with an anti-L^d alloreactive $\alpha\beta$ TCR (Sha 1988), used to investigate positive selection of the T cells by the cognate MHC molecule (Sha 1988).

These first two mice were CD8 specific and in the next year, two more studies showed that the observations for CD8 T cells were transferrable to CD4 T cell selection (Berg 1989, Kaye 1989). Both utilised TCRs specific for pigeon cytochrome c presented by I-E^k although they were generated from different T cell clones and on different mouse backgrounds. Whilst these studies had investigated selection of CD4 and CD8 cells in the thymus, the exact location at which tolerance was induced had not been deduced. Pircher, Burki et al (1989) investigated this through the use of TCR transgenic mice with a double specificity. Mice expressed a TCR specific for both lymphocytic choriomeningitis virus (LCMV) – H-2D^b and minor-lymphocyte stimulatory antigen (MIsa), subsequently appreciated to be mouse mammary tumour virus (MMTV) and, depending on which antigen was involved, differences were seen in the stage of T cell maturation and location in the thymus as to when the TCR-expressing cells could be tolerized (Pircher 1989).

Another early study looked at tolerance using a TCR specific for a circulating antigen and investigated selection in a system with or without the target antigen (Zal 1994). This was used to show 'piggy-backing' of a self-reactive TCR which would usually be deleted with an endogenously coded TCR undergoing positive selection (Zal 1994).

8.4.3 Neo-self antigens

Mice transgenic for TCRs recognizing foreign antigens have been crossed with mice transgenically expressing the foreign antigen as a neo-self antigen, thereby allowing detailed examination of tolerance to be carried out in settings that are not rooted in physiological autoimmunity or infectious disease. The nature of local antigen presentation was elucidated in TCR transgenic models also expressing the cognate LCMV glycoprotein antigen in pancreatic islet cells (Ohashi 1991). The requirement for co-stimulation in this interaction (Harlan 1994) and the ability to break peripheral tolerance in islets (von Herrath 1995) were both observed. Another model, using injection of ovalbumin specific T cells from the MHC class I-restricted OVA specific (OT-I) TCR transgenic mouse into rat insulin promotor (RIP)-mOVA mice expressing membrane bound OVA in the pancreatic islet cells, demonstrated that cross-presentation of antigen in the draining lymph node was an important immunological mechanism (Kurts 1996).

K^b-specific CD8 TCR transgenic mice (Morahan 1989), influenza haemagluttinin TCR (Scott 1994) and hen egg lysozyme CD4 TCR transgenic models (Akkaraju 1997) among others show that factors including amount of the antigen (Ferber 1994), genetic background (Scott 1994) and expression of neo-self antigen in the thymus (Forster 1995, McGargill 2002) play a role in mediation of tolerance. Mechanisms of tolerance in these models have included down regulation of the TCR on self-reactive T cells outside of the thymus (Schonrich 1991), and the generation of Treg cells, selected both by antigen affinity (Jordan 2001), and by either thymic or peripheral antigen expression (Apostolou 2002).

Tregs generated in this way could also be shown to maintain their responsiveness *in vivo* despite appearing anergic *in vitro* (Walker 2003).

8.4.4 Disease Specific TCRs

In contrast to TCR transgenic mice being used to understand general mechanisms of immune development, the design of mice with autoimmune disease-derived specific TCRs has provided new models of disease with specific targets and an increase in the antigen specific T cell population for further analysis. TCR transgenics for antigens thought to be involved in three major autoimmune diseases, MS, type I diabetes and rheumatoid arthritis, have been developed and will be looked at in turn.

8.4.4.1 Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune condition affecting the joints, among other organs and tissues. A much used mouse model for this disease is collagen-induced arthritis where, in the same manner as EAE, a target protein or peptide, in this case type II collagen, is introduced with adjuvant to initiate an autoimmune reaction targeted to the joints. Extending this idea, the first TCR transgenic was created in 1992, with mice expressing just the β chain from an I-A^q-restricted anti-collagen type II T cell (Mori 1992). The introduction of the transgenic β chain and its subsequent association with endogenous α chains, led to an enhanced immune response against type II collagen but was not sufficient to make arthritis-resistant SWR mice susceptible to arthritis (Mori 1992). Extending this idea, $\alpha\beta$ TCR transgenic mice were created, still specific for type II collagen (CII) (Osman 1998). CII-specific T cells were positively selected, not deleted in

the thymus, and were not anergic. Arthritis could be induced with immunization of CII and adjuvant, although no spontaneous disease development was observed. The increased specificity of the immune response was notable as severe disease developed twice as fast in the transgenic mice as in the non-transgenic (Osman 1998).

TCR transgenic mice have also been created for other antigens expressed in the joints. Cartilage proteoglycan (PG) is another candidate target antigen in RA (Glant 1980, Golds 1983, Goodstone 1996) and immunization of BALB/c mice with human cartilage PG (HuPG) induces a disease similar to RA (Glant 1987) by cross-reactivity of the T cells to mouse proteoglycan. Mice expressing a TCR specific for HuPG were created (Berlo 2006) and termed 5/4E8. Transfer of naive cells from these mice could transfer arthritis to recipient animals, demonstrating their role in pathogenesis.

The major TCR transgenic mouse model used in the study of RA, however, is the KRN model. KRN mice possess a TCR specific for bovine pancreas RNase in the context of I-A^k and when they were crossed with NOD mice, their offspring developed a spontaneous, RA-like joint disease (Kouskoff 1996). Sera from these arthritic mice could transfer disease and it was shown that it contained a major antigen from joints and other tissues, glucose-6-phosphate isomerase, whose depletion from sera prevented disease transmission (Matsumoto 1999). The effector phase has been shown to require neutrophils (Wipke 2001, Monach 2010), mast cells (Lee 2002), complement C5a and FcγRIII (Ji 2002, Tsuboi 2010). Since its discovery, this TCR transgenic model has dominated mouse model based research into RA, either through its direct use or the use of KRN T cells. Whilst a great deal is now known about this immune model, its success is a double-edged sword. On the one hand, the more knowledge of this system we have

the more detailed questions we can use it to answer and so the more people will work on it. However, the dominance of this model means that, for what is a poorly understood and heterogeneous human disease, mechanistic studies may become somewhat narrowed to a single mechanistic context.

8.4.4.2 Type I Diabetes

Much research into type I diabetes has been carried out using the NOD strain of mice. These were developed at Shiongi Research Laboratories in Aburhai, Japan and first reported in 1980 (Makino 1980). They spontaneously develop insulin dependent diabetes resulting from T cell infiltration into the Islets of Langerhans and have been considered a genetically, pathologically and immunologically faithful model of the human disease. NOD mice have been used as the base strain for the TCR transgenic mouse lines created to study diabetes with, each providing a mouse usually susceptible to diabetes but with a T cell repertoire enhanced for one antigen specificity.

There are 5 'base-strain' TCR transgenic mouse models used in diabetes research at present, the first TCR transgenic to be developed from the NOD mouse was the BDC2.5 strain. This TCR has α and β chains derived from a NOD mouse recently found to recognize chromogranin A, a pancreatic β cell antigen, presented by I-A^{g7} (Katz 1993, Stadinski 2010). In these mice, there are no signs of transgenic T cell deletion in the thymus and because the NOD mice develop diabetes under normal circumstances, increasing the proportion of T cells derived from a diabetogenic T cell clone, could only accelerate disease progression (Haskins 1990). When the mice were crossed onto a $RAG^{-/-}$ background, thereby eliminating any endogenous T cell repertoire including Tregs,

disease onset and progression was accelerated further (Luhder 1998, Ji 1999). These mice have been used widely in diabetes research as a more susceptible mouse model, increasing the speed with which experiments can be carried out and the reproducibility.

Utilising the same set of T cell clones, another TCR transgenic mouse has been created on the NOD background (Pauza 2004). The BDC6.9/NOD mice show increased susceptibility and speed in developing diabetes relative to the BDC2.5/NOD mouse, but only when the BDC6.9 TCR is on the NOD background. The specific antigen is not present in BALB/c mice and so by replacing the NOD antigen locus on chromosome 6, with a segment from a BALB/c mouse, they have been able to prevent diabetes onset (Pauza 2004). The discovery of this kind of genetic specificity of T cell clones hints at the sort of control potentially present throughout the human population, where development of autoimmunity is controlled by expression of endogenous polymorphic autoantigens.

The third TCR transgenic strain created is the NY4.1/NOD mouse (Schmidt 1997). This, like the BDC2.5/NOD strain, recognizes an islet antigen presented by I-A^{g7} and shows similar thymic selection but develops disease earlier and with an increased incidence in transgenic males. Crossing the NY4.1/NOD mice onto a RAG deficient background brought the onset of diabetes earlier as expected (Verdaguer 1997) but incidence did not reach 100%, and plateaued at 75% in females, and 60% in males. The NY4.1/NOD mouse strain was created from a CD4 T cell clone and so provides information about the MHC class II-restricted component of the immune response. NY8.3/NOD mice were created from a CD8 T cell clone, and so work with them complements that seen in other CD8 models (Lieberman 2003). The NY8.3/NOD mice showed similar disease incidence and onset to the NY4.1/NOD mice but a large difference was seen when the endogenous T

cell repertoire was constrained by crossing them with $RAG2^{-/-}$ mice. In this instance, the cross showed a lower incidence of disease, roughly half of that seen by the NY4.1/$RAG^{-/-}$ cross, suggesting a clear role for CD4 T cells in diabetes development as endogenous TCRs were present on the $CD4^+$ T cells in NY8.3/NOD mice but not in the $RAG2^{-/-}$ cross (Lieberman 2003). Therefore the comparison of two TCR transgenic mice allows a more valuable conclusion to be drawn than seen by looking at one mouse strain alone, and also highlights the importance of looking at T cell clones derived from both CD4 and CD8 when designing TCR transgenic mice.

The final TCR transgenic mouse strain used in diabetes research is specific for $GAD65_{286-300}$, a peptide from a candidate disease autoantigen usually expressed in only the brain and islet cells (Tarbell 2002). Despite this limited expression pattern, these mice showed strong central tolerance as transgenic $CD4^+$ T cells were only selected if also possessing an endogenous TCR α chain as well. These mice are different to the other TCR transgenics derived from the NOD strain, as no development of diabetes was observed, in line with some other data that suggested GAD65-specific T cell were not pathogenic (Tian 1996). The anti-p286 TCR likely plays a protective role in diabetes, as when p286-specific T cells were co-transferred with diabetogenic T cells into a NOD.SCID mouse, the onset of diabetes was delayed (Tarbell 2002).

These TCR transgenic mice have provided valuable information regarding the pathogenesis of diabetes, but they represent a limited repertoire of TCRs and their responses. Recently, a retrogenic approach has been used to develop multiple TCR transgenic mice in a much more time-efficient fashion (Burton 2008). They use defined TCRα and TCRβ proteins introduced by a single 2A peptide-linked multicistronic retroviral

vector into mice by retrovirus-mediated stem cell transfer (Holst 2006). In this study, 17 TCR transgenic mice were tested, specific for known autoantigens (GAD65, insulinoma-associated protein 2, IA2β/phogrin and insulin), unknown islet antigens (BDC6.9, BDC10.1, NY4.1, 1A4) and control antigens (generated from lab hybridomas) (Burton 2008). By developing multiple strains simultaneously, on defined genetic backgrounds, they have been able to directly compare TCRs and their relative role in development of diabetes. Using this system, they have attempted to classify TCRs based upon phenotype in the context of disease; those failing to promote infiltration but able to aid later disease progression, those mediating islet entry but not destruction, and those that mediate islet destruction. These insights shed light on the role each TCR plays and more specifically shows that autoreactivity does not imply pathogenicity.

8.4.4.3 Multiple Sclerosis

EAE, the animal model for MS is induced by the introduction of CNS homogenate, protein or peptides in the presence of a proinflammatory adjuvant. By extension, mice expressing human or mouse-derived transgenic TCRs specific for MBP, MOG or PLP peptides have been used to study these populations of autoreactive T cells, and study other factors influencing disease.

8.4.4.3.1 MBP-specific TCR Transgenic Mice

The first TCR transgenic mice in this field expressed a receptor specific for MBP $Ac_{1\text{-}11}$ presented by $I\text{-}A^{u}$ (Goverman 1993). These mice showed normal thymic development

and T cell selection, responded well to MBP and were initially used to study the role of infectious agents in triggering autoimmune diseases (Goverman 1993, Brabb 1997). These mice developed spontaneous EAE more frequently in the presence of pathogens as observed by comparison of housing the mice under SPF (pathogen free) or conventional conditions. In these cases, the male mice developed spontaneous disease more often than the female mice, in contrast to the human condition.

The second TCR transgenic mice were developed to the same MBP epitope but were derived from a different encephalitogenic T cell clone (Lafaille 1994). In these mice, removal of the non-transgenic T cells (presumably encompassing the Treg repertoire) by crossing with $RAG^{-/-}$ or TCR $\alpha^{-/-}\beta^{-/-}$ mice led to spontaneous EAE in all mice (Lafaille 1994, Olivares-Villagomez 1998) and was independent of microbial flora. This was argued to implicate regulatory T cell activity, as the reintroduction of even a small number of polyclonal, syngenic αβ T cells could prevent the spontaneous EAE (Olivares-Villagomez 1998, Van de Keere 1998). The level of spontaneous disease was also linked to homozygous or heterozygous TCR expression, as homozygous mice showed a higher frequency of spontaneous disease than heterozygous mice, attributed also to the constrained TCR repertoire (Olivares-Villagomez 1998). Using these and the earlier MBP-specific TCR transgenic mice, the importance of localised *in vivo* tolerance was highlighted. It was shown that many non-transgenic T cells traffic to the CNS under normal conditions which suggests that there is a steady state of T cells that traffic through the CNS in a healthy animal and must be prevented from being activated, despite the abundance of antigen (Brabb 2000).

Two further TCR transgenic mice were made with the same T cell specificity, but from a new T-cell clone (1934.4). Their methods of creation differed, with one using a human CD2 cassette to express the TCR genes (Tg4) (Liu 1995), and the other using mouse TCR α and β cassettes (Pearson 1997). Tolerance induction was studied and no spontaneous disease was observed. Using these mice, it was determined that clonal deletion could be induced upon the i.p immunization of a peptide with a higher affinity for the TCR than the unmodified peptide (Liu 1995, Pearson 1997), in the same way as had already been seen in the DO11.10 TCR transgenic mice (Murphy 1990). Administration of a peptide with an intermediate affinity was only able to induce appreciable clonal deletion in mice expressing higher (than physiological) levels of TCR (Liu 1995).

The first MBP-specific TCR transgenic mice for a different epitope, $MBP_{121-150}$, were designed and used to elucidate the role of the target protein in thymic selection. In mice expressing MBP, these T cells were deleted, but in mice that were unable to express MBP (shiverer) they were not (Harrington 1998, Targoni 1998). The clonal deletion appeared proportional with age and therefore MBP accumulation, offering a window for the initiation of autoimmune reactions before tolerance is complete (Huseby 2001b). The presence of MBP Ac_{1-11} TCR transgenic cells that are not deleted in earlier mice models despite their ability to expressing MBP appears to be due to the low stability of the Ac_{1-11}/I-A^u complex (Fairchild 1993, Mason 1995, Harrington 1998).

8.4.4.3.2 TCR Transgenic Mice for other Myelin Epitopes

Transgenic mice for other myelin antigens have also been made. TCR transgenic mice were made from both an encephalitogenic and non-encephalitogenic I-A^k restricted

$PLP_{139-151}$ specific T cell clone (Waldner 2000) as well as from a MOG_{35-55} specific T cell clone (Bettelli 2003). Both strains showed similar thymic selection to the Ac_{1-11} specific mice, with TCR specific T cells neither anergic or deleted in the thymus. In the PLP model, as the number of backcrosses was increased onto the susceptible SJL background, the incidence of spontaneous EAE increased. Interestingly this was the case for TCRs derived both from encephalitogenic and non-encephalitogenic T cell clones (Waldner 2000). The MOG specific mice, called 2D2, could be induced to develop EAE with adjuvant/antigen and pertussis toxin but most interestingly, despite a small number developing spontaneous EAE, there was a much higher incidence of optic neuritis (35%) without EAE, and if the pertussis toxin was removed from the immunization protocol, only optic neuritis was observed at the expense of EAE (Bettelli 2003). This study raises the possibility that TCR transgenics may offer potential for establishing causal relationships between peptide specificity and lesion localisation.

The attempt to model relapsing-remitting MS in a TCR transgenic mouse would draw together both the specificity of the TCR transgenic and the complexity of repeated immunological insults. The SJL/J mouse strain is prone to an EAE course with a relapsing-remitting disease course and using a MOG_{92-106} specific TCR, restricted to $I\text{-}A^{s}$, backcrossed onto the SJL/J strain leads to mice developing a spontaneous RR-EAE (Pollinger 2009). The attacks alter between different CNS tissues and most interestingly, there was no EAE seen in mice depleted of B cells or when crossed to $MOG^{-/-}$ mice. Transgenic T cells expand MOG autoreactive B cells and they produce autoantibodies that recognize a different epitope than the T cells themselves (Pollinger 2009).

8.4.4.3.3 Humanized TCR Transgenic Lines

The limitation of the models presented so far is the reliance on endogenous mouse MHC mediated antigen presentation and the inability to map *in vivo* correlates of TCRs defined from human patient's clones. The logical extension of transgenic mouse models is to include a human element on the other side of the T cell interaction so immune responses will likely be more relevant to the human condition. Humanized transgenic mice were made incorporating a human MBP_{84-102} specific TCR which would recognize peptide presented by HLA-DR15 (Madsen 1999). This system uses the conserved sequences between the human and mouse MBP epitopes such that these T cells will react with the same competence to both the mouse and human peptide. These mice were also bred to express HLA class II DRA*01:01/DRB1*15:01 under the control of the mouse I-E^{α} promoter and the human CD4 molecule under the control of a mouse CD4 enhancer. As with earlier TCR transgenic models, a very low level of spontaneous disease was observed (4%) but crossing onto $RAG^{-/-}$ increased this incidence to 100% (Madsen 1999). The frequency of antigen-specific T cells in this model however appeared to be lower than in any other TCR transgenic model and fewer CD4 cells reacted to MBP peptide. The thymic architecture was also unaffected, a feature not usually seen in mice with a high frequency of transgenic TCR specific cells that are positively selected (Goverman 1997, Pearson 1997). Therefore it is likely that in these mice, the TCR repertoire diversity is similar to normal.

Mice transgenic for the immunodominant $MBP_{111-129}$ epitope in DRB1*0401 individuals were created from a T cell clone from a relapsing-remitting MS patient. DR4 is most commonly found associated with the chronic progressive form of MS and in certain

ethnic groups, most notably from the Mediterranean area (Marrosu 1988). No spontaneous disease was observed in these humanized TCR transgenic mice, but adoptive transfer of TCR transgenic cells into DR4 mice followed by pertussis toxin administration, led to the development of normal EAE and dysphagia (Quandt 2004). Mouse $MBP_{111-129}$ differs from the human MBP epitope by a conservative Arg to Lys substitution in position 122. Not all the T cell clones derived in the development of the mice responded to mouse MBP but the clone selected (MS2-3C8) showed equal responsiveness and a clone not responding was used as a control (Quandt 2004).

The role of B cells in a condition thought to be T cell mediated can be hard to elucidate because of the adjuvants required for initiation already skewing T cell responses. Two groups have crossed their TCR transgenic mouse strains with an IgH^{MOG} strain, originally created by replacing the germline JH locus with a rearranged immunoglobin heavy chain gene derived from a pathogenic monoclonal antibody specific for MOG (Litzenburger 1998). Crossing the 2D2 TCR transgenic mice (Bettelli 2003) led to EAE with optic neuritis and spinal cord lesions. This is also seen in the MOG TCR mouse but the disease is subclinical (Bettelli 2006a, Krishnamoorthy 2006) whilst crossing the TCR_{1640} TCR transgenic mice showed a similar presentation (Pollinger 2009). Lesions were observed in the optic nerve and spinal cord and were reminiscent of those seen in the human condition and it may be that the B cells act to not only secrete autoantibodies but concentrate the low levels of endogenous antigen and present it to the T cells. These studies offer an interesting insight into the potential interplay between B and T cells in mediating the complexity of the human condition and all the variants observed.

As with the TCR transgenic models described for both rheumatoid arthritis and diabetes, the same models have been utilized many times to interrogate different aspects of the human condition. The 2D2 and Tg4 transgenics have been the most widely used and most of the work done is presented in an earlier section of this introduction. They have not only been used to model disease dynamics themselves but their main use has been as a source of TCR restricted T cell populations (O'Connor 2008, Bauer 2009, Zozulya 2009). These mice lines have been used as 'mice prone for autoimmunity' and crossed onto other knockout or knock-in strains including TSP-1_{null} (Yang 2009a), β1-integrin$^{-/-}$ (Bauer 2009), and CD11^{dnR} (Laouar 2008) for the 2D2 mice and CTLA-$4^{-/-}$ (Verhagen 2009) for the Tg4 mice. The finding from these investigations have increased our understanding of MS pathology but it is always worth remembering that with this widespread use of a few models, all findings should be interpreted with the greatest care.

8.4.4.3.4 Line7

The Line7 model of MS was one of several TCR transgenic lines generated in the Altmann lab (in collaboration with Vijay Kuchroo) from the Ob1A12 patient TCR. The Line8 mouse (Ellmerich 2004) was created in the same series of microinjections as the Line7 strain. All of the TCR transgene-positive founder strains were crossed such as to have a null mutation for H2-Aβ (Cosgrove 1991), and introduction of human DR15:01 by a full-length DRB1*15:01 cosmid containing extensive 5' and 3' flanking sequences. The TCR recognizes MBP_{85-99} derived from a human MS patient derived clone, Ob1A12. The TCR contains Vα3.1Jα40 and Vβ2.1Jβ2.1 segments, subcloned into pTαCass and pTβCass

(Kouskoff 1995). The Line8 mice had around 44% of splenic $CD4^+$ T cells expressing the TCR and so presented spontaneous disease in only a small percentage of mice but showed a lack of regular movement as compared to wild type mice. There was no obvious CNS pathology observed with this impairment but immunization with either CFA/antigen or pertussis toxin alone was sufficient to initiate a mild disease. After immunization with CFA/antigen and pertussis toxin all mice developed chronic paralysis with no remission, as in regular EAE, and this disease development correlated with a spread of reactivity of the T cells to other DR15 restricted myelin antigens (Ellmerich 2004).

The Line7 TCR transgenic strain however had a much higher percentage of TCR^+ CD4 (97%) and CD8 (79%) T cells and developed a much higher proportion of spontaneous disease (approx 60%), which rose to 100% if crossed onto a $RAG^{-/-}$ background. Mice developed paralysis after approximately 4 or 5 months of age and inflammation was associated with myelin loss in the cerebellum and spinal cord. Widespread axonal degeneration was seen in mice with chronic, overt paralysis associated with dense areas of lymphocytic infiltration (Ellmerich 2005). In these mice there was also evidence of epitope spread, with responses observed to DR15 restricted epitopes MBP_{38-59}, MOG_{82-96} and αB-$crystallin_{161-175}$ (Ellmerich 2005) emphasising the relevance of this model and its advantages over regular EAE as a more faithful representation of the human condition and ability to study the development of CNS inflammation without the regular biases of the immune adjuvants required in other protocols.

8.5 Aims of the Thesis

The main aim of this thesis is to clarify the relative roles of distinct immune cell subtypes, namely Th1, Th17 and Treg cells, in the pathogenesis of multiple sclerosis. A TCR transgenic mouse model, named the Line7, will be used to further our understanding of the initiation of autoimmunity. The Line7 model has many advantages over other commonly used mouse models in that disease develops spontaneously without the requirement of immune adjuvants. These bring their own biases into the model, and because the Line7 mouse is humanized, all immune responses are directly relevant to the human setting. This reduction in the distance between the mouse model and human disease should allow more accurate prediction of a drug's therapeutic potential saving money and increasing the speed at which it may get into clinical use (Illes 2004).

There is a lot of controversy about the importance of various candidate effector and regulatory cell types in MS pathogenesis, and many immune cell types are implicated in some way. The situation is clearly very complicated and this is further confused by the lack of accessible tissue from human MS patients. It goes without saying that the CNS is unobtainable and the nearest we can get is CSF samples, but these samples do not reflect the site at which disease originates. Mouse models therefore allow target tissue and encephalitogenic cells to be interrogated to determine the cause of disease. EAE however is only partially useful in this aim. The adjuvants used promote Th17 polarization of T cells and so in my view it is not surprising that a large Th17 response is observed. Significant care should be taken when interpreting EAE conclusions regarding mechanisms as it shows more similarities to an acute infection than a chronic autoimmune condition, often resolving naturally in a short period of time. The Line7

model is much more faithful to a chronic autoimmune disease and allows assessment of disease throughout its initiation and progression. This should allow a more accurate determination of the cells involved in the initial development of disease and its subsequent progression. Through careful analysis of this model, my aim is to investigate, in a spontaneous setting, the relative contributions of Th1 and Th17 cells to the initiation of disease.

Without adjuvants dampening regulatory cell function, disease does not develop in other TCR transgenic lines but it is apparent in the Line7 strain. Not all of the mice develop paralysis (60%) and so there must be a determining factor that triggers autoimmunity. In the same way as identical twins show a low concordance of MS in the human condition, the benefit of using these mice is that they are effectively all identical twins. This line provides a unique opportunity to investigate early stages of disease which is impossible in induced studies as the causes of disease development are the substances immunized.

The questions I will address in this thesis are:

- Is there evidence for differential roles of Th1 and Th17 cells in disease initiation and progression?
- Does the Line7 model support or oppose the view that Th17 cells are the most important T cell subset in the development of multiple sclerosis?
- Does disease in the L7 mice progress due to aberrant regulation by Tregs or in the face of competent regulatory function?
- Is there any support for a role of TLR ligation in triggering L7 and MS disease initiation?

Overall, I aim to use the Line7 to reappraise some immunological issues that may have been unduly influenced in the EAE model by dependence on CFA and pertussis toxin. I will contribute my view to the debate regarding Th1 and Th17 cells in MS, and the roles of other effector cells. I will look at potential factors triggering autoimmunity and assess the factors determining how a mouse is able to resist severe disease, putting great emphasis on the role of regulatory T cells in limiting both the initiation of disease but also its progression once started.

9 Materials and Methods

9.1 Non-commercial reagents

1X Tris/Borate/EDTA (TBE) Buffer

10.8g Tris Base (Sigma Aldrich, Poole, UK)

5.5g Boric Acid (Sigma Aldrich, Poole, UK)

0.38g ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Poole, UK)

Up to 1000ml deionised water.

2% Agarose gel

9g Molecular Grade Agarose (Sigma Aldrich, Poole, UK)

Up to 450ml 1X TBE Buffer

Enzyme Linked Immunosorbent Assay (ELISA) wash solution

0.05% Tween 20/Phosphate buffered saline (PBS) pH7.4 (Sigma Aldrich, Poole, UK)

ELISA blocking solution

1% Bovine serum albumin (BSA)/PBS (Sigma Aldrich, Poole, UK)

Complete Dulbecco's Modified Eagle Medium (DMEM)

DMEM (Gibco, Invitrogen, Paisley, UK)

10% Foetal Calf Serum (Biosera, Ringmer, UK)

1% Penicillin/streptomycin (Invitrogen, Paisley, UK)

50µM 2-mercaptoethanol (Gibco, Invitrogen, Paisley, UK)

PBS-T

0.1% Triton/PBS (Sigma Aldrich, Poole, UK)

Tissue lysis buffer

100mM NaCl (Sigma Aldrich, Poole, UK)

10mM Tris-Cl, pH 8 (Sigma Aldrich, Poole, UK)

25mM EDTA, pH 8 (BDH, VWR, Letterworth, UK)

0.5% Sodium dodecyl sulphate (SDS) (BDH, VWR, Letterworth, UK)

FACS buffer

3% FCS/PBS (Biosera, Ringmer, UK)

10mM Sodium Citrate buffer

100mM Citric Acid (Sigma Aldrich, Poole, UK)

100mM Sodium Citrate (Sigma Aldrich, Poole, UK)

Up to 1000ml deionised water

9.2 Animals used in this study

Mice were housed in the Central Biomedical Services centre at the Hammersmith Hospital London, a barrier maintained mouse facility. Mice were kept initially in open cages but subsequently moved into individually ventilated cages (IVCs) as the animal facility was refurbished. The mouse pathogen status was regularly monitored every 3-4 months and screens returned negative for all pathogens with the exception of

Helicobacter hepaticus, *Helicobacter rodentium* and *Tritrichomonas sp* in the original open caged facility. Complete diet was administered to all strains. All studies involving mice underwent ethical review and were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

9.2.1 Line7

Line7 mice were previously generated in the laboratory by Stephan Ellmerich prior to use in this study. Their derivation is described elsewhere (Ellmerich 2004, Ellmerich 2005) but in brief, (C57BL/6 x CBA)F2 oocytes were microinjected with cosmids containing linearised HLA-DRA1*01:01 (cosmid 4.2) (Altmann 1993, Altmann 1995) and HLA-DRB1*15:01 alongside 5' and 3' flanking regulatory sequences (clone 11) (Lock 1988) and one founder (24) from this generation was mated for more than 6 generations with C57BL/6 $A\beta^0$ mice (Cosgrove 1991) to yield DR15 $A\beta^0$ mice. Segments from rearranged Vα3.1Jα40 and Vβ2.1Jβ2.1, derived from the Ob1A12 T cell clone (Ausubel 1997), were amplified by PCR and subcloned into genomic TCR expression cassettes, pTαCass and pTβCass (Kouskoff 1995, van Noort 1995). These cassettes, containing mouse TCR specific regulatory elements, were micro-injected into (C57BL/6 x CBA)F2 fertilised oocytes to generate a number of TCR-positive founders. One of these, termed Line7, was subsequently mated with C57BL/6 DR15 $A\beta^0$ mice for 5 generations to produce the Line7 mice on a C57BL/6 background as used in this study.

At each stage in their derivation, throughout the life of the colony and through this study, genotype was confirmed by PCR of DNA extracted from a tail biopsy using primers pairs to determine the presence of each transgenic element (Table 3) and the respective PCR

protocol. Mice were used in this study across the range of age, according to their clinical score.

9.2.2 Foxp3^{DTR}

Foxp3^{DTR} mice were obtained from Alexander Rudensky, University of Washington. They are C57BL/6 mice that contain the human diphtheria toxin receptor fused to green fluorescent protein DNA, equipped with an internal ribosomal entry site into the 3' untranslated region of the Foxp3 locus. This allows selective depletion of Foxp3^{+} T cells through the addition of diphtheria toxin (Kim 2007). Mice were genotyped by PCR of DNA extracted from a tail biospsy using primer pairs to detect the presence or absence of the introduced DTR-GFP DNA within the Foxp3 locus (Table 3). Mice used in this study were 6-8 weeks of age.

9.2.3 L7Fox

L7Fox mice were created by the mating of Line7 male mice with Foxp3^{DTR} female mice. All offspring were genotyped following the same protocol as Line7 and Foxp3^{DTR} mice (Table 3) and subsequent mating pairs set up according to genotyping results. The mice were bred through 3 generations before use to ensure the correct transgenic combination and genotype confirmed before any experimental procedures carried out. Mice used in this study were 6-8 weeks of age.

9.2.4 C57BL/6

C57BL/6 mice were obtained from (Harlan, Bicester, UK or Charles River, Margate, UK) and housed under the same conditions as other strains for 8 weeks before use.

9.3 Assessment of disease in L7

Line7 mice were scored according to guidelines detailed in Professor Altmann's Home Office Project Licence. Mice were checked weekly from birth and then scored at least twice weekly from first signs of paralysis. Upon reaching score 3, mice were initially weighed and then scored and weighed every other day and upon reaching score 4, mice were weighed and scored twice daily. Weights were recorded to ensure mice were still able to eat, despite physical impairment. The following disease landmarks were used to assign scores:

0 - normal

1 - limp tail

2 - impaired righting reflex or waddling gait

3 - partial hind limb paralysis

4 - total hind limb paralysis

5 - total limb paralysis

6 - moribund

9.4 Nucleic Acid Protocols

9.4.1 gDNA extraction

To extract genomic DNA (gDNA) for analysis of mouse genotype, a small piece of mouse tail was removed and digested in Tissue lysis buffer with proteinase K (0.25mg/ml) (Sigma Aldrich, Poole, UK) at 56^0C overnight. 200µl chloroform was added for phase

separation of the DNA by centrifugation at 12000g for 15min. DNA was precipitated upon addition of the supernatant to 600µl isopropanol and gentle agitation followed by centrifugation at 9000g for 10min. Extracted DNA was washed with 70% ethanol and the pellet air-dried to remove any residual ethanol before being resuspended in RNase free molecular grade water and quantified by picodrop analysis.

9.4.2 RNA extraction

RNA was extracted from mouse tissue using the Trizol Plus RNA Extraction kit supplied by Invitrogen (Paisley, UK) and which is based on the acid phenol method. In brief, mouse tissue was thoroughly homogenised in 1ml Trizol, 0.2ml chloroform added and the mixture vigorously mixed. After a brief incubation, the sample was spun at 12000g for 15 min at 4^0C and the top, clear aqueous layer aspirated. An equal volume of 70% ethanol was added, vortexed to mix, and the solution run through the supplied PureLink spin columns. Subsequent washing steps followed using the wash buffers supplied before elution of the RNA into 50µl RNase free H_2O. The RNA concentration was determined by use of a nanodrop spectrophotometer before further use and stored at -80^0C.

9.5 Primer design

Primers for real-time PCR analysis were designed using Primer3 software (Rozen 2000). Reference sequences (Table 5) were used and primer pairs with hydrolysis probes selected based on the following criteria:

- 20-27bp in length
- 30-70% GC content
- Primer melting temperature 60^{o}C ±3^{o}C
- Hydrolysis probe melting temperature 70^{o}C ±3^{o}C
- Hydrolysis probe dual fluorescence label (5'-FAM 3'-TAMRA)

Primer and probe secondary structure was analysed using NetPrimer software (Premier Biosoft International) to ensure minimal primer dimers and hairpin loops. All oligo peptides were ordered from SigmaGenosys and were resuspended in RNase free molecular grade water.

9.6 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to both establish the genotype of mice and to test primer pairs which would be used for real-time PCR. General reaction mixtures are in Table 1 and Table 2, primers used and cycling conditions are in Table 3.

Reaction mix for genotyping:

Reagent	Final Concentration	Volume per reaction
NH_4 Buffer (10X)	1X	2.5µl
$MgCl_2$ (15mM)	3mM	5µl
dNTPs (1mM each)	0.2mM each	5µl
Forward Primer (10µM)	0.5µM	1.25µl
Reverse Primer (10µM)	0.5µM	1.25µl
BioTaq DNA polymerase (5u/µl)	1unit	0.2µl
gDNA (40ng/µl)	100ng	2.5µl
H_2O	-	7.3µl
Total	-	25µl

Table 1. Genotyping PCR Mix.

Reaction mix for primer testing and general PCR:

Reagent	Final Concentration	Volume per reaction
NH_4 Buffer (10X)	1X	2µl
$MgCl_2$ (50mM)	5mM	2µl
dNTPs (1mM each)	0.2mM each	4µl
Forward Primer (10µM)	0.5µM	1µl
Reverse Primer (10µM)	0.5µM	1µl
BioTaq DNA polymerase (5u/µl)	1unit	0.2µl
cDNA (50ng/µl)	50ng	1µl
H_2O	-	8.8µl
Total	-	20µl

Table 2. Regular PCR Mix.

9.6.1 Primer Pairs and cycling conditions:

Genotyping:

Primer Targets	Primer Direction	Primer Sequence (5' – 3')	Dissociation (temp/s)	Annealing (temp/s)	Extension (temp/s)	Cycles	Product Length
DRα	Sense	CTCCAAGCCCTCTCCCAGAG	94°C/60	55°C/60	72°C/60	32	150
	Antisense	ATGTGCCTTACAGAGGCCCC					
DRβ	Sense	TTCAATGGGACGGAGCGGGTG	94°C/60	55°C/60	72°C/60	32	200
	Antisense	CTGCACTGTGAAGCTCTC					
TCRα	Sense	ATTCAGGTAGAGGCCTTGTCCA	94°C/45	55°C/30	72°C/120	30	200
	Antisense	GAGGTAGTATCCGTAGCACAG					
TCRβ	Sense	GATGGCAACTTCCAATGAGGGCT	94°C/45	60°C/30	72°C/120	30	200
	Antisense	CGCCCCGCTAGTTAGATCTC					
MHC WT	Sense	TCCGCAGGGCATTTCGTGTA	94°C/45	60°C/45	72°C/60	30	200
	Antisense	AGGGAGGTGTGGGTCTCCGG					
MHC KO3	Sense	TCCGCAGGGCATTTCGTGTA	94°C/45	60°C/45	72°C/60	30	500
	Antisense	GAGGATCTCGTCGTGACCCA					
Foxp3 WT	Sense	TGGACCGTAGATGAATTTGAGTT	94°C/40	53°C/40	72°C/30	35	550
	Antisense	CCAGATGTTGTGGGTGAGTG					
Foxp3 DTR	Sense	GGGACCATGAAGCTGCTCCG	94°C/15	59°C/15	72°C/45	35	600
	Antisense	TCAGTGGGAATTAAGTCATGCC					

Table 3. Primer pairs for genotyping of transgenic mice.

9.6.2 Testing primer pairs and general PCR:

50^0C for 2min then 95^0C 10min followed by 40 cycles of 95^0C 30s, varied annealing temperature 30s, 72^0C 30s. The annealing temperature was run as a gradient to test primers but varied according to the specific primer pairs. For all primer pairs in the real-time PCR analysis, the annealing temperature was 60°C.

9.7 Reverse transcription

Reverse transcription of extracted RNA was carried out using the Quantitect Reverse Transcription kit from Qiagen (Crawley, UK). For 1µg RNA, 2µl gDNA wipeout buffer was added to 12µl of H_2O containing 1µg RNA and incubated at 42^0C for 2min. Then 1µl RT primer mix, 1µl RT enzyme and 4µl RT buffer were added and incubated at 42^0C for 15min and 3min at 95^0C. cDNA was stored at -20^0C until use.

9.8 Real-time PCR

Real-time PCR was used to assess relative expression of target genes. Reaction mixture was as in Table 4, primer details are in Table 5.

Reagent	Final Concentration	Volume per reaction
TaqMan Gene Expression Mastermix (2X)	1X	10µl
Forward Primer (10µM)	0.5µM	1µl
Reverse Primer (10µM)	0.5µM	1µl
Hydrolysis Probe (1µM)	0.1µM	2µl
cDNA (50ng/µl)	12.5ng	0.25µl
H_2O	-	5.75µl
Total	-	20µl

Table 4. Real-time PCR Mix.

Samples were run on a Stratagene Mx3000p machine following these cycling conditions: 50^0C for 2min, 95^0C 10min, and 50 cycles of 95^0C for 15s, 60^0C for 15s, 72^0C for 15s.

Gene	Forward Primer	Reverse Primer	Probe (all 5′ FAM – TAMRA 3′)	Product Size	Ref Seq
Innate Immune Markers					
TLR2	CAGACAAAGCGTCAAATCTCAG	CGTTTGCTGAAGAGGACTGTTAT	TTTGGCTCTTCTGGATCTTGGTGGC	87	NM_011905.3
TLR3	CCTCCAAGAACTGCTCTTAGCAA	GGAGAACTCTTTAAGTGGATTTGA	TCCTTGCGTTGCGAAGTGAAGAACT	124	NM_126166.4
TLR4	AATCCCTGCATAGAGGTAGTTCCTA	AAGCTCAGATCTATGTTCTTGGTTG	CCAATGCATGGATCAGAAACTCAGCA	110	NM_021297.2
TLR9	AGTGTCACTTCCTCAATTCTCTGA	ATGTTGGGAGATGGAGGATTC	ATCATTCTCTGCCGCCCAGTTTGTC	104	NM_031178.2
Transcription Factors					
T-bet	ACTTTGAGTCCATGTACGCATCT	AGGATACTGGTTGGATAGAAGAGGT	CACCTGGACCCAACTGTCAACTGCTT	113	NM_019507.1
GATA3	TACCACCTATCCGCCCTATGT	ACACACTCCCTGCCTTCTGT	ACAGCTCTGGACTCTTCCCACCCAG	138	NM_008091.3
RORγt	GTCTGCAAGTCCTTCCGAGAG	ATCTCCCACATTGACTTCTG	CTGCGACTGGAGGACCTTCTACGGC	119	NM_011281.2
Foxp3	ATAGTTCCTTCCCAGAGTTCTTCC	ATGGTAGATTTCATTGAGTGTCCTC	CACCTATGCCACCCTTATCCGATGG	143	NM_054039.1
Cell Lineage Factors					
AHR	CACATCCGCATGATTAAGACTG	GATGTAATCTGGTCTTCCATTTCTG	AAACACAGTCGCTGGAGGTGGGTC	129	NM_013464.4
Cytokines					
IL-1β	ATCAACCAACAAGTGATATTCTCCA	CGTCTTTCATTACACAGGACAGGT	ACAAGGAGAACCAAGCAACGACAAA	121	NM_008361.2
IL-6	GTTCCTCTCTGCAAGAGACTTCC	GTATCCTCTGTGAAGTCTCCTCTCC	CTTGGGACTGATGCTGGTGACAACC	111	NM_031168.1
IL-10	TTAATGCAGGACTTTAAGGGTTACTT	ATTCAAATGCTCCTTGATTTCTG	CCAAGCCTTATCGGAAATGATCCAGTT	123	NM_010548.1
IL-17a	CTGTGTCTCTGATGCTGTTGCT	AAGGGAGTTAAAGACTTTGAGGTTG	AGCTCAGCGTGTCCAAACACTGAGG	149	NM_010552.3
p19	ATGAATCTACTAAGAGAAGAAGAGG ATG	CTGTTGTCCTTGAGTCCTTGTG	CCGTATCCAGTGTGAAGATGGTTGTGA	102	NM_031252.2
p35	AGAGAGACTTCTTCCACAACAAGAG	TCTTCAAGTCCTCATAGATGCTACC	CGTCTTTGATGATGACCCTGTGCCT	100	NM_008351.1
IFNγ	TCATTGAATCACACCTGATTACTACC	TTGACCTCAAACTTGGCAATAC	CTTCTTCAGCAACAGCAAGGCGAA	88	NM_008337.3
iNOS	CTCAGCCCAACAATACAAGATG	GGGATTCTGGAACATTCTGTG	TAAGAGTCACCAAAATGGCTCCCCG	90	NM_010927.3
TGFβ	ATGTTCTTCAATACGTCAGACATTC	TTGCTATATTTCTGGTAGAGTTCCA	TGCAGAGCTGCGCTTGCAGAGATTAA	131	NM_011577.1
TNFα	CTCACACTCAGATCATCTTCTCAAA	CTCCACTTGGTGGTTTGCTA	ACAAGCCTGTAGCCCACGTCGTAGC	75	NM_013693.2
Housekeeping Genes					
GAPDH	GAGAAACCTGCCAAGTATGATGAC	AGACAACCTGGTCCTCAGTGTAG	TCAAGAAGGTGGTGAAGCAGGCATC	100	NM_008084.2
Hprt1	AACCAGGTTATGACCTAGATTTGTTT	CAAGTCTTTCAGTCCTGTCCATAAT	CCTAATCATTATGCCGAGGATTTGGAA	108	NM_013556.2
Tbp	CAGTGCCCAGCATCACTATTT	GCATCCTCTGAATATCTCCTTAGAA	CATGGTGTGTGAAGATAACCCAGAACA	83	NM_013684.3
B2m	CTACTGGGATCGAGACATGTGAT	TGTGTACATTGCTATTTCTTTCTGC	TGCTCTGAAGATTCATTTGAACCTGCT	130	NM_009735.3
Rpl-13a	CCTACCAGAAAGTTTGCTTACCTG	GATCTGCTTCTTCTTCCGATAGTG	TGACAGCCACTCTGGAGGAGAAACG	132	NM_009438.4
YWHAZ	AACTTAACATTGTGGACATCGGATA	TGGATGACAAATGGTCTACTGTGTA	TGAAGCAGAAGCAGGAGAAGGAGGG	131	NM_011740.2
TfrC	AATGGTAACTTAGACCCAGTGGAG	ATTAGCATGGACCAGTTTACCAGA	TCCCGAGGGTTATGTGGCATTCAGT	90	NM_011638.3
Sdha	CTGGTGTGGATGTCACTAAGGAG	CACCTGTCCCTTGTAGTTAGTGG	CACTGTGCATTACAACATGGGTGGG	92	NM_023281.1

Table 5. Primer pairs and hydrolysis probes for real-time PCR.

9.9 Real-time analysis

Analysis of real-time PCR results was carried out using REST software (M. Pfaffl (Technical University Munich)) which derives relative expression values based upon Ct values from a comparison of housekeeping and target genes and their respective efficiencies of amplification.

9.10 Infiltrating cell analysis

9.10.1 Infiltrating cell extraction

To extract infiltrating cells from the CNS, mice were first perfused with PBS to remove blood and avoid any contamination with peripheral blood cells. During terminal anaesthesia, the sternum of the mouse was opened and ribcage lifted to expose the heart, a 25g needle inserted into the left ventricle and PBS slowly injected in. The right atrium was cut to release blood and the flow of PBS continued until no blood could be seen leaving the heart and the liver had lost its colour. After perfusion, the CNS was dissected out and chopped up in 1ml complete DMEM media containing 25µl collagenase (100mg/ml stock) and 10µl DNase I (100mg/ml stock) (Sigma Aldrich, Poole, UK), further disrupted by movement through a 1ml syringe and the homogenate incubated for 2h at 37^{o}C. The homogenate was then passed through a 70µm cell strainer (BD Falcon) washed with complete DMEM to remove residual enzymes. The pellet was resuspended in 4ml 30% Percoll (Sigma Aldrich, Poole, UK) made up with complete DMEM and overlayed on 4ml 70% percoll solution made up with PBS and 3ml HBSS (Gibco, Invitrogen, Paisley, UK) further overlayed on top. This was centrifuged at 900g for 30min

with no brake and reduced acceleration. The floating material between the top two layers was removed and discarded and the desired cells extracted from the interface of the 30 and 70% solutions, washed with complete DMEM and counted before further use.

9.10.2 ICCS staining

Intracellular cytokine staining was carried out on stimulated cells using the BD cytofix/cytoperm solution kit (BD Biosciences, Oxford, UK). Up to 1 x 10^6 cells extracted from the spleen or CNS were stimulated with PMA (50ng/ml) (Sigma Aldrich, Poole, UK) and ionomycin (1µg/ml) (Sigma Aldrich, Poole, UK) for 4h in the presence of Brefeldin A (Sigma Aldrich, Poole, UK) at 50µg/ml in complete DMEM media in a 96well v-bottom plate. At the end of culture, cells were centrifuged at 600g (g equivalent) for 5min and washed three times with cold PBS. Cells were incubated for 10min at 4^{o}C with 100µl Fc Block (eBioscience, Hatfield, UK) (1/1000) in FACS buffer before being incubated with surface staining antibodies (Table 7) 100µl (1/200) in FACS buffer for 20min at 4^{o}C in the dark. Stained cells were washed once with cold PBS and then resuspended thoroughly in 200µl BD cytofix/cytoperm solution and incubated for 30min at 4^{o}C in the dark. At the end of the incubation, cells were washed with 200µl BD permeabilisation buffer and incubated with intracellular antibodies (see Table 7) 100µl (1/100) in BD permeabilisation buffer for 30min at 4^{o}C in the dark. Two subsequent washes with BD perm buffer follow and the cells are finally resuspended in 4% paraformaldehyde (PFA) and kept at 4^{o}C before acquisition.

9.11 Flow cytometry analysis

Flow cytometric analysis of samples was carried out using a BD FACS Aria II running FACSDiva software. Further analysis was carried out using FlowJo software (Tree Star Inc, Ashland, USA).

9.11.1 FACS phenotyping

Some genotyping analysis was carried out by flow cytometric investigation of peripheral blood rather than PCR based assays on genomic DNA. Staining was carried out on 1 x 10^6 cells in which the red blood cells had been lysed, washed twice with PBS/10% FCS and then incubated with the relevant antibodies (see Table 6) for 30min at 4^{o}C, washed twice more and then resuspended in 4% PFA before reading.

9.11.2 Antibody list genotyping and intracellular cytokine staining (ICCS)

Antibody	Company	Fluoro-chrome	Reactivity	Clone	Dilution
B220	BD Biosciences	FITC	Mouse	RA3-6B2	1/50
B220	BD Biosciences	PE	Mouse	RA3-6B2	1/50
I-A/I-E	BD Biosciences	PE	Mouse	M5/114.15.2	1/100
Pan-DRDQDP	BD Biosciences	FITC	Human	TU39	1/50
Vβ2	Serotec	PE	Human	MPB2D5	1/20
CD31	eBioscience	PE	Mouse	390	1/50

Table 6. Antibodies used in genotyping of transgenic mice.

Antibody	Company	Fluorochrome	Reactivity	Clone
CD4	BD Biosciences	V500	Mouse	RM4-5
CD8a	BD Biosciences	V450	Mouse	53-6.7
IL-10	BD Biosciences	PE	Mouse	JES5-16E3
IL-17a	BD Biosciences	Alexa647	Mouse	TC11-18H10
Foxp3	BD Biosciences	Alexa488	Mouse	MF23
NK1.1	BD Biosciences	PE-Cy7	Mouse	PK136
IFNγ	BD Biosciences	Alexa700	Mouse	XMG1.2

Table 7. Antibodies used for surface marker and intracellular cytokine flow cytometry analysis.

Antibody	Company	Fluorochrome	Reactivity	Clone
CD3e	BD Biosciences	V500	Mouse	500A2
NKp46	eBioscience	eFluor450	Mouse	29A1.4
CD49b	eBioscience	FITC	Mouse	DX5
CD8a	BD Biosciences	V450	Mouse	53-6.7

Table 8. Antibodies used for cell repletion analysis.

9.12 Immunocytochemistry

9.12.1 Tissue preparation for histology

For histological analysis of tissue, mice were first fix/perfused with PBS followed by 4% PFA and the entire CNS (brain and length of spinal cord) tissue dissected out. The tissue was submerged in 4% PFA for 4h and then submerged in 30% sucrose at 4^{o}C until the tissue sank in the solution. At this point, the tissue was dried and cut to separate the cortex, cerebellum and spinal cord. Each was mounted in OCT (VWR, Letterworth, UK) individually and snap frozen in cooled isopentane on dry ice and stored at -20^{o}C. 10µm sections of tissue were cut from these blocks on a Leica cm1850 cryostat and mounted onto clear microscope slides (VWR, Letterworth, UK) for further staining analysis.

9.12.2 Haematoxylin and Eosin staining

Haemtoxylin and Eosin (H&E) staining was carried out using the following protocol. The sections were thawed and allowed to air dry for 10min before being rehydrated in PBS for 5min and incubated in 1% Mayer's Haemalum (BDH, VWR, Letterworth, UK) for 30s. The haemalum was differentiated in 3% acid alcohol solution for 10s and washed in running tap water for 30s. Haemalum staining was checked microscopically and if necessary, the steps repeated. Sections were then incubated in (1%) eosin (BDH, VWR, Letterworth, UK) for 5min, washed briefly with running tap water and dehydrated by taking the slides through sequential solutions of 70% ethanol, 100% ethanol, and xylene (1 min in each solution). Slides were left for 5min to clear in xylene and mounted with DPX (Sigma Aldrich, Poole, UK), a coverslip applied and allowed to dry.

9.12.3 Fluorescence staining

For fluorescence staining, slides were thawed and allowed to air dry for 10min and the sections drawn around using a PAP-pen (VWR, Letterworth, UK) to allow smaller volumes of washing buffers and antibody solutions to be used and rehydrated with PBS. For some antibodies antigen retrieval was required and two different protocols were followed. The first was incubation of sections with methanol $^{+}$ 3% H_2O_2 for 5min followed by three washes with PBS-T and the second had the additional step of immersing the sections in citrate buffer, bringing the solution to the boil and simmering for 3min before allowing to cool for 10min and subsequent washing with PBS-T.

After optional antigen retrieval, sections were incubated with relevant primary antibody mixes (see Table 9) in PBS-T containing 3% Normal Goat Serum (Sigma Aldrich, Poole, UK) overnight at room temperature. The following morning, sections were washed 3 times with PBS-T and incubated with secondary antibodies (see Table 10) (1/500) in PBS-T for 2h at room temperature. Sections were then washed again 3 times with PBS-T and incubated with DAPI (Sigma Aldrich, Poole, UK) (1/5000) in PBS-T for 5min, washed 3 further times and mounted with fluorescence mounting medium (Vectorlabs, Peterborough, UK). The coverslips were sealed with nail varnish to prevent the sections drying out and stored at 4^{o}C in the dark.

Antibody	Company	Host species	Reactivity	Clone
CD4	Serotec	Monoclonal Rat	Mouse	RM4-5
CD8	Epitomics	Monoclonal Rabbit	Mouse, Human, Rat	EP1150Y
NK1.1	eBioscience	Monoclonal Mouse	Mouse	PK136

Table 9. Primary antibodies used in immunofluroescence.

Fluorochrome	Company	Host Species	Reactivity
Alexa488	Invitrogen	Goat	Rabbit
Alexa568	Invitrogen	Goat	Rat
Alexa546	Invitrogen	Goat	Mouse
Alexa546	Invitrogen	Goat	Rabbit

Table 10. Secondary antibodies used for immunofluorescence.

9.12.4 Photomicroscopy

H&E images were captured using Oasis scanning software (Microimaging Applications Group, Buckinghamshire, UK) which was connected to a Prior motorised stage attached to a Nikon Eclipse 50i microscope. Fluorescence images were captured using a Nikon e1000 microscope with motorised stage (Prior scientific, Cambridge, UK), digital camera (Photometrics Inc, Tuscon, USA) and ImagePro 7 software (Media cybernetics, Bethesda, USA).

9.13 Treg Suppression Assay

Single cell suspensions of lymphocytes extracted from either the spleen or CNS of 3 mice were pooled together and sorted on a BD FACS Aria II on the basis of their GFP status. GFP^+ Tregs and GFP^- effector T cells (Teff) were separated from each suspension and co-cultured in combinations as per the design of the experiment for 3 days with 30,000 effector cells per well. Tregs were added if necessary at a ratio of 1:1 Treg:Teff. Treg suppression was measured by thymidine incorporation by proliferating cells and cytokine release into the growth medium.

9.14 Proliferation assay

^3H thymidine incorporation was used as a measure of cell proliferation to different stimuli and from different experimental contexts. Cells were cultured in complete DMEM with various concentrations of stimulant for 3-5 days at 37°C with 5% CO_2. Supernatants from the cell culture were aspirated for cytokine analysis and media was replaced alongside 1μCi of thymidine per well. After 8-16h further incubation the culture was frozen at -20°C to prevent the cells proliferating any further and to allow storage of the plate until reading. Plates were harvested using a plate harvester (Tomtec, Hamden, USA) and read using a Wallac Microbeta Trilux system (Perkin Elmer, Cambridge, UK). Counts per minute were recorded and aligned with the cell concentrations for data analysis.

9.15 ELISA

Cytokine release from cultured cells was assessed using an ELISA. ELISA kits were ordered from Mabtech, Sweden and optimised in the lab. Detection limits advertised by the manufacturer were confirmed by limiting dilution of known standards before each kit was used to quantify cytokine release. Detection antibody was coated onto ELISA plates at the relevant concentration (see Table 11) overnight at 4^{o}C or for 2h at 37°C. Plates were washed with PBS before being blocked with 1%BSA in PBS for a minimum of 1 hour at room temperature. Plates were washed with 0.05%Tween in PBS (washing buffer) before supernatants and standards were diluted to useable concentrations and allowed to incubate in the plates overnight at 4^{o}C. Plates were washed again with washing buffer in PBS before the detection antibody was added in 1%BSA/PBS at the relevant concentration (see Table 11) and incubated for 1 hour at room temperature. Streptavidin at 1/1000 dilution was added after further washes with washing buffer and incubated at room temperature for half an hour. One final washing step followed and the substrate for the horseradish peroxidase (HRP) was introduced to the plate. After between 10min and 1 hour, 1M H_2SO_4 was added to stop the reaction and the absorbance of each well read on a μQuant ELISA reader (Biotek Instruments Inc, Potton, UK) using KCJunior software. The data for each well was compared to the standard curve to obtain a concentration of cytokine and multiplied by any dilution factor to achieve the final concentrations and normalise between runs.

Species	Target Cytokine	Coating Buffer	Coating Concentration	Detection Concentration	Detection Limit
Human	TNFα	$NaHCO_3$ pH 8.3	1/500	1/1000	29pg/ml
Mouse	IFNγ	$NaHCO_3$ pH 8.3	1/1000	1/2000	3.16pg/ml
Mouse	IL-10	$NaHCO_3$ pH 8.3	1/250	1/2500	20pg/ml
Mouse	IL-17a	PBS pH 7.4	1/500	1/1000	5pg/ml

Table 11. Conditions for cytokine ELISA.

9.16 TLR agonist immunization

TLR agonists from TLR2 agonist kit (Invivogen, Nottingham, UK) were resuspended using molecular grade H_2O and diluted to the desired concentration in PBS (see Table 12). 100µl of the solution was injected i.p. into the mice twice weekly for three weeks.

First run:

Agonist	Concentration	Total per injection
LPS	10µg/ml	1µg
LM-MS	1µg/ml	0.1µg
LTA	10µg/ml	1µg

Second Run:

LTA	50µg/ml	5µg

Table 12. TLR agonist immunization concentrations.

9.17 Whole Blood Assay

9.17.1 Methodology

To assess reactivity of patients and controls to TLR ligands, plates were set up containing 50µl selections of TLR ligands in RPMI (Gibco, Invitrogen, Paisley, UK) + 10% FCS, from various Invivogen TLR and NBOD agonist kits, and stored at -20^{o}C until use (See Table 14). Whole blood from patients and controls was extracted into BD Vacutainer© plus plastic K_2EDTA tubes (BD Biosciences, Oxford, UK) to prevent clotting and diluted 1/5 in RPMI +

10% FCS before use. The stimulant plates were thawed prior to use and 50µl aliquots of diluted whole blood were added to the 50µl of stimulant mix to provide a 1/10 final dilution of the whole blood, and the plates incubated at 37°C 5% CO_2 for 24h. After incubation, the plates were centrifuged at 300g for 10min and the supernatants aspirated for secreted cytokine analysis by ELISA.

9.17.2 Ethical Consideration and Demographics of MS and Control Patient Recruitment

Informed consent was received from all persons donating blood for use in this study. MS patients used in this study presented to MS clinics hosted by Dr Richard Nicholas and Dr Omar Malik at the Charing Cross Hospital, London where details of potential uses of their blood was described and consent taken for this purpose. Healthy controls were recruited to age and sex-match the MS patients and consent was taken in the same way to MS patients. The demographics of both populations are shown below.

	MS Patients	Healthy Controls
Number	10	8
Average Age	40.5	43.4
Standard Deviation	12.3	11.7
Gender Contribution	All female	All female
Clinical Presentation	All RR-MS	n/a
Treatments	3 off treatment for >2 months 4 Natalizumab 4 Interferon-β	n/a

Table 13. MS Patient and Healthy Control Demographics

TLR Agonist	Agonist for	Concentration
γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP)	NBOD1	1µg/ml
Salmonella typhimurium Flagellin	TLR5	1µg/ml
Fibroblast Stimulating Ligand-1 (FSL1)	TLR6/2	10ng/ml
Heat Killed *Listeria monocytogenes* (HKLM)	TLR2	10^8 cells
Imiquimod	TLR7	1µg/ml
Lipomannan from *Mycobaterium smegmatis* (LM-MS)	TLR2	10ng/ml
Lipopolysaccharide from *Esherichia coli* K12 (LPS *E.coli* K12)	TLR4	100ng/ml
Porphyromopnas gingivalis Lipopolysaccharide (LPS)	TLR2	1µg/ml
Staphylococcus aureus Lipoteichoic acid (LTA)	TLR2	100ng/ml
Muramyl Dipeptide (MDP)	NBOD2	10µg/ml
Synthetic Lipopeptide Pam_3Cys-Ser-$(Lys)_4$ (Pam3CSK4)	TLR1/2	1µg/ml
Insoluble Peptidoglycan from *E. coli* K12 (PGN-ECndi)	NBOD1/2	1µg/ml
Peptidoglycan from *S. aureus* (PGN)	TLR2	1µg/ml
Phytohaemagglutinin (PHA)		20µg/ml
Polyinosinic:polycytidylic acid (PolyI:C)	TLR3	1µg/ml
20-mer GU-rich Single-Stranded RNA Oligonucleotide (ssRNA40)	TLR8	1µg/ml

Table 14. TLR and NBOD agonist panel for whole blood assay.

9.18 Treg depletion and other antibody depletions

For Treg depletion in the Foxp3^{DTR} mice or L7Fox cross, 100µl diphtheria toxin (50µg/kg) (Sigma Aldrich, Poole, UK) was injected at 12.5µg/ml in PBS i.p. per dose. For CD8 depletion, 100µl YTS169 antibody (Cobbold 1990) at 1mg/ml in PBS was injected i.p per dose. For NK1.1 depletion, 200µl anti-NK1.1 (eBioscience, Hatfield, UK) at 1mg/ml in PBS was injected i.p per dose.

9.19 Statistical Analysis

9.19.1 Real-time PCR

Statistical analysis of real-time PCR data was done by using randomisation tests with pairwise reallocation. This analysis is in built in the REST software (M. Pfaffl (Technical University Munich)) used to generate relative expression values and is displayed through the use of a p value for which significance is measured as being $p<0.05$. Randomisation tests do not assume a normal distribution in the data, rather that the allocation of results into different groups is random. They then determine how likely the difference observed between the two groups could be explained by this allocation through the analysis of over 5000 iterations of other random pairs of data. Significant results are shown in two ways; * illustrates significance with regard to the baseline expression (C57BL/6 mouse), ** illustrates significance of expression of a gene between disease scores.

9.19.2 FACS and ELISA Analysis

Statistical analysis was carried out on means generated from the analysis of multiple mice within specific experimental groups, determined by the design of the investigation. The means are assumed to come from normally distributed populations and so comparison of the means was done using a two-tailed Student's T test with Welch's correction. This allows comparison of means without assurance of equal variance, necessary as many means were calculated from limited sample sizes. A two-tailed test was used in favour of a one-tailed as the null hypothesis was that there was no significant difference between the two groups without a consideration of which direction the difference may be. Significant differences are highlighted on each graph with the corresponding significance level; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ANOVA analysis was not used as interest was in differences between distinct disease landmarks rather than investigating whether the disease landmarks together would explain the variance observed.

10 Characterisation of the Line7 model

10.1 Introduction

In recent years, a great deal of attention has focused on the role of Th17 cells in the development of autoimmunity. From the initial studies showing the importance of IL-23 (Cua 2003) and IL-17 in EAE (Langrish 2005, Komiyama 2006) those that showed IFNγ to be dispensible for EAE (Ferber 1996), and the subsequent confirmation findings that Th17 cells could transfer EAE (Langrish 2005). There may have been a resulting tendency in the field to attribute a central role of Th17 cells as necessary and sufficient for EAE, and by extrapolation, MS (Cua 2003, Hofstetter 2005, Komiyama 2006, Reboldi 2009). However, in this same period, many conflicting and results have been generated that make such a view contentious. Examples of observation that do not readily fit a simple Th17 model are the requirement of T-bet for encephalitogenicity of both Th1 and Th17 cells (Yang 2009b), that the majority of CNS infiltrating CD4 cells are exTh17 Th1 cells (Hirota 2011) and the identification of T-bet^{+}RORγt^{+} cells (Abromson-Leeman 2009). As the original idea of Th1 and Th2 cells survived for around 20 years before it was shown to be inadequate, other ideas should be entertained despite the vast support for one cell type over another.

Animal models are utilized in MS research because they provide what is lacking from human studies, namely direct investigation of diseased organs at differential disease states. Human studies rely mainly on post-mortem brain tissue and blood samples taken from patients, with some work done with CSF samples, although even these are not directly from the site of inflammation and CNS damage.

The largest difficulty faced when using animal models is understanding which aspects can be directly transferred to interpretation of the human condition (Sriram 2005, Steinman 2006) and which are just artefacts of either the animal physiology or the model itself. EAE in this way provides many difficulties in drawing conclusions with regard to human MS: not only is it a very severe, experimentally-induced condition but it is carried out in such a wide range of different genetic backgrounds and with a wide range of neurological antigens that results from one study are not always directly comparable with another (Lassmann 2007). That being said, some EAE models have proved to be more successful and more widely used than others, dominating the published research, and leading some observers to look no further when considering the human condition.

A resulting tendency to allow findings from a very narrow part of the disease model spectrum to dominate the literature may be to the detriment of achieving a strong understanding of the human disease which is far more complicated than the animal model. Inducing disease in an EAE model necessarily requires the introduction of a large number of biases into the immune system. The impact that each aspect of the model required for EAE induction, such as the nature and effects of the CFA, pertussis toxin and antigen used (Veldhoen 2006a, Veldhoen 2006b, Li 2007) has on the final outcome is often ignored in the interpretation of the generated results and therefore should be properly controlled when delineating a mechanism of action in the human context. A specific example is the promotion of Th17 cells through the use of pertussis toxin (Chen 2007) and CFA (Tigno-Aranjuez 2009).

Therefore there is a need to investigate spontaneous mouse disease models, without manipulation of the innate-adaptive interface through pro-inflammatory adjuvants or

other injections, to facilitate interpretation of the generated results. The Line7 mouse model was designed with these intentions, to generate a more relevant model to the human condition, and to provide data to contribute to the immunological debate (Ellmerich 2005). The model benefits from the ability to select mice from different disease scores, representing the distinct stages of disease progression. By separating mice according to disease landmarks, it should be possible to understand which cells are important at which stages and whether one cell type is proximal to another in the initiation of disease.

The first priority with such a model is to fully characterize the immunological developments throughout disease progression, rather than just a simple pre-disease and peak disease comparison allowed by most EAE models because of the rate of progression.

A combination of techniques are required to build up a full picture of the developments at each stage in disease progression, and whilst not directly concluding the mechanism of action, the importance of cell types can be inferred from the findings. A combination of real-time PCR, immunofluorescence staining, and flow-cytometric analysis were employed on mice at different stages of disease. The aim has been thus to generate a dataset illustrating the progressive activation of immune cells through the disease process, encompassing direct observation of infiltrating cells and direct interrogation of the polarization of the cellular infiltrate respectively. Real-time PCR is a very sensitive method for detecting changes in the transcription of genes, indicative of activation and inferred to lead to correlate with protein levels. In this context, whole tissue could be interrogated to show changes in inflammatory gene products and immune cell polarizing

factors. Whilst results cannot be attributed to specific cells, it can implicate increases in activation of one cell type at the expense of another and also the activated environment of the diseased tissue.

Direct observation of infiltrating cells is the clearest way to show the presence of immune cells at the site of damage. Analysis by immunofluorescence and flow cytometry provides an important checkpoint to implicate cells in the pathogenesis of disease. Both can highlight the importance or unimportance of infiltrating cell populations based upon their localization in the inflamed tissue and detected cytokine production. This can be attributed to individual cells rather than gross measurement of the whole tissue to implicate the determinant factors in disease progression. In this way a full picture of the model can be drawn, illuminating the important questions to ask rather than interrogating the model with pre-conceived views.

10.2 Results

10.2.1 Development of Paralysis

The Line7 transgenic model exhibit spontaneous ascending paralysis that passes through distinct symptomatic landmarks that allow assessment of severity and comparison between mice in which disease has progressed to different extents. In order to gain an understanding of the progression of disease, mice were scored daily according to the guidelines in the Materials and Methods section and in accordance with requirements of Professor Altmann's Home Office Project Licence under the Animals (Scientific Procedures) Act 1986. Disease scores were collated for each mouse and transposed relative to the date of birth of each mouse. An average for each day was calculated and plotted as a point on a scatter plot to illustrate the progression of disease over time. Not all mice were retained in the cohort through to one year of age through requirements for experiments and so they were removed from the averaging process at that time. Their scores however were used to contribute to the average for the period of time they were alive. Mice not developing signs of disease were not included in the calculation so that the progression is relevant to the 'average' mouse developing signs of disease and not the whole colony as this would generate an unrealistic profile for the mice that did develop signs of disease.

The progression of paralysis scores of the affected mice is shown in Figure 1. It shows that first signs of disease are observed in mice at approximately 3 months of age, although other mice in the colony continue to present first signs of disease through to one year of age (Figure 1A). Female mice present initial disease signs at a marginally

faster rate than male mice although this is not a significant difference. After the initial signs, disease progresses at variable rates in individual mice, which results in the average disease score to follow a gradual progression (Figure 1B). The progression of disease in the affected miceis not associated with any weight loss (data not shown). Around 40% of mice do not develop signs of disease at all and these remain unaffected through to a year of age (Ellmerich 2005). These mice are termed score 0 (old) or 0o to distinguish these mice from the pre-disease young score 0 mice which are termed 0y (0 young). It has been noted in other spontaneous models that disease is profoundly affected by the microbiological status of the animal facility (Goverman 1999). For only the disease progression, Line7 tissue cryosection analysis and real-time PCR transcriptional analysis experiments, mice were housed in the H1 facility at the MRC Clinical Sciences Centre and the following infections were commonly detected on screening; *Helicobacter hepaticus* bacteria, *Helicobacter rodentium* bacteria and *Tritrichomonas sp* parasite. For all other experiments, mice were housed in the H2 facility in the Burlington Danes Building, Hammersmith Hospital Campus and all screens returned were negative for any pathogens.

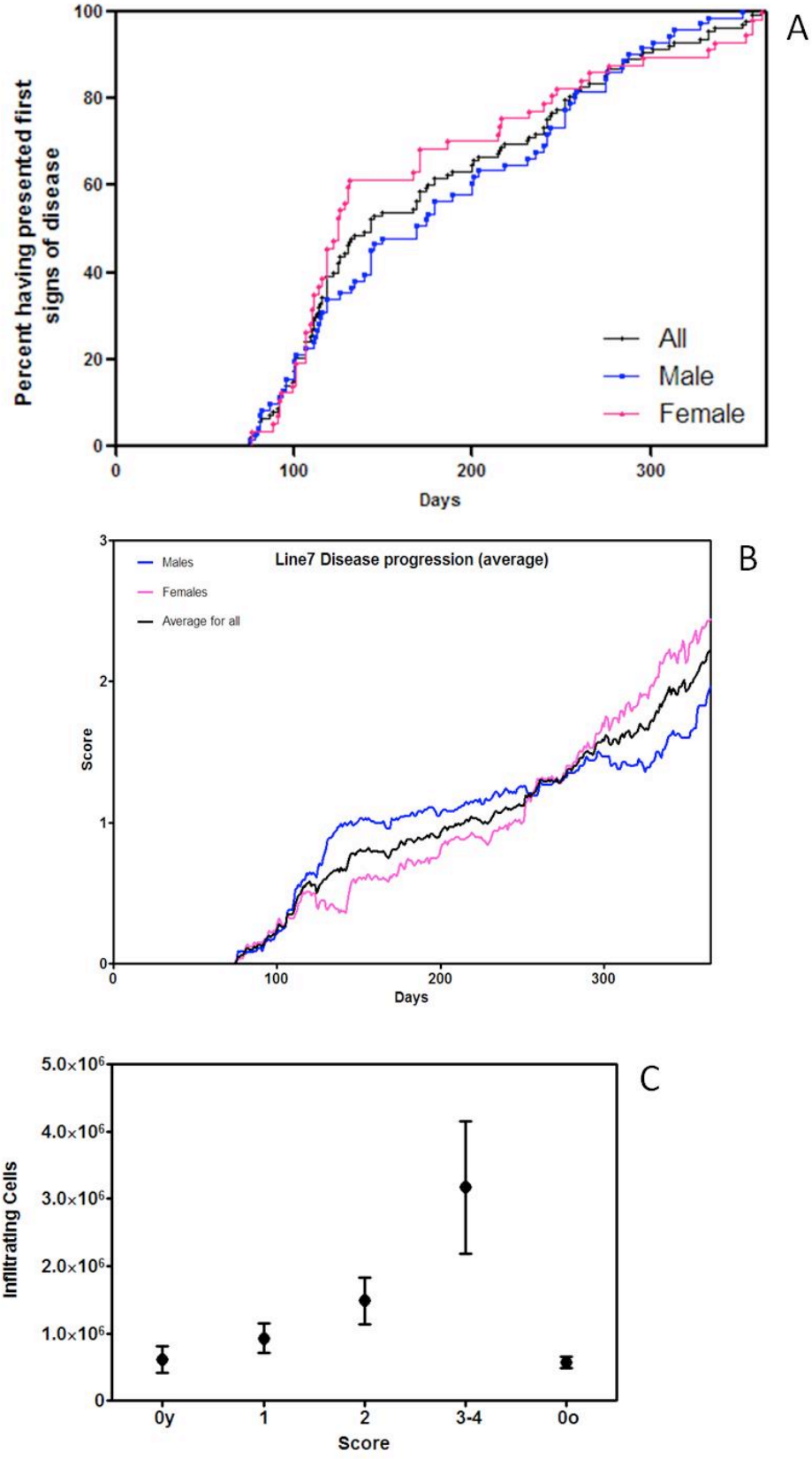


Figure 1. Spontaneous disease progression in the Line7 mouse.

A – Variable presentation of first signs of disease occurs throughout the first year of life. The day when first signs of disease were observed was recorded and displayed as a cumulative percentage of all mice presenting disease signs. B – Scores from all mice presenting signs of disease averaged to generate 'standard' progression of disease as measured by increasing physical symptoms. For A and B, mice housed in H1 mouse facility. n=137 total. C – Counts of infiltrating cells as extracted using density gradients. Scores at time of extraction averaged and shown with SEM. Mice at various disease scores are compared to young, pre-diseased mice (0y) and old, unaffected mice which have resisted signs of disease past 8 months of age (oo). Mice housed in H2 mouse facility. n=26 total.

Any mouse not presenting signs of disease by the age of 8 months will only present signs on very rare occasions and so these are treated as non-affected mice in further experiments. Interestingly, these unaffected mice are all male and all female mice develop paralysis, albeit at differing severities.

The progression pattern of male and female mice is also slightly different, with male mice typically presenting earlier than female mice. First signs of disease are observed at younger ages on average in male mice although the further progression of disease is slower in males than females (Figure 1A). This illustrates a sex-dependent difference albeit not at the same ratio as seen in the human disease.

10.2.2 Visualization of Cellular Infiltration

Progression of signs of disease in the Line7, as with EAE, is associated with increasing cellular infiltration into the spinal cord. By developing a technique for extraction of lymphocytes from the CNS, it became possible to investigate the infiltrating cell populations at each stage of disease and determine whether a development of signs of disease could be attributed to one or many cell types. The protocol is described in the materials and methods section but in brief involved an enzymatic digestion followed by density gradient. The technique proved sufficiently successful as to allow investigation of mice individually, rather than requiring pooled cell populations, and means that averages could be calculated for mice at different disease severities from multiple experiments reducing the error introduced by recording whole samples from each disease score on only one day.

Infiltrating cells extracted from CNS of animals at various disease scores were counted using a haemocytometer to determine the size of the infiltrating population (Figure 1B) before being further stimulated and incubated with fluorescently conjugated antibodies. The infiltration was found to correlate with the severity of signs of disease, such that a larger infiltrating population was associated with more severe signs and the relationship appears to be increasing in an approximate exponential manner as disease develops (Figure 1B). Despite not presenting signs of disease, there are detectable levels of cells infiltrating the CNS of both young mice (less than 2 months) and old mice (over 8 months) with a score 0 but these infiltrate levels are lower than in any affected mice (Figure 1B).

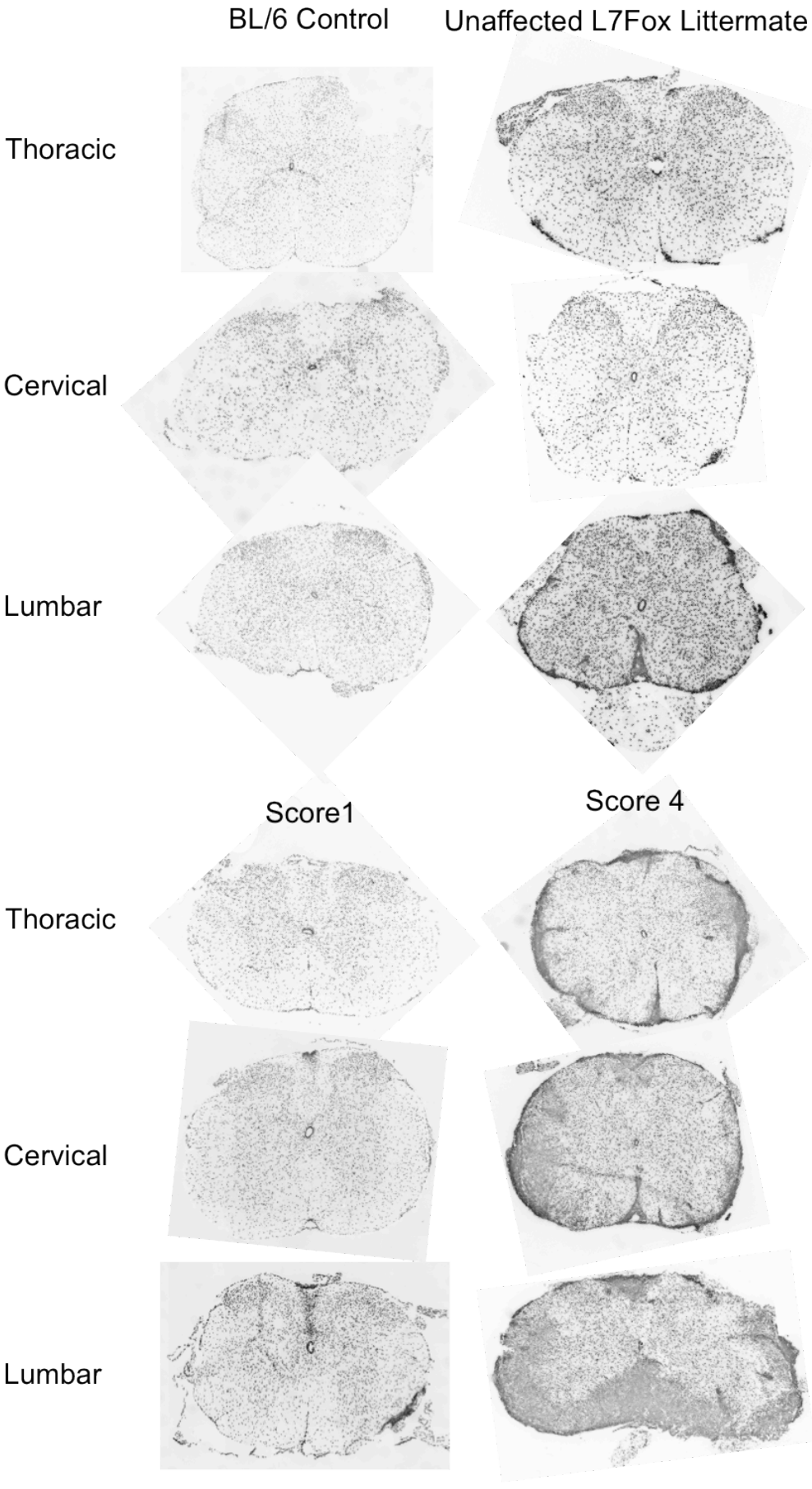

BL/6 Control
Unaffected L7Fox Littermate
Thoracic
Cervical
Lumbar
Score1
Score 4
Thoracic
Cervical
Lumbar

Figure 2. Progression of disease associated with increased cellular infiltration ascending the spinal cord.

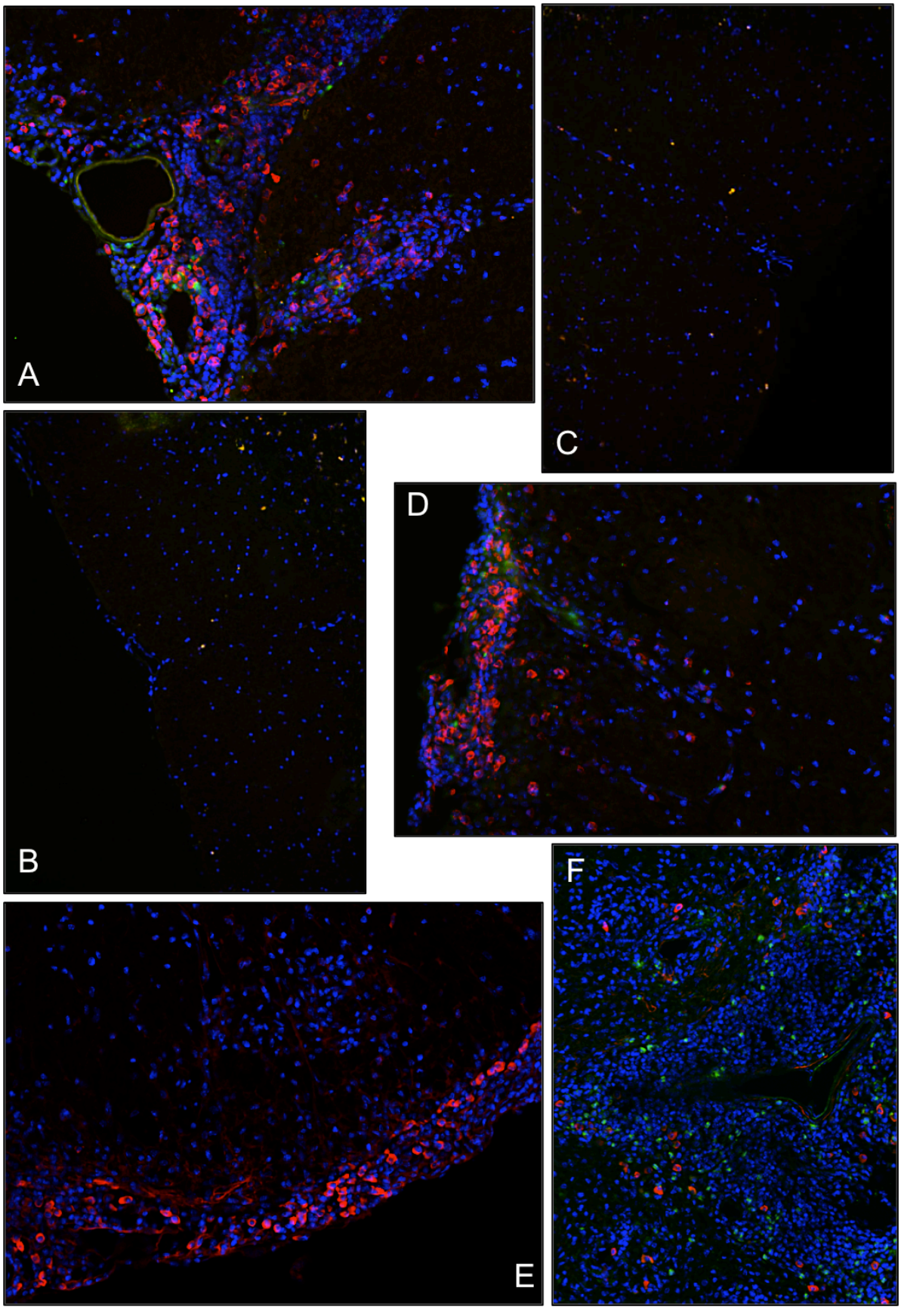
10µm fix/perfused tissue sections from mice at distinct disease landmarks were stained with DAPI to indicate nuclei. Images are tiled, inverted views of 10X fields of view. Tiling was done using ImagePro 7 software and images inverted using Adobe Photoshop software. Representative images of at least 3 mice from each disease score.

Staining of cryosections with DAPI to visualize DNA, was carried out to show the location and timing of cellular infiltration in the model. This simple observation does not provide any information with regard to cell phenotype but allows aggregations of cells to be seen and provides a supporting observation to the cell counts derived from the cell extraction protocol. The image was reduced to black and white to improve the visualisation of individual cells. Multiple 10X single frame of views were tiled together to generate views of whole tissue sections using ImagePro software (Media Cybernetics). The density of cellular infiltration rendered a quantitative measure impossible as cells could be seen overlapping with each other and would not be individually distinguishable, necessary for counting. Consideration of these images showed infiltration typically in the lumbar regions and ascending to the thoracic and cervical regions when the severity of disease increases (Figure 2). Control tissue also stained showed little cellular infiltration above the normal density of neuronal cells present. Infiltrating cells could be seen to accumulate in the meningeal regions of the spinal cord and infiltrate through the dorsal

and ventral fissures as well as direct localised infiltration directly into the tissue with no obvious pattern of location (Figure 3).

These same cryosections were also stained with antibodies directed towards CD4 and CD8 (Figure 3A, B, C, D, F), and NK1.1 (E). Primary antibodies directed against cell surface markers (and raised in different species if used in conjunction with another primary antibody) were initially incubated with the sections to adhere to specific cell surface markers before sections were washed to remove unbound antibody from the slide. Fluorescently conjugated secondary antibodies, directed against the primary antibodies, were then used to visualise the location of the primary antibodies as they amplify the signal making it easier to detect under the microscope. This staining was carried out to determine the composition of the cellular infiltrate and identify any different infiltration patterns for each cell type. Non-overlapping fluorescent molecules were used on the secondary antibodies in each analysis so that the different cellular makers could be distinguished by the wavelength of light required to excite and the subsequent wavelength of the emitted light observed. A typical example if two antibodies were to be used would be peak excitations at 495nm and 590nm with peak emissions at 519nm and 617nm respectively (Alexa488 and Alexa594 dyes). Therefore one marker would appear green whilst the other red. Minimal fluorescence contamination would also be seen, resulting from the overlap of the emission spectrum of one dye with the excitation wavelength of the other. All three cell types; CD4, CD8 and NK, could be identified in the infiltrating population. The initial description of this model by Ellmerich and colleagues (Ellmerich 2005) had noted that, despite expressing an HLA-DR-restricted TCR, this

particular line (but not other founders from the same pronuclear microinjections), selects into both the CD4 and CD8 SP populations. Representative images are shown in Figure 3 showing all these cell types infiltrating. Most notably cells were seen in large numbers throughout the meningeal tissue of the spinal cord and infiltration could be observed occurring through the dorsal fissure (Figure 3A). Similar patterns of infiltration were observed through the ventral fissure of the spinal cord (Figure 3F) and comparative sections taken from control C57BL/6 mice showed no meningeal aggregation of cells or infiltration through either the dorsal or ventral fissures (Figure 3B,C). Despite this large abundance of infiltrating cells, no specific pattern of infiltration could be attributable for each cell type. There were seemingly equal distributions among all infiltrating locations and no temporal infiltration of one cell type before the others (Figure 3). Infiltrations containing both CD4 and CD8 cells could be seen occurring in the tissue away from the fissures in specific locations (Figure 3A, D) and also in a more widespread diffusion (Figure 3E). Overall, these date provide an illustration of the cellular infiltration and confirmation that CD4, CD8 and NK cells are present, co-localised in infiltration populations. These pictures provide circumstantial evidence for a role in mediating CNS damage but not direct evidence. No conclusion could be drawn with regard to importance in controlling infiltration or mediation of neuronal damage at this stage and other techniques are required to investigate these processes further.


A
C
B
D
F
E

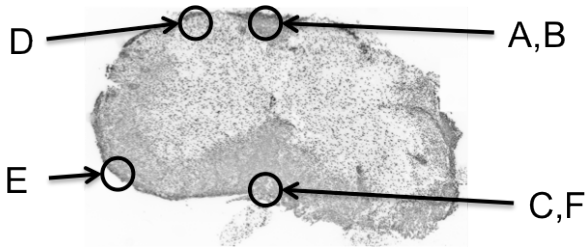


Figure 3. Cellular infiltration into the spinal cord of Line7 mice.

L7 and mice from distinct disease landmarks were fix/perfused and 10μm sections cut. Representative 10X pictures are shown from indicated locations in the lumbar spinal cord. A – Entry of cells into the dorsal fissure from the meningeal space and emanating from vascular structures inside the white matter. Score 4 Line7, CD4 (red) CD8 (green) DAPI (blue). B – View of dorsal fissure in C57BL/6 mouse showing no infiltration and accumulation of cells, staining as (A). C – View of ventral fissure in C57BL/6 showing no infiltration and accumulation of cells, staining as (A). D – CD4 and CD8 cells accumulating in meningeal tissue and isolated infiltration events in a score 4 Line7, staining as (A). E – NK cells accumulating throughout meningeal tissue in a score 4 Line7 mouse, NK1.1 (red) DAPI (blue). F – Large numbers of infiltrating cells surrounding vascular tissue in the ventral fissure of a score 3 Line7, CD4 (red) CD8 (green) DAPI (blue).

10.2.3 Analysis of Infiltrating Cell Populations

The protocol for extraction and isolation of CNS-infiltrating cells was developed such that cells could be investigated from individual mice without the need to pool samples. Using this method it was possible to take mice from each disease score and investigate the composition of the infiltrating cell population. Even proportions of male and female mice

were taken at each disease score to control for any gender bias. After extraction, cells were stimulated using the non-specific stimulant of PMA and ionomycin. PMA acts to activate PKC pathways whilst ionomycin increases the intracellular calcium levels, acting together to stimulate the release of cytokines. It is assumed that the cytokines released in response to this stimulation are indicative of those primed released in the *in vivo* environment, as the stimulation does not affect the polarisation of the cells. The stimulation was carried out in the presence of Brefeldin A, a protein transport inhibitor that prevents the movement of proteins from the endoplasmic reticulum to the golgi apparatus, and therefore keeps cytokines destined for release sequestered within the cells producing them. After surface staining and cell fixation, it is then possible to permeablise cells and stain for the intracellular cytokines to determine the polarisation of the cell population extracted. Two-tailed t tests with Welch's correction for unequal variance were used to analyse differences in means of cell proportions resulting from this experiment.

Surface staining of cells shows that the largest population of infiltrating lymphocytes are in fact NK cells, as defined here by being cells in the lymphocyte gate that are $NK1.1^{+}$. Unfortunately due to the number of antibodies used in this analysis and the limited range of fluorescent molecules available, it was not possible to further confirm this population with other markers such as NKp46. They consistently make up around 50% of the infiltrating lymphocyte population but do not significantly change in proportion as disease progresses although there is a higher proportion observed in mice resisting development of signs of disease compared to mice at early stage of disease (Figure 4).

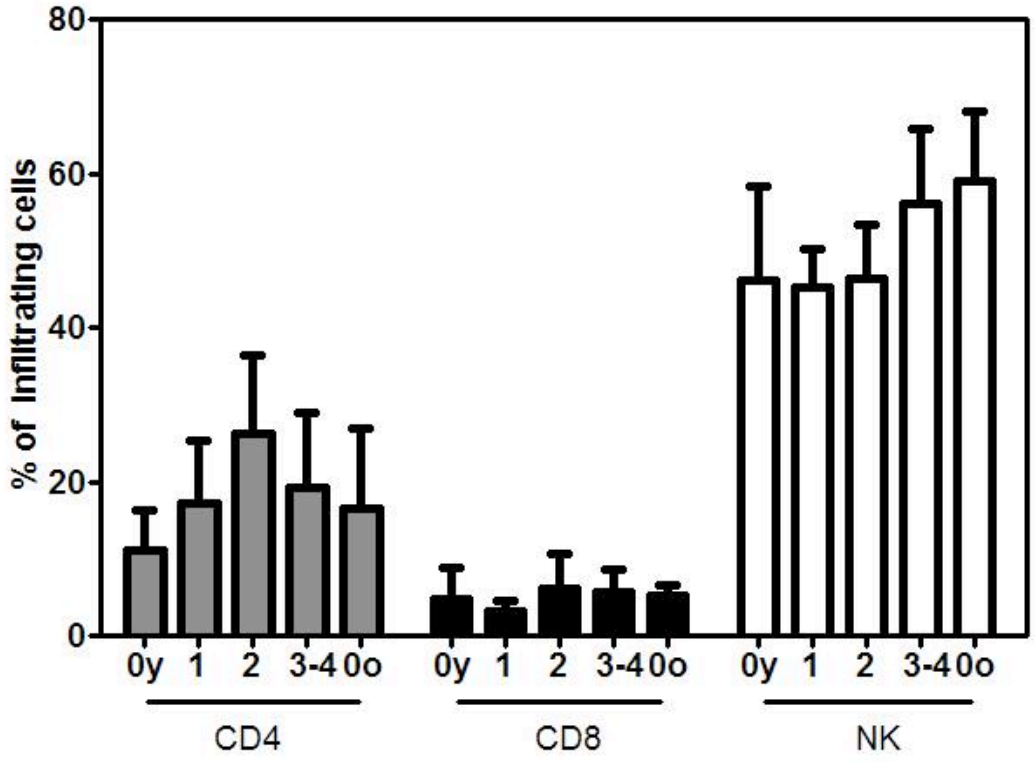


Figure 4. Phenotypic analysis of infiltrating lymphocyte population across spectrum of disease in Line7 mice.

*Cells were extracted from perfused L7 CNS tissue by enzymatic digestion and separation on a percoll density gradient. Cells were incubated with antibodies for cell surface markers CD4, CD8 and NK1.1 and read on a BD FACS Aria II, gated on the lymphocyte population and percentage distributions calculated for each mouse. Data presented is average of mice at that disease score with SEM (n=18 total). * shows statistical significance between cell populations of different disease scores ($p<0.05$) (two-tailed T test).*

There is a smaller (5-10%) but consistent population of infiltrating $CD8^+$ cells across all disease scores, but it is the $CD4^+$ population that correlates with initiation of disease signs. Through the early development of symptoms (scores 0-2), the CD4 population

increases as a proportion of infiltrating lymphocytes. At the peak of disease an increased infiltration of NK cells lowers the proportion of the infiltrate that are $CD4^+$, although neither of these changes show significance upon statistical analysis. The mice remaining unaffected show a similar infiltration pattern to the pre-diseased mice in all cell types (Figure 4).

In addition to surface staining, the non-specific stimulation, in the presence of Brefeldin A allowed intracellular staining to be conducted for IFNγ and IL-17, to interrogate cells within the lymphocyte gate as to which cytokines they were producing. Much controversy is directed at the composition of the cellular infiltrate of autoimmune conditions with the relative contribution of Th1 and Th17 cells unclear. Interestingly, the proportion of the infiltrate that are $IFN\gamma^+$ mirrors the pattern of $CD4^+$ cells, with the proportion of infiltrating cells increasing with disease score at early stages, through the initiation of disease signs, but falling off to early levels at peak of disease (Figure 5A). A significant increase in $IFN\gamma^+$ cells is seen at early disease scores relative to pre-disease (Figure 5A). In contrast, the $IL\text{-}17^+$ cells appear in their highest proportion only at the peak of disease. Through disease initiation and early progression they are present in lower proportions than $IFN\gamma^+$ cells and in fact are first consistently detectable in mice at score 2, when disease has already been initiated and other T cells have infiltrated the CNS (Figure 5A). Analysis of mice that resisted autoimmunity showed populations of both $IFN\gamma^+$ and $IL\text{-}17^+$ single positive cells infiltrating the CNS to a greater level than pre-disease mice, although the total infiltrating population in both groups is very small and so is not representative of significant infiltration.

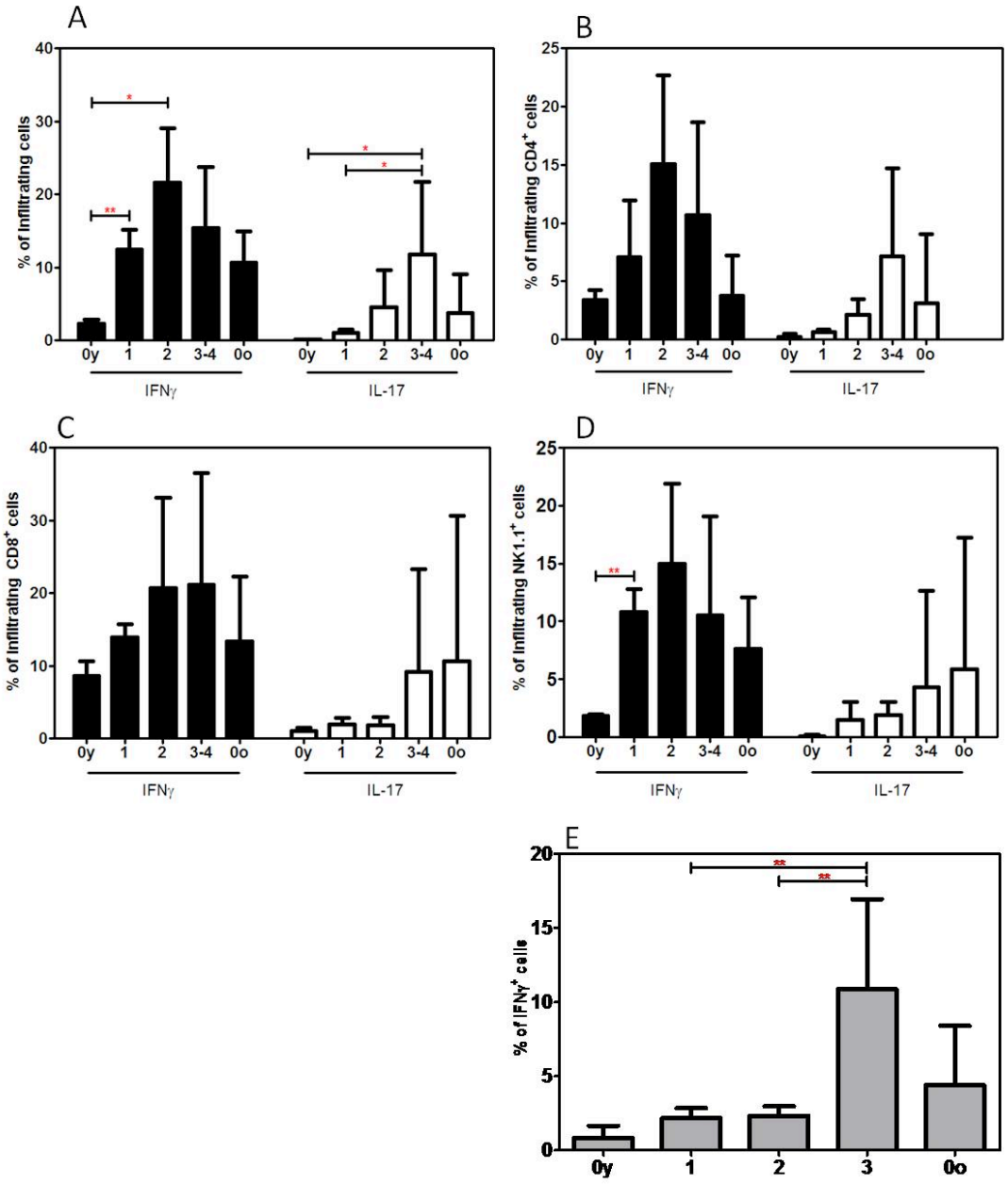


Figure 5. IFNγ precedes IL-17 production in disease initiation.

CNS infiltrating cells were extracted from perfused L7 CNS tissue by enzymatic digestion and separation on a percoll density gradient. Intracellular and surface antibody staining was carried out on extracted cells from the CNS after stimulation with PMA/ionomycin for 4h with BFA and read using a BD FACS Aria II. Data presented is average of all mice at that disease score (n=18 total). IFNγ and IL-17 secreting cells gated on the total

*lymphocyte (A), CD4 (B), CD8 (C), NK (D) cell population at each disease severity. Lymphocytes were gated initially according to cell surface markers and positive cells interrogated to determine the size of cytokine positive populations. Proportion of IFNγ$^+$ cells also positive for IL-17 shown at each disease score (E). Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

In addition to analysis of each surface or cytokine marker alone, the cytokine production within each lymphocyte population was also investigated. This was achieved through gating on each surface marker using FlowJo software, to show only those cells positive for each cell surface marker and then the subsequent analysis of cytokine production within these populations. The patterns of cytokine production in the total lymphocyte gate are recapitulated by analysis within gates of individual surface markers; CD4, CD8 and NK1.1. All three show an increase in IFNγ$^+$ cells as disease initiates and IL-17$^+$ cells appearing around the peak of disease (Figure 5B,C,D). The peak of IFNγ production is however not significantly different to any other disease score in either of the CD4 or CD8 populations, and only within the NK population is a significantly higher proportion of IFNγ$^+$ cells seen at early disease (score 1) compared to pre-disease (score 0y). The patterns of IFNγ$^+$ cells resemble that seen in the total lymphocyte population with an increase in proportion as disease develops before reducing in proportion at peak disease. This would suggest that no one cell type forms the dominant contributor to IFNγ release and rather that they all contribute to the observed cytokine release.

There is no significantly increased IL-17 release at any disease score relative to another. In both CD8 and NK cell populations, the highest proportion of IL-17$^+$ cells are seen at peak disease but there is a large degree of variation between mice, with only one showing a substantial population, meaning that this does not achieve statistical significance as compared to pre-diseased mice. In these two cell types the proportion at peak disease is similar to that seen in mice that fail to develop signs of disease, suggesting a potentially protective role for these cells. It is only in the CD4 population that a correlation with disease severity is observed. As with the total lymphocyte population, no IL-17$^+$ cells can be observed until disease is established (score 2) whilst the peak is reached at highest disease scores. The population of CD4$^+$IL-17$^+$ cells in mice not developing signs of disease is similar to that of CD4$^+$IFNγ$^+$ cells, although in neither case significantly lower than peak disease levels.

The increase in IL-17$^+$ cells at peak disease appears to be associated with IFNγ co-production. Through analysis of double positive cells (IL-17$^+$ cells within the IFNγ$^+$ gate), the proportion can be seen to peak at high disease having been a significantly smaller population at earlier stages of disease (Figure 5E). This would suggest that a proportion of the IL-17 production identified in this model comes from cells already producing IFNγ and it may be that the remaining IL-17 comes from cells that no longer produce IFNγ but it is impossible to tell in this analysis (Hirota 2011).

Analysis of peripheral lymphocyte activation and cellular proportion (in the same manner as CNS infiltrating cells) by analysis of splenocytes is important to determine whether these changes in cellular dynamics and activation is a result of organ-specific or systemic changes. Flow cytometric analysis shows that there is no change in overall proportions

of cellular composition, apart from a slight increase in proportion of $CD4^+$ cells at peak disease (Figure 6A). In the spleen, CD4 cells make up the highest proportion of cells at all disease states, with lower levels of NK cells than seen in the CNS infiltrate. There is no change in the proportion of CD8 or NK cells between disease scores and although the increase in proportion of $CD4^+$ cells is seen at peak disease, it is not statistically significant to pre-disease or indeed, those mice which resist development of paralysis (Figure 6A). There is no change in peripheral IL-17 production throughout disease progression or increase in IFNγ release correlating with disease progression in contrast to the observations from the CNS (Figure 6B).

Further analysis of cytokine production from within the CD4, CD8 and NK lymphocyte divisions all show a consistently higher proportion of $IFN\gamma^+$ cells than $IL\text{-}17^+$ (Figure 6C,D,E). No variation is seen within the $IL\text{-}17^+$ population of each group as disease progresses. The proportion of NK cells $IFN\gamma^+$ does not show any significant variation despite a trend towards decreasing as disease develops. A slightly higher proportion of $IFN\gamma^+$ cells within the CD4 gate of affected mice is not statistically significant compared to mice without signs of disease. The only statistically significant difference is a lower proportion of $CD4^+IFN\gamma^+$ cells in mice resisting disease than those before signs of disease could develop. In the early stages of disease (score 1 and 2), a trend towards an increase in the proportion of CD8 cells producing IFNγ could be observed compared to pre-disease mice, but this had reduced by peak disease although remaining higher than pre-disease. Interestingly, the proportion of $CD8^+IFN\gamma^+$ cells was higher in mice resisting signs of

disease than those before, suggesting that such an increase may just be in the normal development of the mouse and not important to this disease.

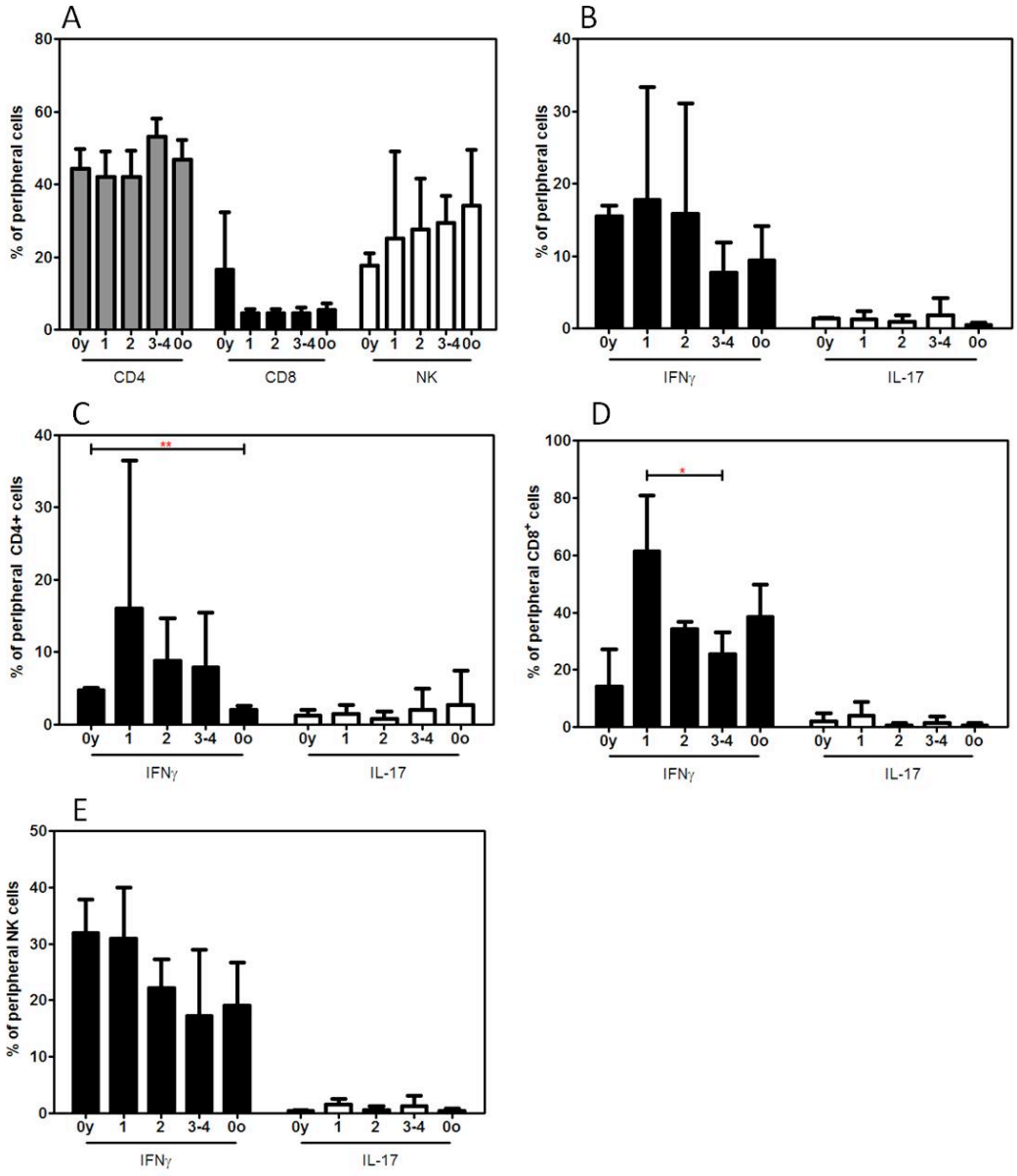


Figure 6. No variation in peripheral cellular composition across disease progression.

Lymphocytes were extracted from the spleen of L7 mice across all stages of disease and were stimulated with PMA/ionomycin for 4h with BFA. Spleen derived lymphocytes were then stained with surface and intracellular antibodies before being read on a BD FACS

*Aria II. A – Surface staining of total lymphocyte populations. IFNγ and IL-17 positive cells as measured from total lymphocyte (B), CD4 (C), CD8 (D), and NK (E) cell populations. Lymphocytes were gated initially according to cell surface markers and positive cells interrogated to determine the size of cytokine positive populations. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

10.2.4 Transcriptional Profile

In addition to the phenotypic analysis of the cellular infiltrates, the transcriptional profile of CNS and peripheral tissue was assessed across the duration of disease signs. Gene expression from 6 mice (3 male and 3 female) were measured for target genes run alongside 2 housekeeping genes to act as reference marks. Ct values derived for all samples run were compared to other groups by using REST software to generate results in the form of relative expression. REST analysis also incorporates randomisation tests and a pairwise reallocation to determine statistical significance between relative expression values. Measurement of the level of transcription is an indication of the first stage in the process of activating a gene. mRNA is transcribed from DNA sequence before being translated to produce the final protein released into the cell. Comparing the expression of genes can allow the determination of whether a gene plays an important role in a process through comparison of its expression to a baseline. In this instance the baseline was created by measuring transcription levels in C57BL/6 mice housed under the same conditions as the Line7 mice of differing disease progressions.

Real-time PCR was used to measure the relative expression of transcription factor and cytokine genes derived from both the inflamed CNS and spleen tissue. Whole tissue was dissected out of mice and immediately homogenized in Trizol to preserve RNA integrity. RNA was extracted from tissue and suspended in RNase free water to allow ease of use in PCR reactions. Details of the primer and probe pairs used are in Table 5 in the materials and methods section. Primers and probe pairs were designed to match sequence spanning exon-exon boundaries of mRNA meaning that they would not anneal to gDNA sequence but only transcribed and spliced mRNA sequence. As an additional control, housekeeping gene transcription was measured in representative samples from all disease scores. The two most consistently non-varying housekeeping genes were then selected for each tissue and run alongside the target genes for every sample.

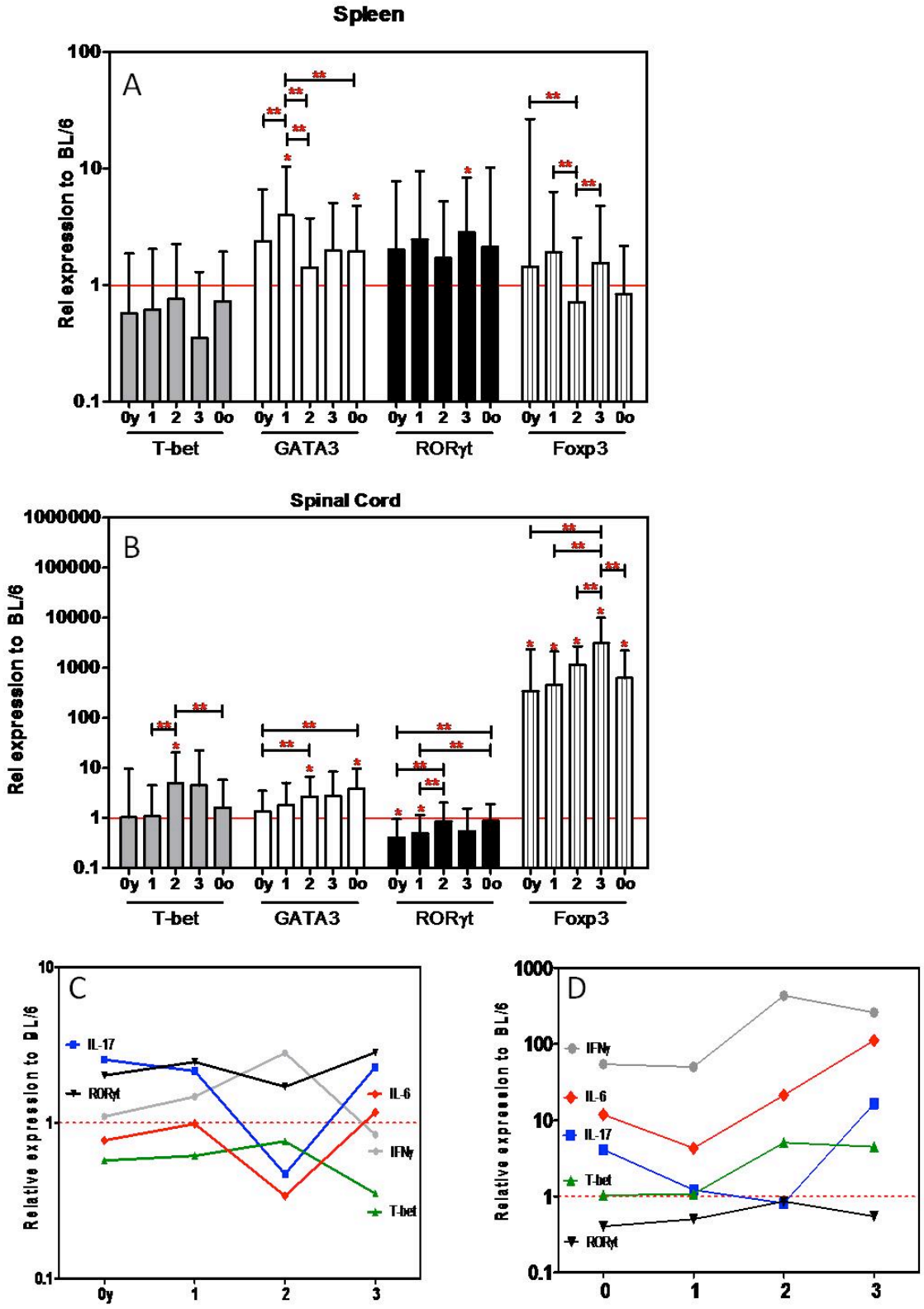


Figure 7. Relative expression profile of Line7 mice across disease progression as compared to C57BL/6 controls.

*mRNA was extracted from spleen and spinal cord tissue of L7 mice taken at distinct disease landmarks. Following reverse transcription of mRNA to cDNA, real-time PCR was used to quantify relative expression of selected gene transcripts using individually designed hydrolysis probe based assays. Expression of the major transcription factors helper T cell subsets in the spleen (A) and spinal cord (B) shown with * to show significance compared to control ($p<0.05$) and ** to show significance between scores ($p<0.05$ (randomisation analysis). Summarised in C and D. n=33 total, 6 mice per disease score (3 male and 3 female) and 3 C57BL/6 controls (all female).*

In the spleen, representative of the periphery, there is relatively consistent expression of all T helper cell transcription factors similar to those seen in unaffected C57BL/6 mice (Figure 7A,C). T-bet is expressed at similar levels throughout disease progression and is comparable to the baseline expression observed in a C57BL/6 mouse. RORγt mimics this pattern although does show significantly increased expression at peak disease relative to a C57BL/6 mouse but not to any other Line7 disease severity. GATA3 is highest during early disease and significantly higher than both baseline expression and expression seen at all other scores except peak disease. Whilst GATA3 expression is significantly lower in mice that resist disease than those in the early stages, the older mice do show significantly higher GATA3 expression than the baseline C57BL/6 mouse. Foxp3 expression fluctuates around the levels observed in a C57BL/6 mouse but never reaches statistical significance. When expression between disease scores is compared however, score 2 mice are seen to have lower expression than both score 1, 3 and pre-diseased mice.

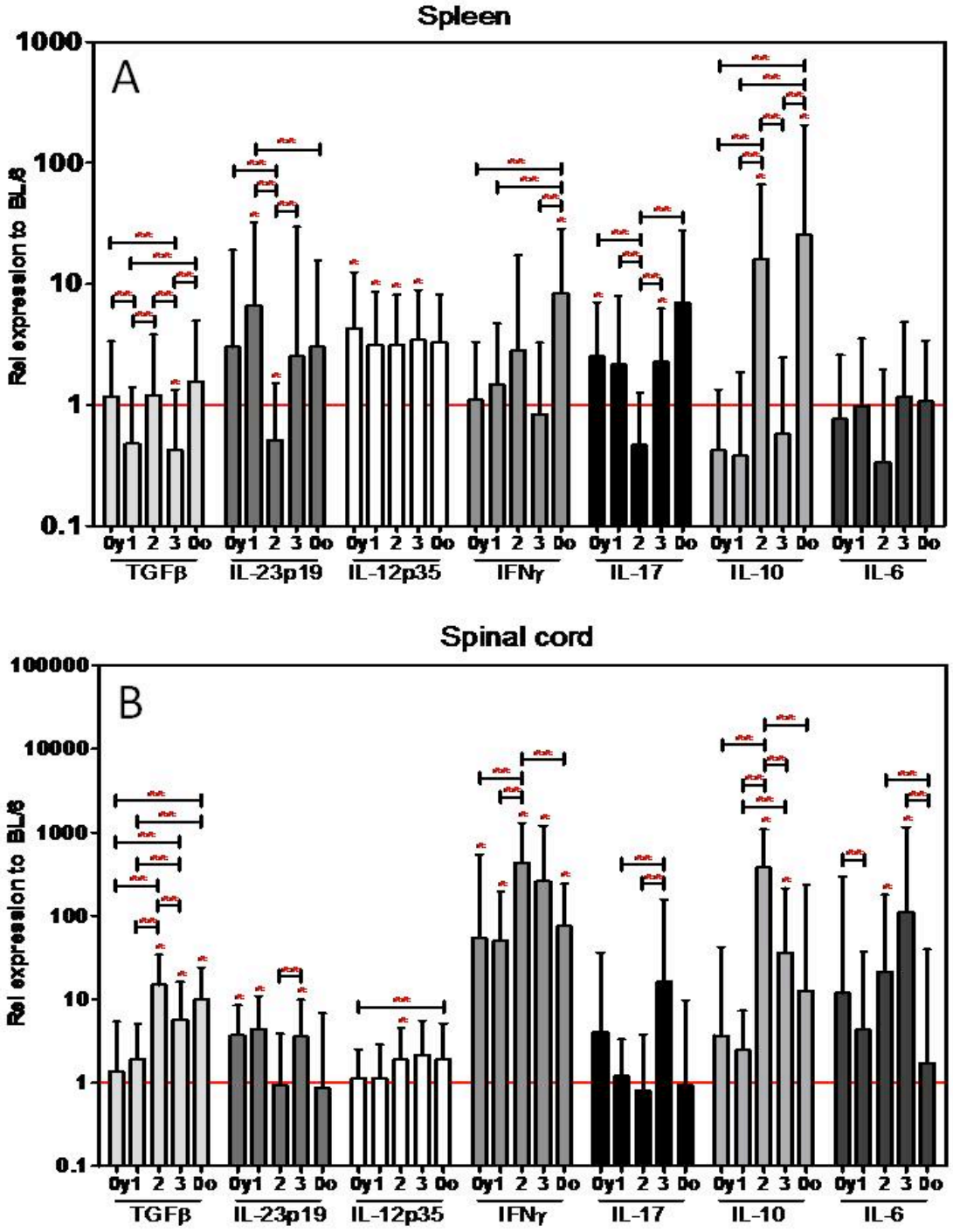


Figure 8. Relative expression profile of Line7 mice across disease progression as compared to C57BL/6 controls.

mRNA was extracted from spleen and spinal cord tissue of L7 mice taken at distinct disease landmarks. Following reverse transcription of mRNA to cDNA, real-time PCR was

*used to quantify relative expression of selected gene transcripts using individually designed hydrolysis probe based assays. Expression of representative cytokines for the differentiation and expansion of major helper T cell subsets in the spleen (A) and spinal cord (B) shown with * to show significance compared to control ($p<0.05$) and ** to show significance between scores ($p<0.05$) (randomisation analysis). Summarised in Figure 7 C and D. n=33 total, 6 mice per disease score (3 male and 3 female) and 3 C57BL/6 controls (all female).*

Similar patterns are not seen in the spinal cord. GATA3 expression steadily increases from a level comparable to that seen at baseline to a significantly higher expression in moderately affected mice at score 2. Mice that resist paralysis however, have the highest spinal cord GATA3 expression, with significantly higher expression compared to both baseline and pre-disease mice. RORγt expression is noticeably dampened, only comparable levels to BL/6 mice at high disease (scores 2 and 3) and in mice resisting signs of disease despite the large cellular infiltration compared to the C57BL/6 mice. By contrast, T-bet is upregulated in the CNS in disease, with a significant increase evident from mice of score 2 (Figure 7B,D). Foxp3 expression is significantly higher than in mice at baseline and shows significantly higher transcription at peak disease with regard to that seen at all other disease scores.

In addition to analysis of transcription factors, further analysis of cytokine expression was carried out to confirm the *ex vivo* flow cytometry data (Figure 8). In the periphery, no consistent pattern of either IL-17 or IFNγ expression correlated with disease progression.

IFNγ expression remained at the level of C57BL/6 mice throughout disease progression but was observed expressed at higher levels in mice that did not develop signs of disease. Peripheral Line7 pre and peak-disease expression of IL-17 was higher than in C57BL/6 controls although it was significantly lower at mid-disease than at any other disease severity. The expression of cytokines that have a role in either Th1, Th17 or Treg polarization were also investigated. IL-6 and IL-23p19 are important cytokines to differentiate and maintain the Th17 phenotype whilst IL-12p35 is important to the Th1 phenotype. TGFβ and IL-10 are in general anti-inflammatory cytokines and aid in the differentiation and mechanism of action of Tregs respectively. IL-6 did not vary throughout disease and was transcribed at comparable levels to baseline. IL-23p19 expression did not correlate with disease symptoms, and only showed a significant dip in expression, centered on mid-disease when it was expressed at lower than baseline levels. IL-12p35 expression was consistently significantly higher than baseline although there was no variation between disease severities. Expression of TGFβ fluctuated around baseline levels. Differences were observed between consecutive disease severities such that expression was significantly different between mice scoring 0 and 1, 1 and 2, and 2 and 3, representative of an inconsistent expression profile. IL-10 expression showed peaks at mid-disease and in unaffected mice, which were significantly higher than all other scores and baseline C57BL/6 expression. Therefore, expression of cytokines in the periphery did not show a clear pattern that reflected disease progression. However, as with the flow cytometric analysis on splenocytes, this result is not surprising as the inflammation in CNS restricted and rather shows that the inflammation is limited to the CNS and not a systemic condition.

Investigation of transcription in the spinal cord would provide more informative data and due to the rapidity of sample collection, allows a direct look at the inflamed tissue without any potential manipulation of phenotype by *in vitro* conditioning. Higher than baseline expression is to be expected in this tissue due to the larger cellular infiltrate in affected mice. There would also be expected to be increases in transcription similar to the increases in infiltration if cells are either migrating in already polarized or become polarized once they reach the site of inflammation.

In the spinal cord, IFNγ was expressed consistently higher than baseline and showed a peak of expression relative to other disease scores in a similar pattern to the flow cytometry data. Significantly lower expression was seen in mice of pre or early-disease and also in mice that resisted signs of disease, but in all cases, expression was significantly elevated above baseline levels, likely representative of the cellular infiltrate in all mice. In a similar way, IL-17 transcription mirrored that seen from the FACS analysis as the only peak in transcription was observed at the highest disease severity. However, even at this peak, expression levels were not significantly different to baseline despite the large cellular infiltrate. These data confirm the findings from the cell extraction and acts to endorse the reliability of the *in vitro* stimulation.

As with the spleen analysis, expression of cytokines important in polarizing and maintenance of T helper subsets was investigated. IL-6, important in polarizing Th17 cells was only observed at levels above baseline in mid and high disease mice. In this way it could be seen to precede the IL-17 expression and would be indicative of the development of a polarizing environment. Significantly increased expression with respect to baseline was not seen in mice in pre or early disease stages but expression of

IL-23p19, thought to be required to maintain the Th17 phenotype, was increased at these times. Expression returned to baseline during mid disease but significantly rose again at peak disease signs. Both cytokines were expressed at baseline levels in mice resisting disease. IL-12p35 showed a trend towards increased expression with increased disease severity but was only observed at greater than baseline levels at mid disease. There was however significantly higher transcription in the spinal cord of mice resisting signs of disease than those prior to development of signs, although the increase is not particularly large.

The patterns of IL-10 and TGFβ expression were very similar. Expression of both IL-10 and TGFβ peaked at score 2 and both were expressed significantly higher than baseline and early stages of disease in the spinal cord at the largest disease scores (2 and 3). Baseline levels of expression were observed in pre and early disease mice and high transcription was observed in both in mice resisting signs of disease although it only showed statistical significance for TGFβ. This high transcription of anti-inflammatory cytokines is presumably a response in the inflamed tissue to try and limit further inflammation. Overall, the cytokine profile in the mice resisting signs of disease appeared to indicate a controlled level of sub-clinical disease.

Transcriptional profile data is summarized in Figure 7C and D. These show the expression changes as lines against disease severity, such that a positive gradient is representative of an increase in expression as disease develops whilst a flat line represents no change with disease. Only the most important genes involved in Th1 and Th17 polarization have been included to help highlight the differences. From these it shows that there is relatively little change in gene expression with disease in the spleen (Figure 7C) as all the

lines remain close to horizontal from pre-disease to peak disease. In the CNS however, Th17 genes (IL-17, RORγt and IL-6) do not begin to increase until after disease is established. RORγt expression never reaches comparable C57BL/6 levels whilst IL-6 increases after score 1 and IL-17 is only observed increasing after score 2. In contrast, both T-bet and IFNγ peak at score 2 similar to the FACS data on infiltrating cells and show higher relative expression than their respective Th17 genes.

10.2.5 Treg Infiltration

A previous observation made in the lab was a paradoxically high proportion of $CD4^+CD25^+$ cells, presumed to be Tregs, extracted from the CNS of affected mice; (a previous project student in the lab had indicated that around a third of the lymphocyte infiltrate may comprise Tregs; (Felicity Holt, MSc Thesis, Imperial College). The observation of a strong Treg component was confirmed though analysis of Foxp3 transcription, as measured by real-time PCR, correlating directly with disease progression (Figure 9A). Increased transcription was observed above levels seen in C57BL/6 mice and this increased as the mice became more affected, reaching a peak transcription at peak disease, and also being at lower levels in those mice never affected (Figure 7B).

By crossing the Line7 line with the $Foxp3^{DTR}$ line, first described by the Rudensky lab (Kim 2007) to generate a new line, termed L7Fox, it was possible to directly show Treg cells in the CNS. Tregs were observed as GFP^+ cells seen both in the lymphocyte gate of the cellular infiltrate, by flow cytometry (Figure 1B) and by immunofluorescence on cryosections (Figure 10).

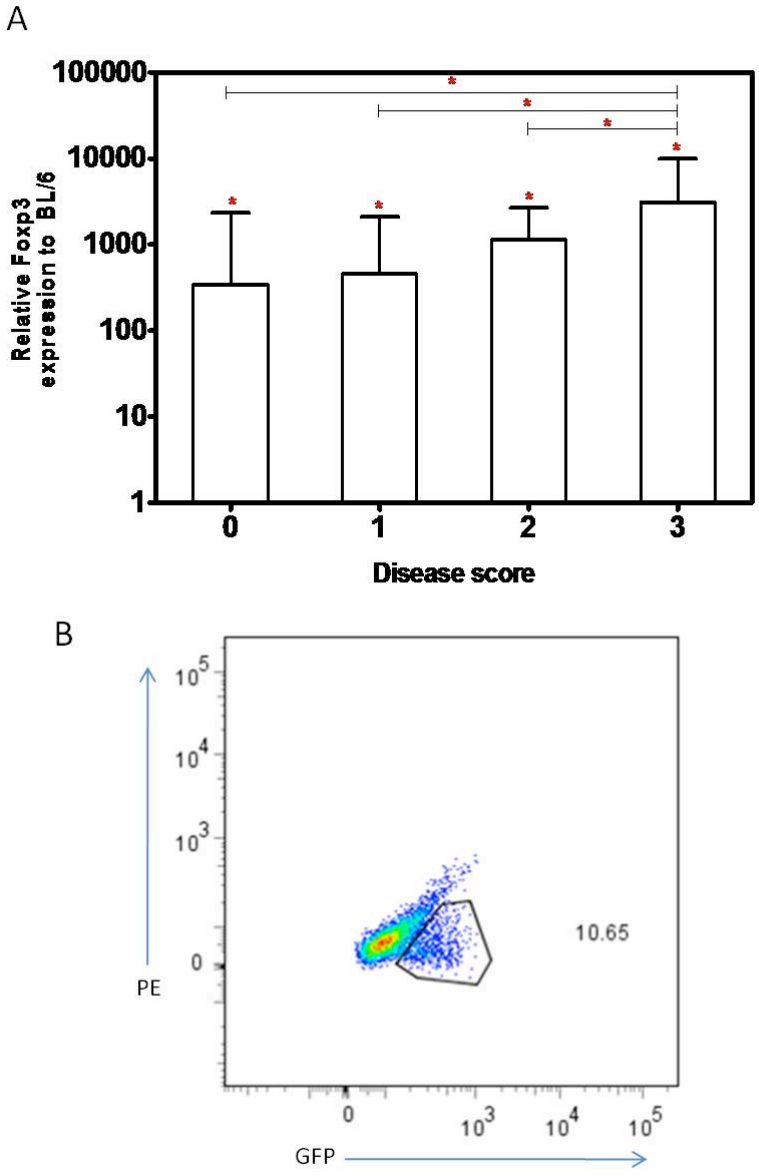


Figure 9. Infiltration of Tregs into CNS correlating with increased disease severity.

*A – Real-time PCR assessment of Foxp3 mRNA transcript expression in spinal cord of mice at different stages of disease. mRNA was extracted from spinal cord tissue, reverse transcribed to cDNA before real-time PCR analysis with an individually designed hydrolysis probe assay. Shown with * to show significance compared to control ($p<0.05$) and ** to show significance between scores ($p<0.05$) (randomisation analysis). n=27 total, 6 mice per disease score (3 male and 3 female) and 3 C57BL/6 controls (all female).*

B – Representative image of GFP$^+$ cells extracted from CNS of score 3 L7Fox mouse. Lymphocytes were extracted from CNS of L7 mice following perfusion, enzymatic digestion and separation on percoll density gradients. Percentage of total lymphocyte population is shown. No PE antibody was used to stain cells but the PE axis was used to eliminate autofluorescent cells.

A typical flow cytometry plot is shown illustrating the GFP$^+$ cell population within the cellular infiltrate. Using this approach, the percentage of Tregs is reduced from the original estimates of around 30% to around 10%, but this can be explained by using Foxp3 as a marker rather than CD25 and the elimination of activated cells from the Treg gate. The Tregs cells show an infiltration pattern similar to other infiltrating cell populations, infiltrating initially through the lower regions of the spinal cord and entering via the dorsal and ventral fissures (Figure 10A, B, C). They show some aggregation in the meningeal tissue and in the regions where other cells are infiltrating. Such a pattern of infiltration was not observed in an unaffected L7Fox littermate illustrating that the Tregs do migrate to the site of inflammation and are not purely resident there (Figure 10C). In addition, Tregs are also found throughout the spinal cord in areas with large cellular infiltrates (Figure 10E, F, G), evenly dispersed throughout the tissue. They can be seen occupying the same locations as NK (Figure 10B) and CD8 (Figure 10D, F) effector cell populations during infiltration, illustrating the potential that they may act directly to inhibit inflammation. As with the earlier immunofluorescence, this confirms a Treg presence in the CNS and supports previous observations but requires further investigation to determine functionality.

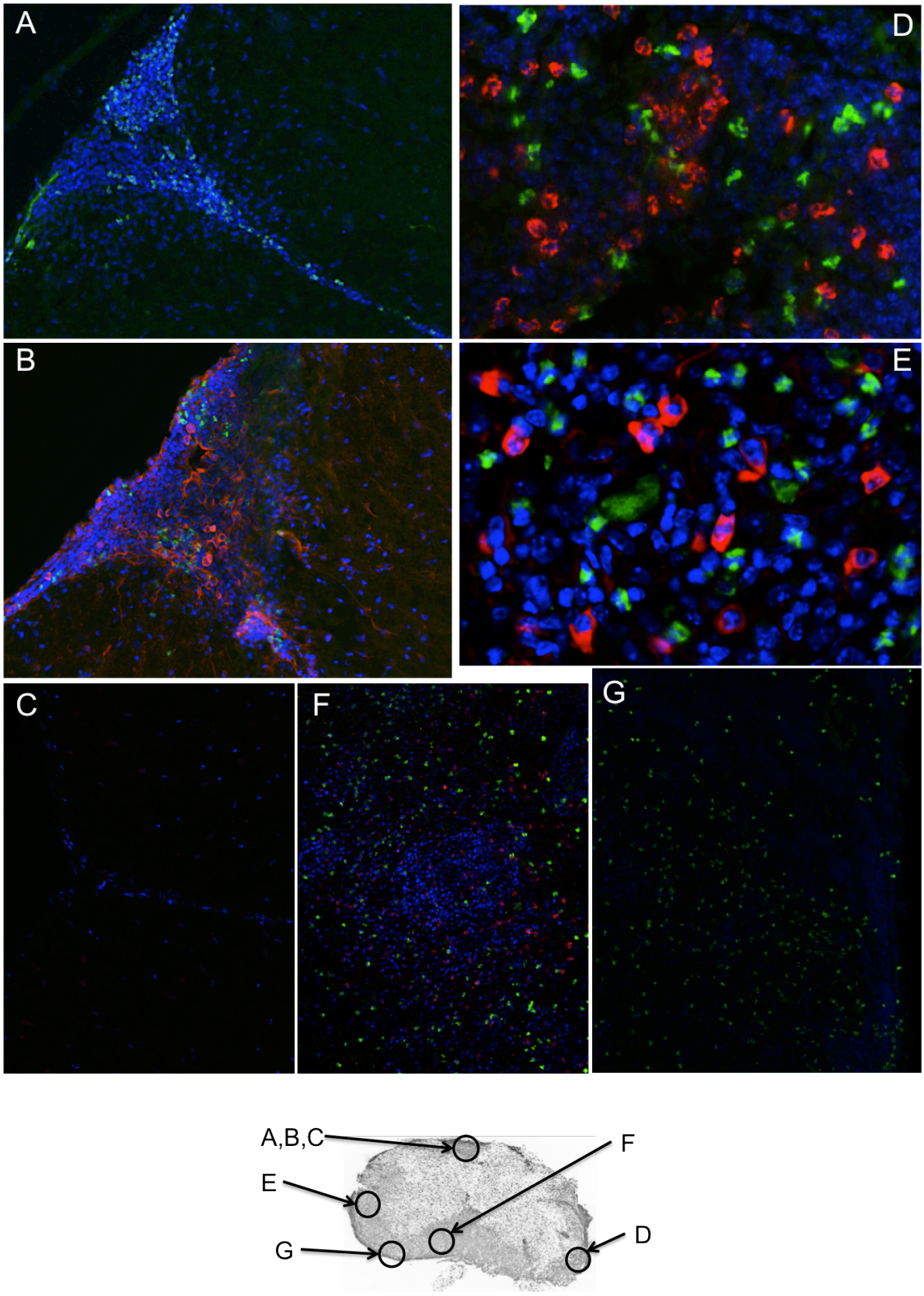


Figure 10. CNS infiltration of Tregs.

L7Fox mice from distinct disease landmarks were fix/perfused and 10μm sections cut. Representative 10X pictures are shown from indicated locations in the lumbar spinal cord. A – Treg accumulation and infiltration from meningeal tissue in the dorsal fissure, 10X view of score 4 L7Fox, GFP (green) DAPI (blue). B – Tregs found at same location as infiltrating NK cells in the dorsal fissure, at 10X magnification, of a score 4 L7Fox mouse, NK1.1 (red) GFP (green) DAPI (blue). C – 10X view of dorsal fissure from unaffected L7Fox littermate showing no accumulation of Tregs in mice with no presentation of symptoms, CD8 (red) GFP (green) DAPI (blue). D – Large population of Tregs co-localising with infiltrating CD8 T cells, at 20X magnification in a score 4 L7Fox mouse, CD8 (red) GFP (green) DAPI (blue). E – Infiltrating $CD4^+$ Tregs alongside GFP-$CD4^+$ T cells in the white matter, at 20X magnification, in a score 4 L7Fox mouse, CD4 (red) GFP (green) DAPI (blue). F – Tregs throughout the white matter surrounding large aggregation of infiltrating effector cells, at 10X magnification, in a score 4 L7Fox mouse, CD8 (red) GFP (green) DAPI (blue). G – Large distribution of Tregs throughout white matter, not constrained to the meningeal tissue, at 10X magnification, in a score 4 L7Fox mouse, staining as (A).

10.3 Discussion

The basic immunological characterization of a model is crucial in order to lay the foundations for further experiments, especially in a spontaneous model. Appreciation of how disease develops without any interference from outside factors enables unbiased interpretations to be drawn and highlights the large effects that immune adjuvants can have on our understanding of autoimmunity. While a number of other labs have published spontaneous, TCR transgenic models of EAE-like disease (Goverman 1993, Lafaille 1994, Waldner 2000), the model used here is the only one in which there is a high level of spontaneous disease in a model driven by a TCR derived from a human MS patient.

Evidence for a role for cells in the development of disease is provided by the correlation of disease severity with cellular infiltration. This simple count supports a direct role for infiltrating cells in mediating neuroinflammation, a process that can be prevented by limitation of cellular infiltration, as observed in mice resisting disease development. Cytokine positive cells could be extracted from the CNS of non-diseased old mice illustrating that *in situ* activation was occurring but the lack of substantial cell numbers likely prevented development of autoimmunity.

MS is thought to be a CD4-mediated disease by extrapolation from work in EAE (Ben-Nun 1981, Pettinelli 1981), and analysis of the cellular dynamics in the Line7 mouse would support this conclusion. Infiltration of $CD4^+$ cells correlated with disease initiation suggesting they were directly involved in the process, and this would not be surprising as the model utilizes an HLA class II restricted TCR and and also carries the cognate HLA-DR15 presenting molecule (Ellmerich 2005). However, what was surprising was the large

proportion of the cellular infiltrate that was NK1.1^{+}. These are assumed to be NK cells, although the contribution of NKT cells to this population was not addressed in this study. A major role of NKT cells is the production of a wide range of cytokines and so it could be cytokine production is derived from NKT rather than NK cells and this would require further investigation.

NK cells have been found in demyelinating MS lesions (Traugott 1985) but the scale of the infiltration was unexpected. The role these cells play in the disease dynamics was not investigated at this point but the case could be made that their infiltration is not directly linked to disease progression as no change in their proportion of the infiltrate was observed. The activation state of the NK1.1^{+} cells infiltrating, as measured by IFNγ production, however correlates with disease initiation and progression. It has been shown that the secretion of IFNγ from NK or NKT cells can play a role in driving Th1 cell polarization (Martin-Fontecha 2004, Morandi 2006) or exacerbate existing inflammation (Wilson 2003, Kent 2005), which in this model may act to amplify the activity of Th1 cells in the early stage of disease. Despite the proportion of the infiltrate not increasing, the number of NK cells infiltrating does increase as disease progresses. NK cells have been shown to possess cytotoxic activity directed at neurons (Backstrom 2003) oligodendrocytes, astrocytes and microglial cells (Antel 1998, Morse 2001, Saikali 2007) so it could be that they are directly responsible for immune damage although their migration pattern does not highlight a leading role.

In contrast, CNS infiltrating NK cells have been seen to produce neuroprotective factors such as brain-derived neurotrophic growth factor and neurotropin-3 (Hammarberg 2000)

during EAE. The infiltration, albeit at a consistent rate could be a reaction to the inflammation and NK cells are acting to protect the CNS from immune-mediated damage. The infiltration is very high despite, and despite not correlating with disease severity; $NK1.1^+$ cells still make up the largest infiltrating lymphocyte population. Both protective and pathogenic roles have been suggested for NK cells although it is impossible at this stage to delineate which is the most important in this context. NK cells in the CNS have been shown to control the magnitude of local inflammatory and autoimmune responses against myelin (Hao 2010) and so it is likely their role in Line7 mice encompasses a balance between both this protective role and an indirect pathogenic role, mediated through release of pro-inflammatory cytokines.

Analysis of the dynamics of cytokine positive cells was the most informative result from this model. The discovery that, without the use of any immune adjuvants or polarized cell populations, $IL\text{-}17^+$ cells were only observed at peak disease and not seen in any number at scores before that, suggested that they were not involved in the initiation of autoimmunity. This is in stark contrast to other studies which have concluded that it is Th17 cells which initiate autoimmunity (Hofstetter 2005, Langrish 2005, Komiyama 2006). Whilst a disregard for the importance of Th17 cells in Line7 disease as a whole would be naïve, this evidence would support the conclusion that any Th17 cells were only involved in the progression of a previously initiated autoimmune attack. The observation of IL-17 derived from $IFN\gamma^+$ cells at high disease also adds a twist on interpretation of results as it would support a potential change in cytokine profile from the same cells. In other studies, $T\text{-}bet^+ROR\gamma t^+$ cells have been identified (Abromson-Leeman 2009) and it is likely that these are also positive for those transcription factors too. This hints at the

plasticity of T helper cells switching from a Th1 to Th17 phenotype, a dynamic not recognised as being possible from work in other studies (Shi 2008, Wei 2009).

IFNγ^+ cells on the contrary were readily identified in early diseased mice whilst not evident before, a pattern indicative of direct involvement. The peak infiltration, as measured by proportion of the infiltrate, is at score 2 (mid-disease) and the proportion tails off at peak disease. Cellular infiltration however continues to increase and so whilst a decrease in their proportion suggests a lack of involvement, the absolute numbers of IFNγ^+ infiltrating cells actually continued to increase. Not all of the IFNγ was derived from Th1 cells though. Cytokine positive cells were observed within CD4, CD8 and NK cell populations as disease progressed supporting a level of redundancy in the production of pro-inflammatory cytokines. However, it is not clear whether each mediates neuroinflamation, or whether the production of IFNγ by NK cells for example, may act to aid in the polarisation of Th1 cells in the CNS as seen by other studies (Martin-Fontecha 2004, Morandi 2006).

Transcriptional analysis has a very important role to play in qualifying flow cytometry data as it is a direct measure of gene activation within the inflamed tissue. Observation of spinal cord transcripts across disease mirrored the pattern seen from the FACS data, showing IL-17 transcription appearing only once disease had already initiated and peaking at high disease. T-bet and IFNγ transcription was detectable and peaked earlier, adding further support to their importance in the early stages of disease. Studies have suggested T-bet defines the encephalitogenicity of T cells, and these findings would support that conclusion (Gocke 2007, Yang 2009b). The highest CNS transcription of GATA3, a Th2 transcription factor, in mice that resisted signs of disease provides indirect

support for the importance of Th1 or Th17 cells in mediation of disease. In these mice, a Th2 response would act to limit the Th1 or Th17 activation, suggesting potentially that a localised anti-inflammatory boost may act to limit CNS autoimmunity. However, previous studies have shown MBP-specific Th2 cells to be capable of causing EAE (Lafaille 1997), and so any steps in that direction should be taken with care.

Very noticeable was the lack transcription of any Th17 polarizing cytokines and RORγt in the spinal cord until disease had established. Previous studies in RORγt$^{-/-}$ mice showed mild EAE however, illustrating that RORγt is not required for EAE induction but more likely exacerbates it (Ivanov 2006). IL-6 transcription was first seen at score 2 and steadily increased, followed by IL-17 expression. IL-17 and IL-6 mRNA has been observed in lesions in other studies (Lock 2002) but because they were only observed in post-mortem tissue and at peak of disease, such observations would also have been seen in the Line7 if the earlier scoring mice were ignored. Despite high IL-23 expression at early and peak disease stages, however, RORγt expression did not surpass that seen in a non-inflamed C57BL/6 CNS. Therefore, the CNS cytokine environment appears conducive to Th17 expansion but not differentiation until peak disease symptoms. It also suggests that IL-23 may play multiple roles in the course of disease. In the initial stages it is upregulated in an attempt to limit infiltration (Langowski 2006) whilst the upregulation at peak disease is to aid in the expansion of the Th17 population (Stritesky 2008).

These data indicated that the failure to identify IL-17^{+} cells was not an artifact but representative of the local environment. Taken alongside the flow cytometry data, they would suggest a mechanism whereby Th1 cells (T-bet^{+}IFNγ^{+}) are involved in the initiation of an autoimmune attack and Th17 cells (RORγt^{+}IL-17^{+}) are only involved in the

progression, and subsequent development of such an attack. Th1 cells have been shown to accumulate in both the human and mouse CNS (Traugott 1988, Olsson 1990, Link 1992, Merrill 1992) and this data would support other findings concluding that Th17 cells require Th1 cells to infiltrate the CNS (O'Connor 2008). It would add weight to the argument that the importance of Th17 cells in autoimmunity is perhaps confounded by reliance on the immune adjuvants used in active EAE (Chen 2006) and the impossibility of generating pure Th17 cell cultures free from Th1 cells used for passive EAE (Langrish 2005, McGeachy 2007). No transcriptional predisposition towards Th1 or Th17 phenotypes could be observed in the periphery throughout disease, further supporting the importance and relevance of these differences within the target organ of inflammation.

In addition to the dynamics of pro-inflammatory T cells in disease development, the Line 7 model is supportive of an important role for Tregs in autoimmunity. Whilst not investigating the functionality of Tregs at this stage, the observation that they accumulate in the inflamed CNS demonstrates the presence of an anti-inflammatory response occurring throughout the lifespan of the mice. The accumulation is proportional to both the age and disease severity score of the mice, indicative of a continual migration or proliferation of Tregs as disease is progressing. Increases in Foxp3 transcription in the periphery are not seen throughout disease and levels remain comparable to unaffected C57BL/6 mice, demonstrating that this is not a systemic Treg upregulation. Tregs have been seen to accumulate in the CNS of other EAE models and have been associated with the recovery of animals from signs of disease (McGeachy 2005, Liu 2006b, Korn 2007b). These observations are supported by this model but the

recovery is not seen, despite the increase in Treg numbers. Tregs readily infiltrate alongside autoreactive cells, and the observable increase in CNS Foxp3 expression, implying that regulation is occurring continually throughout disease progression.

In Line 7 mice, autoimmunity progresses without an adjuvant required to impair the function of Tregs. This would suggest impaired function of Tregs in the Line 7, especially as migration does not appear to be impaired. In one study the absence of Tregs in the MS lesion was attributed to poor migration, something not seen in this model (Tzartos 2008). Without direct testing however, it would be premature to come to this conclusion and so this issue will be addressed in the next chapter through the use of the newly created L7Fox line which offers great benefits in visualisation and extraction of Tregs over previous attempts in an autoimmune setting.

11 Role of Tregs and Effector Cells

11.1 Introduction

Previous work in the laboratory had shown the presence of abundant $CD4^+CD25^+$ lymphocytes in the CNS of a heavily diseased Line7 mice, in line with other studies in both mice and humans (Felicity Holt, MSc Thesis Imperial College London), and confirmed by the work described here in the preceding chapter. This somewhat paradoxical increase in Treg infiltration led to outstanding questions regarding the functionality of the Tregs. MS has been considered a disease of aberrant regulation (Viglietta 2004) yet considerable numbers of Tregs clearly enter the CNS during disease; are they inherently dysfunctional, or perhaps simply fail to control the overwhelming force of pathogenic cells?

Functionality of Tregs, as measured by *in vitro* suppression assays of mitogenesis, was impaired in the context of MS. Tregs, sorted as $CD4^+CD25^+$, derived from MS patients' blood demonstrated a reduction in ability to suppress stimulated effector cells, either by myelin peptides or more general stimulations (Viglietta 2004, Haas 2005, Kumar 2006, Feger 2007, Venken 2008a, Frisullo 2009). Accumulation of Tregs in CSF (Venken 2008b), the closest source available to the inflamed tissue, and reduction in Foxp3 levels on a per cell basis (Huan 2005, Venken 2008b) adds support to the conclusion that their function may be impaired.

Depletion of the Treg population in mice has been shown to increase disease severity (Zhang 2004b), decrease the threshold of antigen required (Stephens 2005), and inhibit the recovery of animals (Korn 2007b) in the EAE model. Conversely, the introduction of

Tregs prior to EAE induction prevents the development of disease (Olivares-Villagomez 1998) and even if administered alongside autoreactive T cell blasts, reduces disease severity (Kohm 2002). These findings point to an important role of Tregs in preventing the initiation and subsequent severity of autoimmune reactions, but direct studies in human patients are limited by the source of Tregs. Findings from human blood-derived Tregs are not directly transferable to the situation in the inflamed tissue, rather are suggestive of a more general context of autoimmunity.

The limitation that Tregs cannot be extracted from the site of inflammation during disease from human tissue can be overcome through the use of mouse models. It has already been demonstrated by us and other groups that Tregs accumulate in the CNS throughout CNS autoimmunity (McGeachy 2005, Liu 2006b, Korn 2007b), allowing the investigation of these cells once extracted from the inflamed CNS. Tregs taken during the recovery phase of EAE were shown to be capable of suppressing MOG-specific proliferation and release of IFNγ *in vitro* (O'Connor 2007), however another group extracted Tregs from peak disease and did not demonstrate any suppressive action on CNS-derived effector T cells (Korn 2007b). Therefore Tregs have been implicated in bringing about the natural recovery seen in some EAE models, whilst the inflamed CNS has been shown to drive the proliferation of Treg cells (Korn 2007b, O'Connor 2007), likely in an attempt to control the inflammation.

Up until now, the investigation into Treg function has been limited by the ability to specifically identify and extract Tregs from desired samples. Initially Tregs were identified as being $CD4^{+}CD25^{+}$ until the discovery of Foxp3 as the characteristic transcription factor (Fontenot 2003, Hori 2004) allowed better identification of these

cells and exclusion of contaminating populations. However, the limitation of Foxp3 is that it resides in the nucleus and so it is unavailable to use as a marker for sorting and then further culture of Tregs. CD25 (IL-2Rα) is not present solely on Tregs but also on activated T cells (Leonard 1985, Thèze 1996) meaning that the sorted populations of Tregs could include effector cells, compromising suppression studies and leading to false conclusions. Studies aiming to deplete Tregs also had to rely on CD25, and used a depleting antibody, PC61 to remove any $CD25^{+}$ cells. There is debate as to the effectiveness of PC61 in depleting Treg populations (Kohm 2006, Stephens 2006, Zelenay 2006), as despite the removal of $CD25^{hi}$ cells, cells that were $Foxp3^{+}$ remained (Kohm 2006). In addition, the contribution of removal of activated cells could not be excluded from the reduction in disease signs and prevention of autoimmunity observed. There have also been recent attempts to deplete Tregs through the use of a depleting GITR antibody (Coe 2010). These provide a potential depleting mechanism in place of the use of anti-CD25 antibodies. The suppressive function of Tregs is unaffected whilst ligation with DTA-1 antibody simply selects Tregs for depletion but this protocol still relies on surface markers and GITR, similar to CD25, is not restricted to Treg cells.

Therefore the L7Fox strain was created in order to allow targeted depletion of Tregs without affecting any other cell populations. The administration of diphtheria toxin depletes the Treg population and allows direct observation of the progression of autoimmunity in these animals without the regulatory component of the immune system present (Kim 2009). The added advantage of using these mice is the GFP expression from the disrupted Foxp3 locus (Kim 2007). This allows ease of identification of Tregs and most importantly the ability to sort Tregs without risk of contamination from effector cell

populations. The added advantage to using this protocol is the absence of immune adjuvants required to initiate disease. The use of such adjuvants has been shown to impair Treg function (Cassan 2006, Chen 2006) and so the aim of such work will be to identify whether the impairment of function at peak EAE was a result of the immunization protocol or a representative state of autoimmunity.

In addition to observations of the impact of Tregs on the course of disease, the removal of Tregs will highlight which effector cells are under the strongest Treg control in the autoimmune context. This will implicate protective or pathogenic roles for these cells in the development of paralysis in the Line7 model. In addition, the use of depleting antibodies will be used to specifically remove CD8 or NK cells to add additional data with regard to their roles. Mixed results have been generated from previous depletion and knockout studies in EAE. Depletion of NK cells has been shown to exacerbate EAE (Zhang 1997a, Matsumoto 1998, Xu 2005) or reduce severity (Winkler-Pickett 2008) whilst use of $CD8^{-/-}$ mice has lead to a reduction in CNS inflammation (Abdul-Majid 2003). Therefore the results generated in the Line7 model would contribute to the debate regarding their roles.

Identification of Treg control on either population combined with less severe disease upon cellular depletion would implicate a pathogenic role in disease initiation. In this way, the degree of Treg control over disease initiation and progression can be determined and combined with data highlighting the relative importance of effector cell types in mediating this autoimmunity.

11.2 Results

11.2.1 Unabated Development of Paralysis without the presence of Tregs

In order to look at the role that Tregs might play in limiting spontaneous disease in the Line7 mice, a new line was created called L7Fox. This was created by crossing Line7 with Foxp3^{DTR} mice generated by Alexander Rudensky (University of Washington). Line7 mice were mated with Foxp3^{DTR} mice for at least three generations to provide mice homozygous for both the Line7 and Foxp3^{DTR} transgenes. The presence of transgenes was confirmed by PCR analysis as outlined in the materials and methods.

Foxp3^{DTR} mice express GFP in the presence of Foxp3, under the control of a repressible promoter. The promoter is reactive to the presence of diphtheria toxin, such that the addition of DT depletes the Treg population. L7Fox mice share the characteristics of both founder lines and allow investigation of Treg dynamics within a model of autoimmunity.

DT was administered every other day in young (8 week old) L7Fox mice, beginning on day 0. 3 male and 6 female mice were used in both the DT and PS control group. This age of mice is before they present signs of disease under normal circumstances, and the regimen of DT administration was to maintain complete depletion of the Treg population. Previous work had shown that depletion of Tregs was achieved for 2 days after the initial dose of DT, (Kim 2007) and after consideration of other depletion experiments investigating Treg return that were carried out in the lab, regular doses very other day were administered to allow this depletion to be extended. Ablation of Tregs was near complete with less than 0.3% of total lymphocytes remaining Foxp3^{+} in mice upon removal from the study (Figure 11D). The ablation of Tregs allowed disease to

progress unabated in 100% of mice compare to 0% of control mice of this age given PBS (Figure 11A).

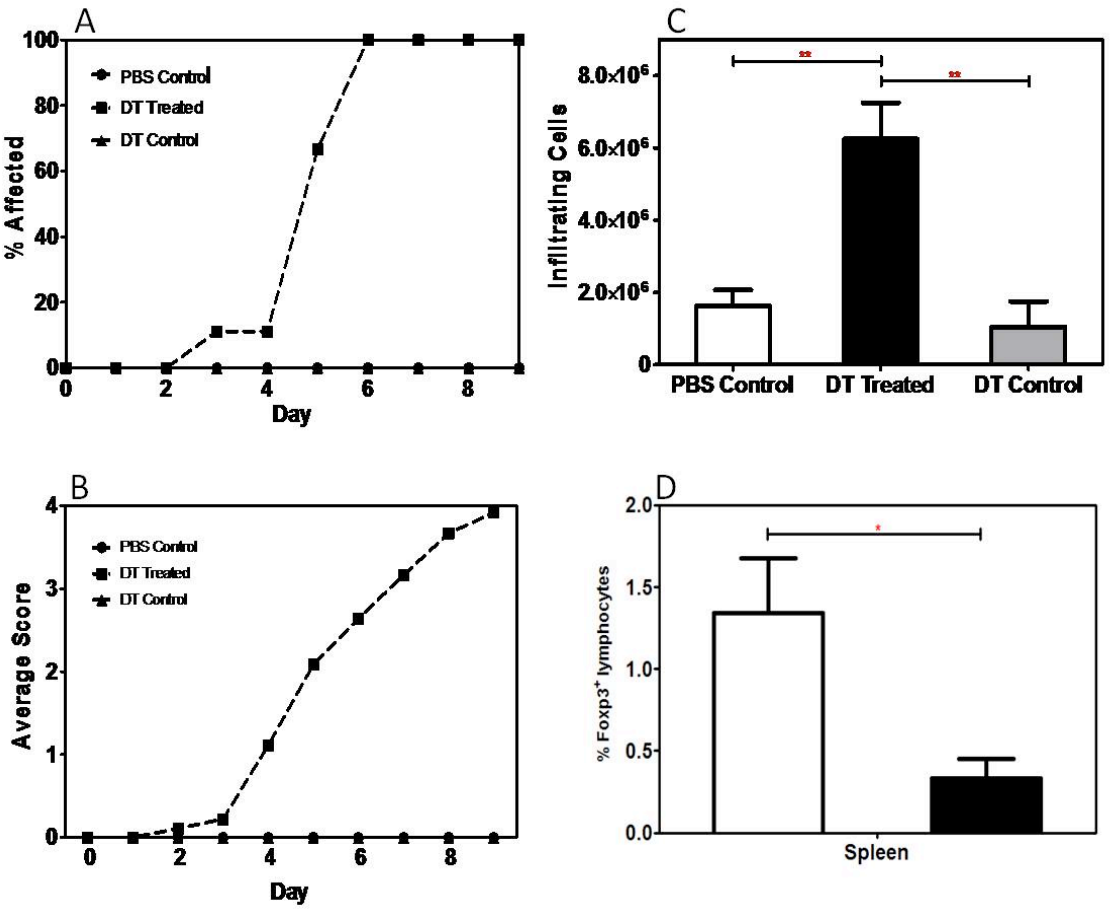


Figure 11. Complete removal of Tregs leads to acute progression of cellular infiltration and disease symptoms.

A – Percentage of mice affected in the three groups; L7Fox administered DT, L7Fox administered PBS, L7 administered DT, through the length of the study. B – Scores of all mice in the study averaged within groups to generate mean disease progression. (n=22 total). C – Counts of CNS infiltrating cells taken when mice were removed from the study. CNS infiltrating cells were extracted following perfusion, enzymatic digestion of CNS tissue and lymphocytes separated using percoll density gradients. PBS controls were

*taken at the same time as affected mice and DT control mice were taken at the conclusion of the study. (n=10 total). D – Counts of splenic Foxp3^{+} lymphocytes based upon GFP expression in spleen of DT treated (black) and matched PBS control (white) L7Fox mice when they were removed from the study. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

Mice were observed everyday and their score recorded. An average score for mice in the study was calculated by adding the scores of all mice and dividing by the number of mice in the study. Mice were culled and samples taken at the point where full hind limb paralysis had been achieved and the paralysis was serious enough to risk the animals survival. Upon removal from the study, the final score of the mouse was used for each subsequent day to calculate the average for the study. This was necessary to provide a realistic average score for the study, not assuming that disease scores would increase but not losing the impact of a mouse that had progressed through to full disease on the average for the colony. The first signs of disease were seen after as few as 2 days after the initial dose of DT, but all mice had presented within a week and by day 9, all mice had progressed through to full hind limb paralysis (B). As a further control, DT was administered to 4 Line7 mice (2 male, 2 female) lacking the Foxp3^{DTR} construct, to control for any effects of DT itself. These mice were 4 months of age, at the time when signs of disease would be expected to be presented. In these circumstances, no presentation or enhancement of disease signs was observed, and the cellular infiltration was comparable

to PBS controls. Removal of Tregs led to mass infiltration of cells into the CNS at significantly higher levels than compared to both the PBS and DT controls (Figure 11C).

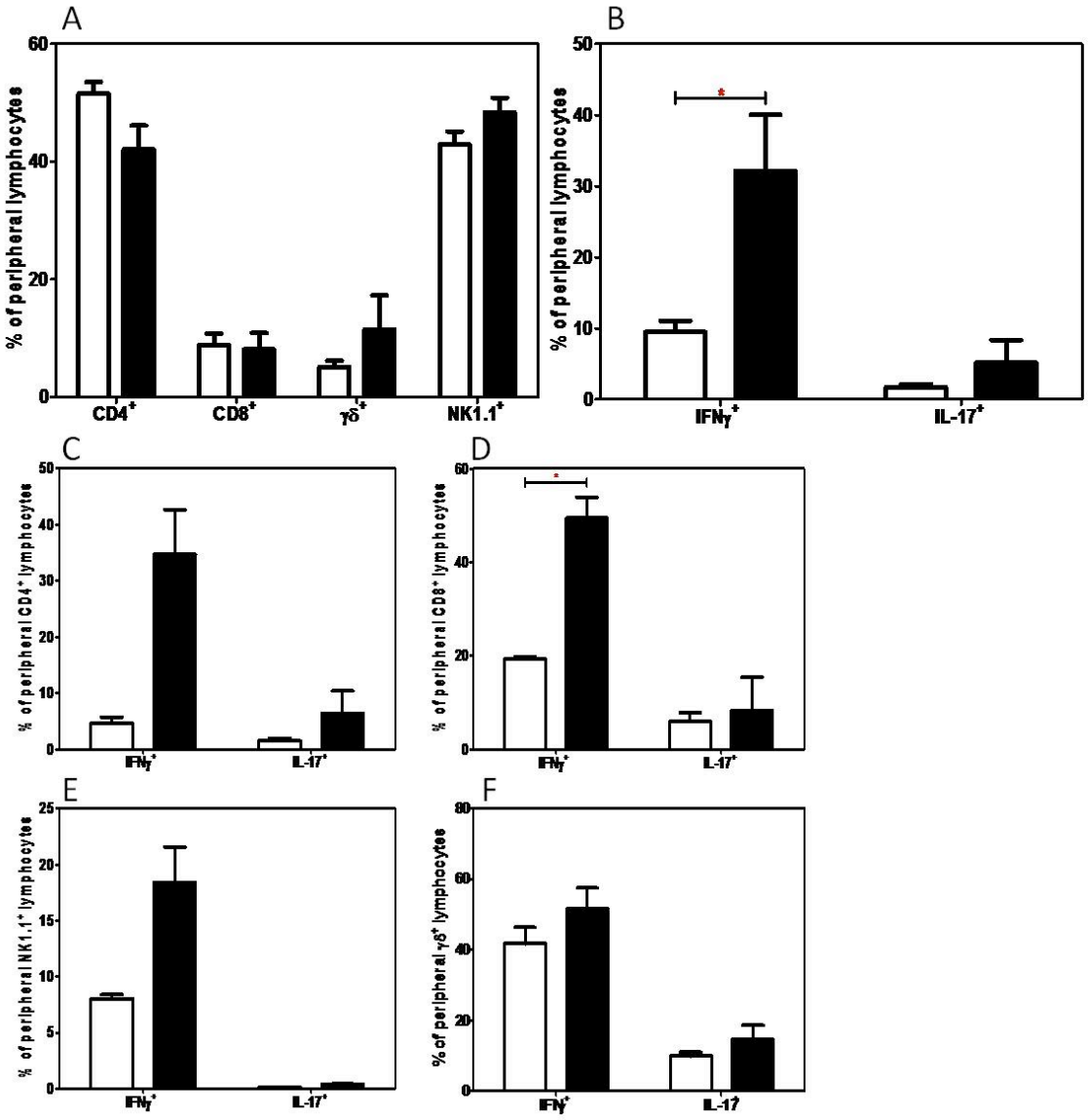


Figure 12. Peripheral cell dynamics after Treg depletion.

Surface and intracellular cytokine staining of peripheral lymphocytes extracted from spleens of affected DT treated mice (black) and matched PBS controls (white) after stimulation for 4h with PMA/ionomycin and BFA. Cells were extracted from mice when

*they were removed from the study. A – surface staining of total spleen-derived lymphocytes. IFNγ and IL-17 secreting cells as shown as percentage of total lymphocyte (B), CD4 (C), CD8 (D), NK (E), and γδ (F) cell populations. (n=3 per group). Lymphocytes were gated initially according to cell surface markers and positive cells interrogated to determine the size of cytokine positive populations. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

The functional effect of Treg ablation was then assessed by flow cytometric analysis of splenic and CNS-infiltrating lymphocytes, looking for any resulting changes in intracellular cytokine staining from CD4, CD8, NK and γδ cells. The same extraction protocol was utilised as described before, allowing individual mice to be investigated separately. Upon removal of an affected DT administered mouse, a sex matched corresponding PBS control mouse was also removed. This was to control for both the reagents used and the time since the last injection had been administered. Lymphocyte populations were non-specifically stimulated with PMA and ionomycin in the presence of BFA. They were stained with fluorescently conjugated antibodies for cell surface markers and permeabilised to allow the use of antibodies for intracellularly sequestered cyokine production. Two-tailed t tests were used with Welch's correction for unequal variances to compare means between cellular populations. After removal of Tregs, there was no significant variation in the proportion of CD4, CD8, γδ and NK cell populations in the periphery (Figure 12A) although there is a slight decrease in the proportion of CD4 cells and minor increases in the NK cell and γδ cell proportions (Figure 12A). The decrease in

the CD4 population could be explained as the removal of Tregs whilst the other proportions will adjust in reaction. At a functional level, as expected, control was released on cytokine production. IFNγ and IL-17 secreting cell populations increase in the absence of Tregs, but only the increase in IFNγ population shows statistical significance (Figure 12B). The IFNγ response shows a larger increase than IL-17, reaching around a third of lymphocytes producing IFNγ, and is represented in the statistical analysis. By analyzing the cytokine positive cells after selection of populations positive for each cell surface marker, it could be seen that the CD4, CD8 and NK cell populations all demonstrated a similar significant increase in IFNγ production although due to small sample numbers, only CD8 reached significance. γδ cells demonstrated a trend towards an increase of IFNγ production but this did not achieve statistical significance (Figure 12F). Greater proportions of positive cells can be seen in all analyzed cell populations with respect to release of IFNγ as compared to IL-17. There is a trend towards enhancement of IL-17 in both the CD4 and γδ populations although not statistically significant (Figure 12C,F). With respect to the peripheral response, the findings appeared to suggest that the impact of Treg ablation on disease exacerbation was to a far greater extent attributable to the release from regulation of IFNγ than IL-17 responses. Note that, in this model, since the TCR is carried on a RAG-sufficient background there is a full repertoire of HLA-DR15-restricted responses to other antigens apart from MBP, including epitope spread to other myelin antigens (Ellmerich 2004).

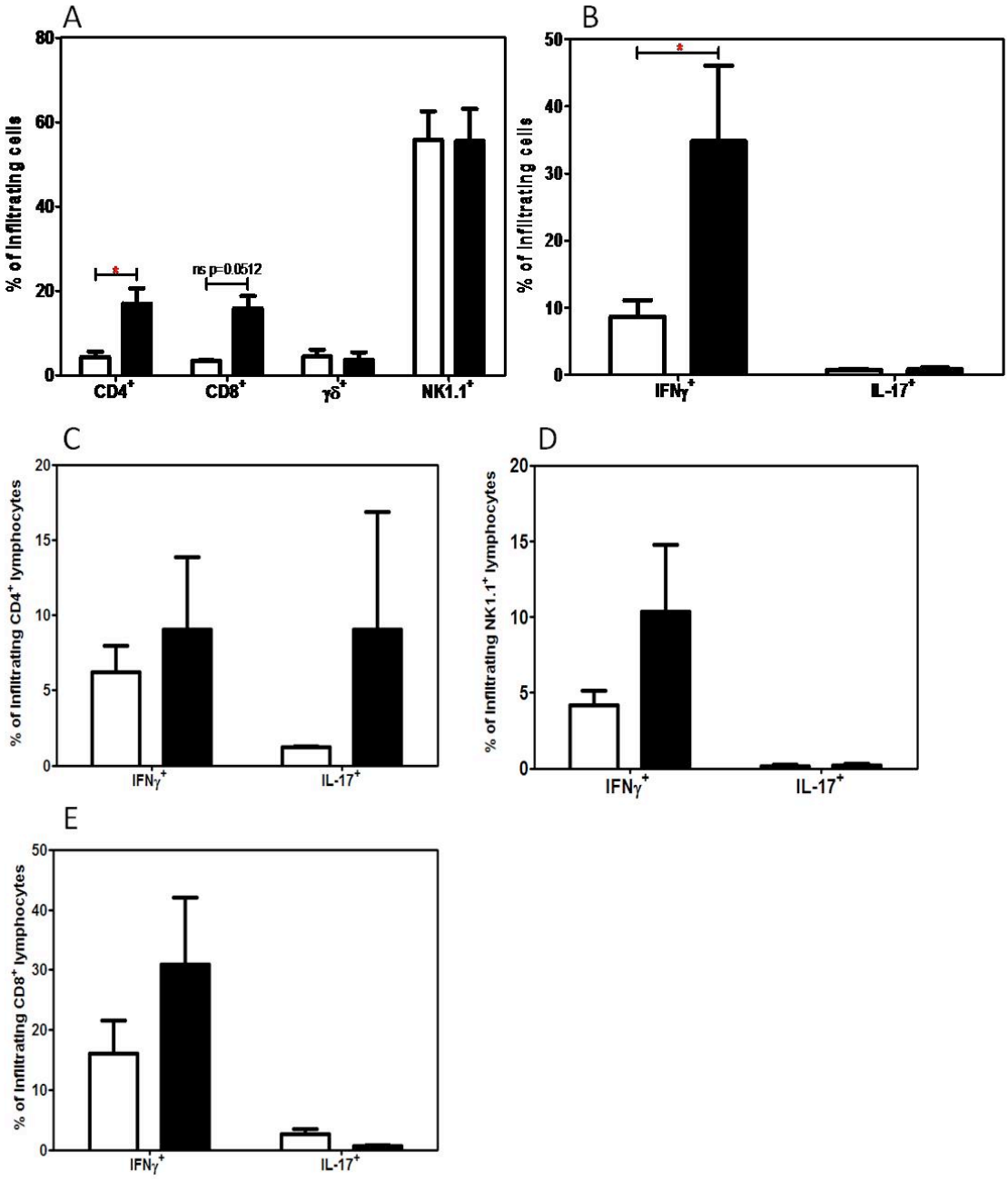


Figure 13. Characteristics of CNS lymphocyte infiltrates after Treg depletion.

Surface and intracellular cytokine staining of CNS infiltrating lymphocytes in affected DT treated mice (black) and matched PBS controls (white) after stimulation for 4h with PMA/ionomycin and BFA. Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density

*gradients when mice were removed from the study. A – surface staining of total CNS infiltrating lymphocytes. IFNγ and IL-17 secreting cells are shown as percentage of total lymphocyte (B), CD4 (C), CD8 (D), and NK (E) cell populations. (n=3 per group). Lymphocytes were gated initially according to cell surface markers and positive cells interrogated to determine the size of cytokine positive populations. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

This regulation appears to be relevant to cytokine production from CD4, CD8 and NK cell populations but not cellular proliferation, as this was not seen to increase in the absence of regulation. In the CNS, ablation of $Foxp3^+$ cells can be seen to impact on cellular infiltration across the BBB. Both CD4 and CD8 cell infiltration were seen to increase in the absence of Tregs, each reaching close to 20% of the total infiltrating population. Only the increase in CD4 cells showed statistical significance although the increase in CD8 cells approached significance (Figure 13A). The rate at which this increase in proportion occurs makes it unlikely that it is simply an increase in proliferation of resident lymphocytes but rather migration across the BBB. However, in this experiment such an explanation cannot be ruled out and investigations using tracking of peripheral cells would be required to differentiate between these two possibilities.

NK cells still make up the largest population of infiltrating cells but they do not show any increase in proportion upon removal of Tregs. This consistency is mirrored by the γδ cell population, and neither cell population demonstrate significantly different proportions between control and Treg depleted cellular infiltrates.

IFNγ$^+$ cells can be readily identified in the infiltrate and are present at significantly higher levels than in Treg competent mice. There are very few IL-17$^+$ cells observed in the CNS, in contrast to the peripheral picture, and even upon removal of Tregs, there is no significant increase in cytokine production as measured by number of IL-17$^+$ cells (Figure 13B). No evidence was therefore found that triggering of disease exacerbation through loss of Tregs had correlated either with increased infiltration by IL-17$^+$ cells or by increased activation to secrete IL-17 by those cells present. In the absence of Tregs, there is a trend towards control of IFNγ production from both CD8 and NK cells being released, as the proportion of cytokine positive cells increased, although neither observation was statistically significant. Very little production of IL-17 could be observed from either CD8 or NK cell populations irregardless of Treg presence or absence (Figure 13D,E). CD4 cells were seen to secrete both IFNγ and IL-17, although at low total levels and not in significantly different proportions compared to when Tregs were present (Figure 13C,D,E). Trends towards increases in activation were seen but these were not statistically significant.

Analysis of the cell surface markers within the IFNγ$^+$ cell population was carried out to determine whether the removal of Tregs affected the source of the released IFNγ observed. The proportion of cytokine release increases significantly by around 20%, from the CD8 cell population (Figure 14A) in response to the removal of Tregs and progression of symptoms. Smaller proportions of IFNγ release were observed from the γδ and NK cell populations. The γδ cell observation shows a statistically significant decrease whereas there is only a trend observed in the NK cell derived IFNγ release. No difference in

proportion of IFNγ derived from CD4 cells was observed and this remained around 10% of the cytokine release.

Observation of double cytokine positive cells as a proportion of each cytokine positive population shows a significant decrease in the number of IFNγ^{+} cells also producing IL-17 after removal of Tregs (Figure 14B). This is in contrast to results at similar disease severities in Line7 mice that progressed through disease without any interference. In these mice, IFNγ^{+} cells began to release IL-17 at high disease scores. These observations show no evidence that that release was due to Treg control however, and it is more likely that other factors influenced the phenotypic development of those cells. The same analysis of the IL-17^{+} cell population shows an increase in the proportion of IL-17^{+} cells also releasing IFNγ but this was not statistically significant. It should be noted that there are very few of these cells to put this in context (Figure 14B) but supports evidence that Treg control over IFNγ production is most important in development of disease in Line7 mice.

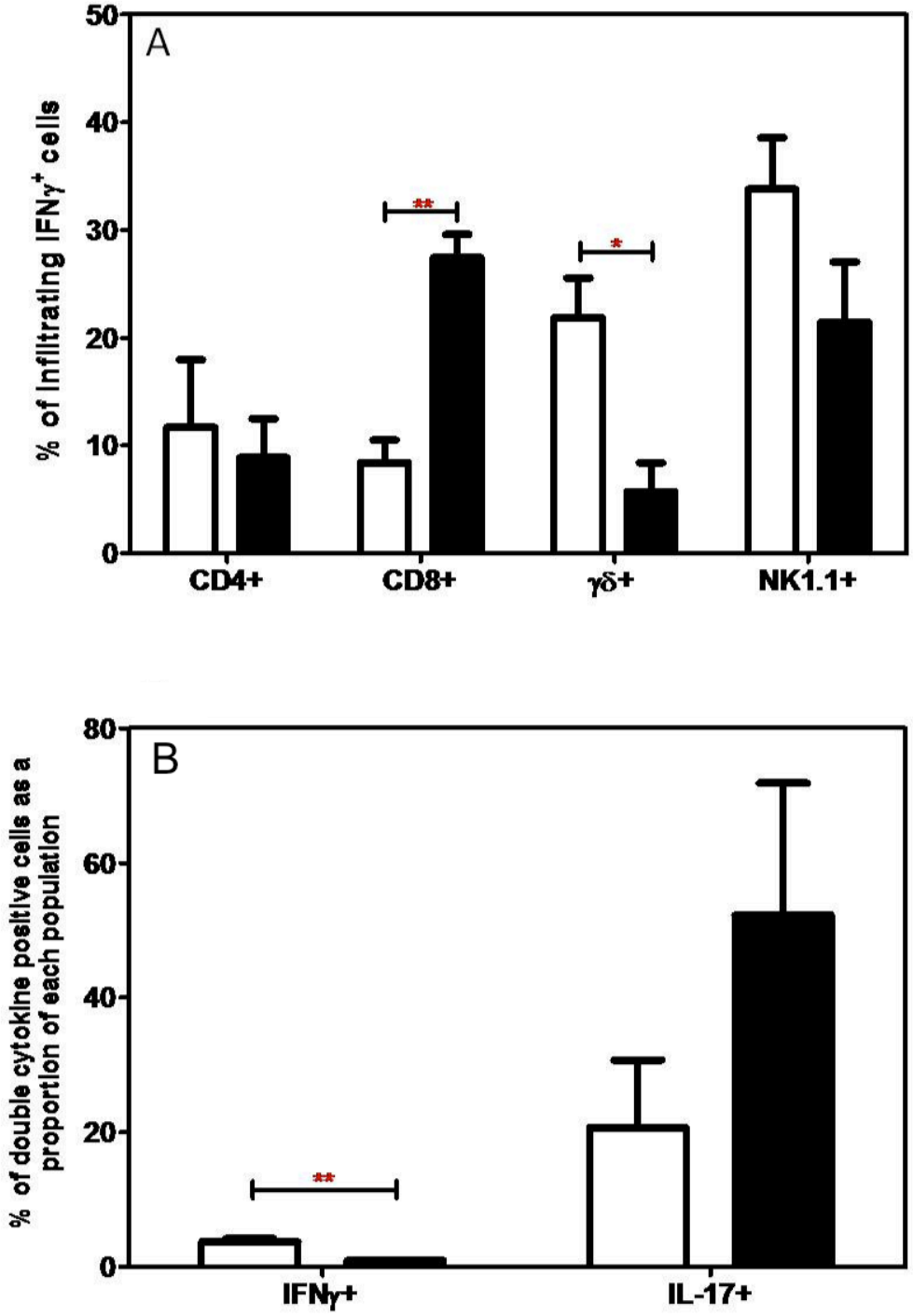


Figure 14. CNS IFNγ derived from infiltrating CD8$^+$ cells.

Composition of cytokine positive cells extracted from the CNS of DT treated (black) and matched PBS control (white) mice after stimulation for 4h with PMA/ionomycin and BFA.

*Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. A – Surface markers as shown as percentage of IFNγ^{+} population. Lymphocytes were gated initially on IFNγ and positive cells interrogated for presence of cell surface markers. B – Proportion of cytokine positive infiltrating cells also producing the other cytokine (IFNγ and IL-17 only). Lymphocytes were gated on IFNγ and IL-17 and positive cells interrogated for production of the other cytokine. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01 (two-tailed T test).*

11.2.2 Return of Tregs Halts Disease Progression

Through work with another PhD student in the lab (Deborah Chong) we had clear data on the kinetics of Treg reconstitution following the DT administration. To assess the impact of Tregs returning to an inflamed CNS following a period of ablation, Tregs were depleted through use of DT but only one or two doses (day 0 and day 2) were given to allow the Treg population to reconstitute as the DT was processed and removed from the mice. 4, 6-8 week old L7Fox mice were used per group (3 male, 1 female). Age matched Foxp3^{DTR} mice were used to quantify Treg depletion and repletion following the protocol used to treat L7Fox mice. DT was administered to 48 mice, split into 2 equal groups, receiving either one dose of DT or two doses on alternate days. Untreated mice were taken to provide a baseline and 3 mice removed from each DT treated group on alternate days. Size of Treg population was determined by measurement of GFP^{+} cells derived from the

spleen and an average taken from the three mice at each time point. From this it could be shown that two doses of DT were sufficient to maintain a depleted Treg population until day 6 (Figure 15A). By this time 3 out of 4 mice had already progressed through to full hind limb paralysis in a similar way to the previous continual Treg depletion experiment.

Only one mouse survived and managed to limit paralysis scores (Figure 15A). In contrast, one administration of DT was sufficient to deplete Treg cells but by day 4, they began to return and had returned to baseline levels by day 8 (Figure 15B). In these mice receiving one dose of DT, the removal of Treg population allowed disease to initiate, with symptoms presented between days 4 and 6. Disease progressed until further presentation of disease signs halted by day 9 (Figure 15B) and after this point mice were stable albeit with mild to heavy impairment. The progression of disease and return of the Treg population overlapped almost perfectly (Figure 15B).

Age and sex-matched controls were administered PBS according to the same immunization regime as a control for both disease development and cellular dynamics. As with the previous Treg depletion experiment, mice were removed from the study and samples taken when the development of paralysis threatened their survival. Upon the removal of an affected mouse, a matched control was also taken to control for reagents and duration since the last injection had been administered. Infiltrating and peripheral cells were extracted from the CNS and spleen respectively following the earlier described protocols. The size of the infiltrating cell population was counted using a

haemocytometer and cells stimulated and stained with antibodies in the same manner as previous experiments. Comparison of mean cell populations was done using two-tailed t tests with Welch's correction for unequal variances in this experiment.

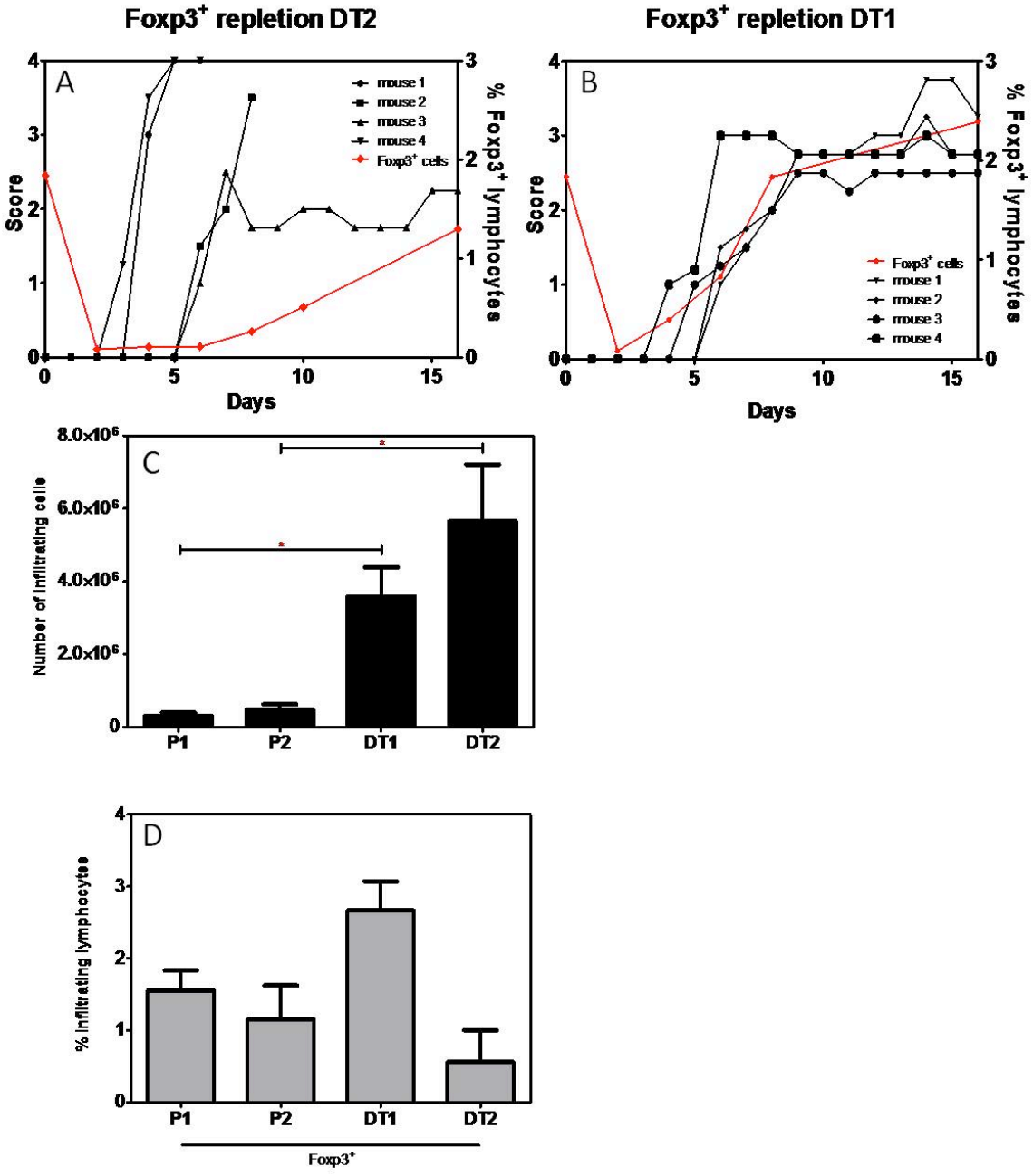


Figure 15. Return of Treg population to CNS of mice halts disease progression.

*L7Fox mice received either two doses (A) or one dose (B) of DT to limit Treg numbers, (as illustrated in the periphery by the red line tracking their recovery (n=3 per point)). Individual score progressions of each mouse in the study are shown in the respective graphs. All PBS control L7Fox mice receiving the same dosing regimen remained unaffected (not shown on graph). Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Counts of infiltrating cells made when each mouse was removed from the study, matched PBS control mice taken alongside affected DT treated mice (C) and shown as mean with SEM. Count of GFP^+ cells shown as percentage of the total lymphocytic infiltrate shown (D). n=4 per group. P1 and P2 grouping signifies control L7Fox mice receiving 1 or 2 doses of PBS respectively, DT1 and DT2 grouping signifies L7Fox mice receiving 1 or 2 doses of DT respectively to deplete Tregs. Asterisks denote statistical significance between cell populations of indicated disease scores; * $p<0.05$ (two-tailed T test).*

Maintaining a low Treg population allowed a significantly higher infiltration of cells into the CNS (Figure 15C) compared to respective PBS treated controls. The infiltration was higher in mice receiving two doses of DT demonstrating that the period of time without a Treg presence affects the level of cellular infiltration allowed. However, upon their return, Tregs readily infiltrated into the CNS as measured by FACS analysis of GFP^+ cells in the cellular infiltrate (Figure 15D). This provides evidence of an unimpaired migratory

potential upon the reconstitution of the Treg population and that this infiltration may be an important factor in the deceleration of disease progression.

As with continual depletion of Tregs, CD4 and CD8 infiltration into the CNS significantly increased in the absence of Tregs (Figure 16F). In mice receiving either dosing regime of DT the proportion of the infiltrating population increased. Whilst the CD8 proportion increased significantly in response to any duration of Treg depletion, in the longer depletion protocol, the proportion of CD4 cells in the infiltrate was not significantly increased compared to control mice. This significance is likely representative of a high variance in the control mice as the limited size of this population means a greater influence for individual cells. There was no change in γδ T cell infiltration but a significant increase was observed in NK cell infiltration after only one dose of DT. Similar to CD4, there was no significant increase in infiltration seen after two doses of either DT compared to PBS (Figure 16F) but as the spread of results was small and no difference was observed in NK cell infiltration in response to complete Treg ablation (Fig 13A), this result is supported by other results.

In response to the depletion of Tregs by two doses of DT, control of both IFNγ and IL-17 production from splenocytes was released (Figure 16A). Reconstitution of the Treg population, after only one dose of DT, allowed a regain of control over peripheral cytokine release demonstrating the functionality of Tregs upon their return (Figure 16A). In the CNS, control over cytokine release was limited to IFNγ with significantly more cytokine positive cells seen infiltrating after any depletion of the Treg population.

No change was observed in the IL-17^{+} cell population after a depletion of the Treg population (Figure 16B) in a similar manner to previous Treg depletion experiments

(Figure 13B). Mice receiving two doses of DT that progressed through to full disease showed a significantly higher number of IFNγ^{+} cells than those with stable disease after only one dose of DT (Figure 16B) showing evidence for a partial recovery of control.

Within the CD4 and CD8 infiltrating cell populations, there were significant increases of IFNγ production after Treg depletion, regardless of dosing strategy and a trend towards an increase was observed in the NK cell population (Figure 16C,D,E). No differences were observed between DT dosing regimes in any of these cell types. In these populations, no change was observed in IL-17 production, demonstrating no evidence for their involvement in disease progression or stabilization.

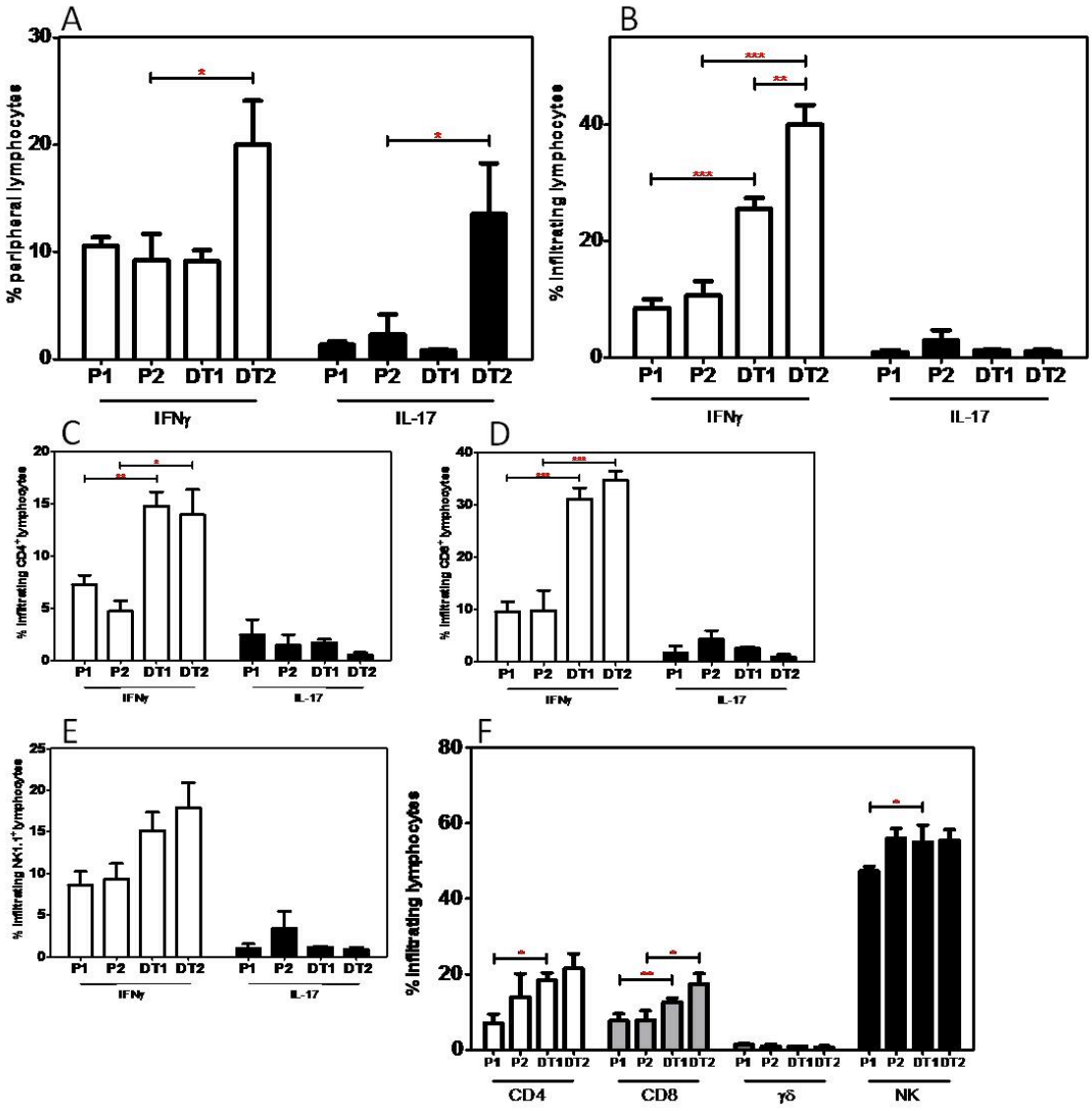


Figure 16. Altered cell dynamics after temporal Treg depletion.

Intracellular cytokine staining of peripheral (A) and infiltrating (B) lymphocytes extracted from affected DT treated mice (one dose - DT1, 2 doses - DT2) after stimulation with PMA/ionomycin and BFA with matched PBS controls (P1, P2). Shown as percentages from the total lymphocyte (A,B), infiltrating CD4 (C), infiltrating CD8 (D), and infiltrating NK (E) cell populations. Surface staining of total infiltrating lymphocyte population (F) also shown. n=4 per group. Infiltrating lymphocytes were extracted from the CNS of perfused

*animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. Lymphocytes were gated initially according to cell surface markers and positive cells interrogated to determine the size of cytokine positive populations. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01, *** p<0.001 (two-tailed T test).*

Analysis within the IFNγ$^+$ cell population showed that as with a continual depletion of Tregs, an increase was observed in the proportion of these cells positive for CD8$^+$ (Figure 17A) which reached significance after 2 doses of DT and approached significance after only one dose compared to their controls. A significant decrease was observed within the NK cell population and a trend within the γδ cell population, although not from both dosing regimens. The decrease in γδ cell derived IFNγ was from only one dose of DT whereas a decrease in NK cell derived IFNγ after two doses was likely a result of sample size, as very high proportions were derived from small IFNγ$^+$ cell populations within small total infiltrating populations in the CNS of unaffected PBS control mice (Figure 17A). No difference was observed in the proportion of CD4$^+$ cells producing IFNγ (Figure 17A) as seen in the previous depletion experiment (Figure 14A).

No significant difference was observed in the proportion of double cytokine positive cells within the IFNγ$^+$ population in contrast to the previous depletion experiment (Figure 17B). There was however, a significant increase in the proportion of IL-17$^+$ cells also releasing IFNγ (Figure 17B). After either dosing regime the proportion of double positive

cells significantly increased but as with the previous experiment, these results were from very small cytokine positive cell populations and so should be treated with caution.

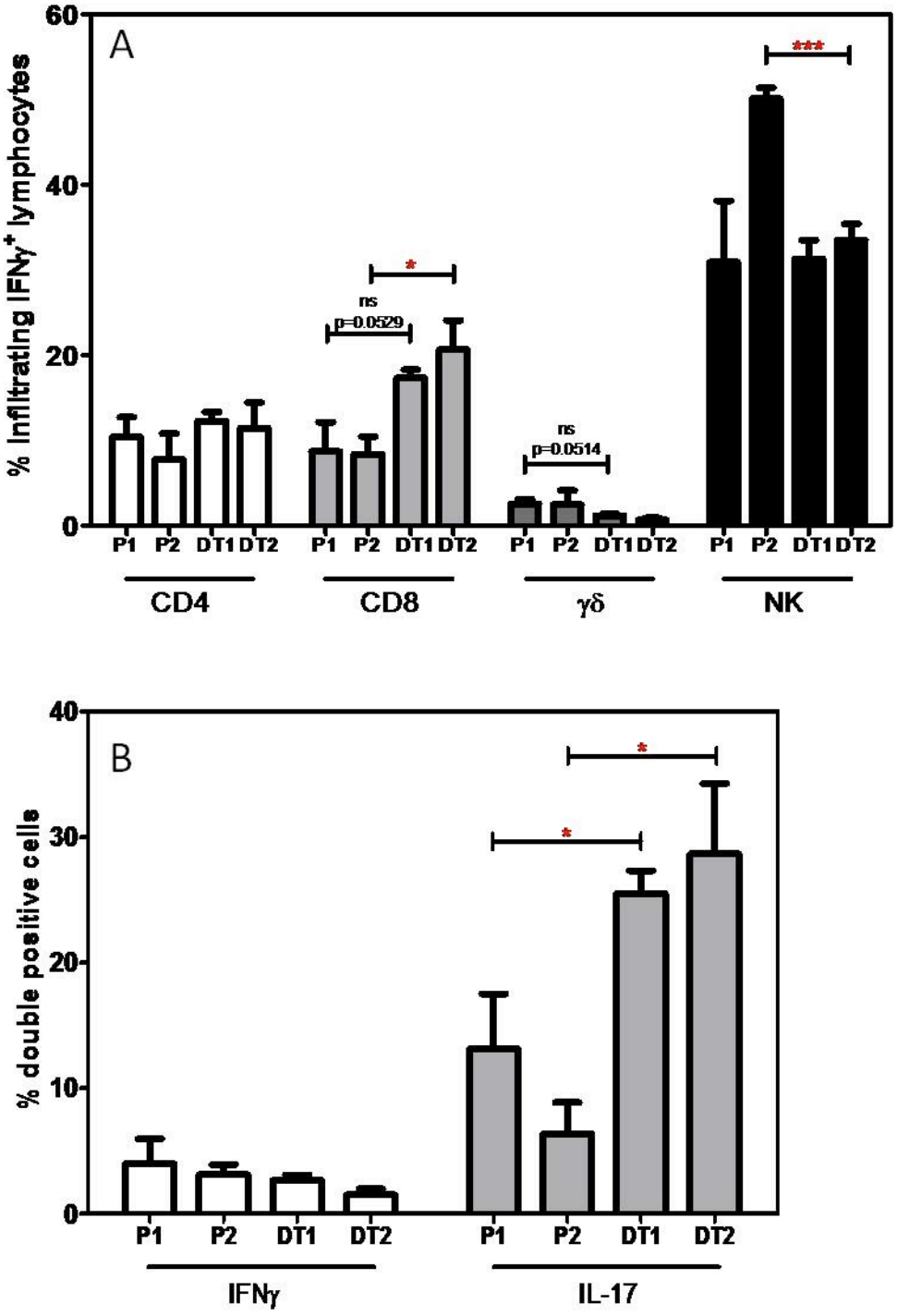


Figure 17. Derivation of CNS localized cytokine production.

*Composition of cytokine positive cells extracted from the CNS of DT treated (one dose - DT1, 2 doses - DT2) and matched PBS control (P1, P2) mice after stimulation for 4h with PMA/ionomycin and BFA. n=4 per group. Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. A – Surface markers as shown as percentage of IFNγ$^+$ population. Lymphocytes were gated initially on IFNγ and positive cells interrogated for presence of cell surface markers. B – Proportion of cytokine positive infiltrating cells also producing the other cytokine (IFNγ and IL-17 only). Lymphocytes were gated on IFNγ and IL-17 and positive cells interrogated for production of the other cytokine. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01, *** p<0.001 (two-tailed T test).*

11.2.3 Tregs Remain Functional Throughout Disease Development

The main issue unresolved at this point was whether the Tregs were acting directly on the effector cells or whether the stopping of disease progression was associated with other indirect factors related to the return of the Tregs. It was also of interest to determine whether Tregs retained their functionality throughout disease progression or whether progression under normal circumstances occurred because of aberrant regulation.

In order to assess Treg functionality, cells were extracted from both the spleen and CNS from 3 (1 male, 2 female) high scoring mice (2-3) and sorted on the basis of Foxp3 expression; Tregs being GFP$^+$ and effector cells GFP$^-$. Cells extracted from each mouse

were pooled together to provide sufficient cells for the experiment. The sorting was carried out using a BD FACS Aria II running FACSDiva software and high purity mask. The sort generated 99% pure populations of Tregs and effector T cells as can be seen by the FACS plots of cell populations pre and post-sorting (Figure 18D,E,F,G,H). There is no CNS post-sort GFP^+ plot as this population was limited in number and no cells could be spared from the suppression experiment.

Effector cells were cultured either with or without Tregs in the presence of 2.5μg/ml MBP_{85-99}, and cellular proliferation was assessed by thymidine incorporation after 3 days. Media only and unstimulated effector cell cultures are shown on the graph to provide illustration that proliferation and cytokine release was significantly higher than background levels. Two-tailed t tests were used to compare means derived from this experiment. Significant proliferation was observed upon stimulation with MBP_{85-99} with effectors derived from both the spleen and CNS (Figure 18A). Upon addition of Tregs, effector cell proliferation was significantly reduced in all combinations tested demonstrating the functionality of Tregs even at high disease scores (Figure 18A). Effector cells were cultured with Tregs derived from the same organ (spleen or CNS) to demonstrate functionality *in vivo*. In addition, peripherally derived Tregs were cultured with effector T cells from the CNS to show that these cells have not escaped regulation and are likely active despite regulation *in vivo*.

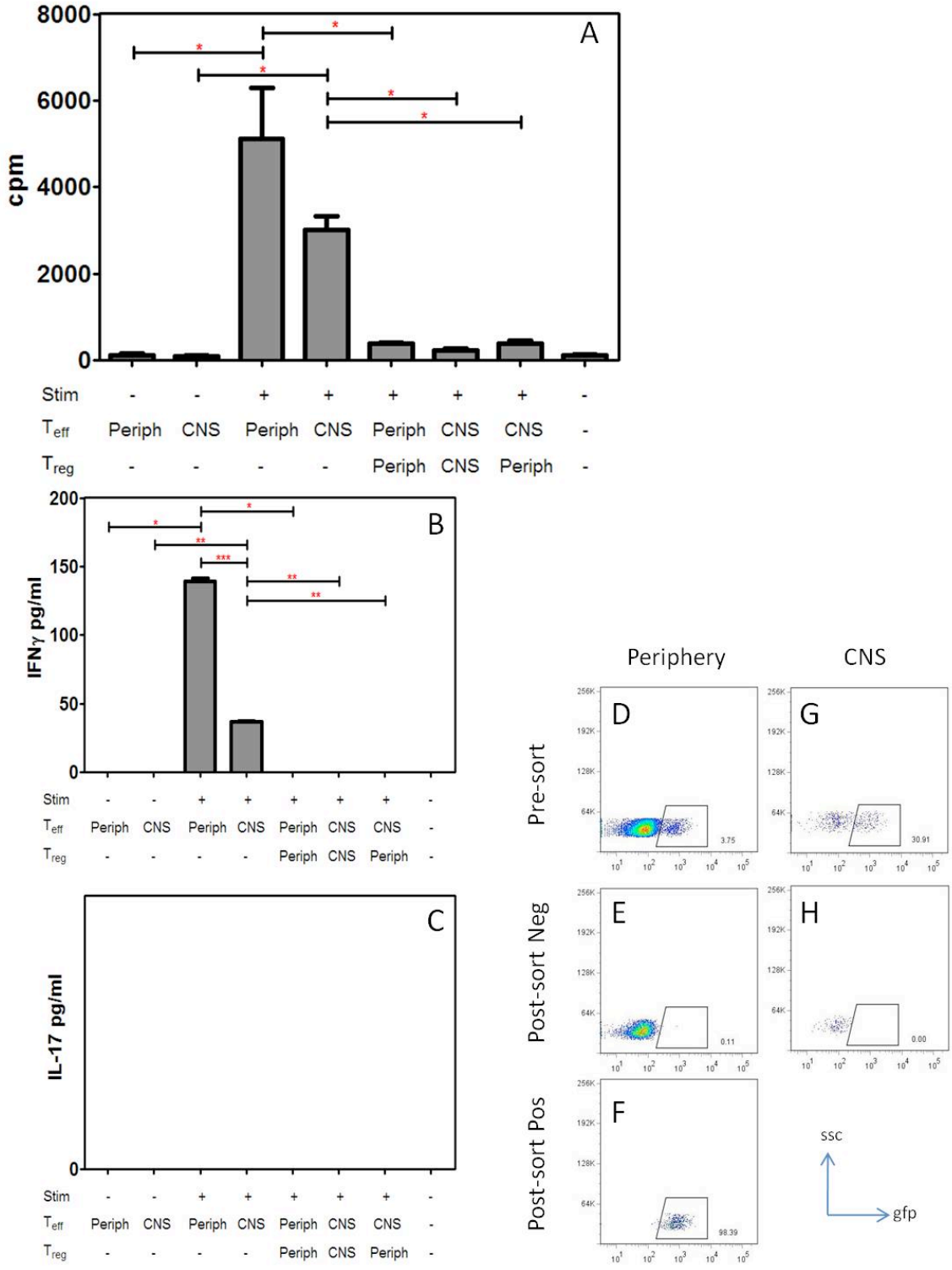


Figure 18. Tregs retain suppressive ability throughout the mouse even at high disease.

GFP$^+$ Tregs and GFP$^-$ T effector cells were sorted using a BD FACS Aria II from extracted CNS and spleen-derived cell suspensions (n=3 pooled). Infiltrating lymphocytes were

*extracted from the CNS of perfused score 2-3 L7Fox mice by enzymatic digestion followed by separation on percoll density gradients, peripheral lymphocytes were extracted from the spleens of the same L7Fox mice. Effector cells were cultured in the presence or absence of MBP_{85-99} peptide stimulation (2.5μg/ml) and regulatory cells at a 1:1 ratio. Origin of cells outlined beneath each figure. Proliferation was assessed by thymidine incorporation (A) (30,000 effector cells per well) and cytokine secretion by ELISA (B,C). Bars indicate mean from triplicate cultures ± SEM. Asterisks denote statistical significance between cell populations of indicated disease scores; * p<0.05, ** p<0.01, *** p<0.001 (two-tailed T test). Purity of sorting was very high with mixed populations (D,G) at the start and pure effector cell (E,H) and regulatory (F) populations after. Percentages are from total cell population.*

Cytokine release from these cell cultures was also measured by ELISA. After stimulation, only IFNγ was released with no IL-17 detectable (Figure 18B,C). There was a significantly lower production of IFNγ from CNS effector cells than peripheral effector cells (Figure 18B) upon stimulation with MBP_{85-99}, although the release of cytokine from both was significantly reduced to undetectable levels upon culture in the presence of Tregs (Figure 18B). This demonstrated that in addition to suppression of cellular proliferation, Tregs were able to suppress activation of effector cells, as measured by pro-inflammatory cytokine production and these mechanisms together explain the halting of disease progression upon their return.

11.2.4 Role of CD8 and NK Cells

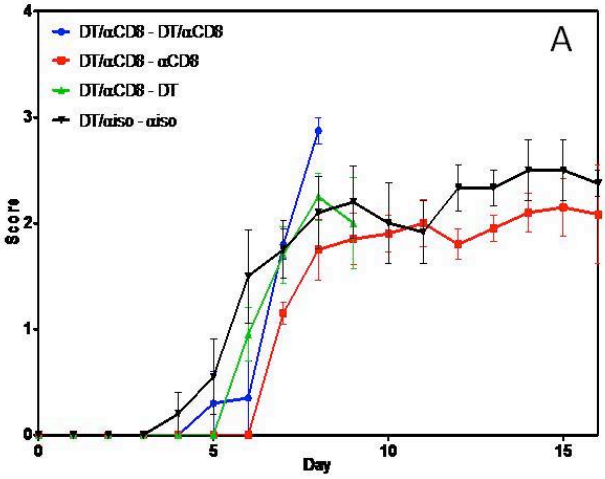
An attempt was made to shed light on the role that CD8 and NK cells may play in disease initiation and progression. Depleting antibodies for CD8 and NK1.1 were given to mice in order to remove these cell populations and assess the impact on disease progression. 5, 6-8 week old L7Fox mice were included in each group (2 male, 3 female). Antibodies were administered alongside a regimen of DT in order to increase the speed at which signs of disease developed. CD8 cells showed increased cytokine production and increase infiltration upon removal of Tregs implicating a pathogenic role in disease progression. If these results represent the true function, disease initiation or severity should expect to be reduced compared to controls. The infiltration of NK cells however, did not increase in response to Treg depletion, despite showing increased cytokine production. They make up the largest proportion of the cellular infiltrate but so far there is no conclusive evidence as to whether they fulfill a protective or pathogenic role. If NK cells are merely bystander cells then no difference would be expected in disease progression whereas roles promoting or preventing disease should appear in relation to controls. A group receiving an isotype control antibody in place of a depleting antibody was included in the study to act as a control for antibody administration. In this group only one dose of DT was administered and the second dose isotype control antibody alone.

The regimens of depleting antibody and DT were selected to provide movement to both accelerate or delay the development of disease signs. They are described for CD8 but apply identically for NK cells. Three combinations of dosing were designed, with every mouse receiving injections on day 0 and day 2. The first dose for all groups contained

both DT and the αCD8 to remove Tregs and the $CD8^+$ cells. The second dose contained either αCD8 alone, DT alone, or both together. This would generate mice in which cells returned at different times, hopefully impacting on the development of disease. If $CD8^+$ cells played a pathogenic role then a delay in development of disease signs would be expected in mice receiving two doses of αCD8 in which CD8 cells were absent past the return of Tregs. If however they were protective, then development of disease would be expected to be accelerated in these mice. Statistical significances were determined by comparison of means by a two-tailed T test with Welch's correction for unequal variance. In order to determine the extent of depletion and rate of return of CD8 or NK cells, depleting antibodies were administered to $Foxp3^{DTR}$ mice as either a single dose, or two doses on two days apart. 24 (12 male, 12 female) mice received αCD8 split between the two dosing regimes and 24 (12 male, 12 female) received αNK1.1. 3 mice receiving the single dose were culled on days 2, 6, 11 and 16 after immunization, whilst 3 mice receiving two doses were culled on days 4, 8, 12 and 16 after the initial immunization. 3 mice that did not receive an injection were taken to provide a baseline reading. Spleen cells were extracted from these mice and stained with antibodies to determine size of the originally depleted cell populations. Mice receiving αCD8 were identified using another CD8 antibody, specific for a different epitope whilst NK cells were identified as being CD3-$NKp46^+$ as the αNK1.1 antibody used in other experiments was specific for the same epitope as the depleting antibody. Results are displayed in Figure 19C and D, and showed that despite a reduction in proportion of both cell types, neither was completely depleted. CD8 cells showed around a 75% reduction, whilst the population of NK cells was reduced by around 80%. Residual low levels remained throughout the life of the

experiment, and neither cell phenotype was observed to return to baseline levels by the end of the study (Figure 19C,D). There was no discernable difference in depletion efficiency between one and two dose regimes, meaning the intended difference in temporal repletion did not occur.

The disease curves reflect the incomplete depletion and no obvious change was seen between mice administered one or two doses of either depleting antibody or the isotype control (Figure 19A,B). Average scores for mice in each group were calculated by addition of the scores of mice within each group divided by the total mice still alive on each day. Mice were taken for samples as in previous experiments when their paralysis threatened their survival but their scores were not included in the generation of an average score after their removal. No significant delay in development of symptoms was observed in any group and there was no significant reduction in severity of symptoms from any dosing regimen.



Group/dosing regime	Mice surviving to end of study
DT/αCD8 – DT/αCD8	0/5
DT/αCD8 – αCD8	5/5
DT/αCD8 – DT	1/5
DT/αNK1.1 – DT/αNK1.1	2/5
DT/αNK1.1 – αNK1.1	5/5
DT/αNK1.1 – DT	1/5
DT/isotype- isotype	3/5

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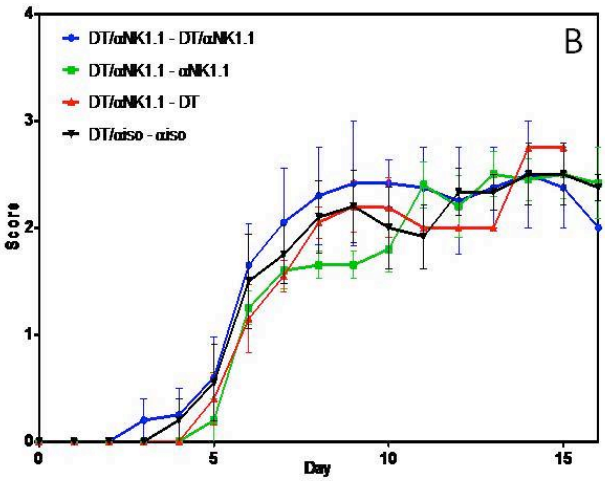

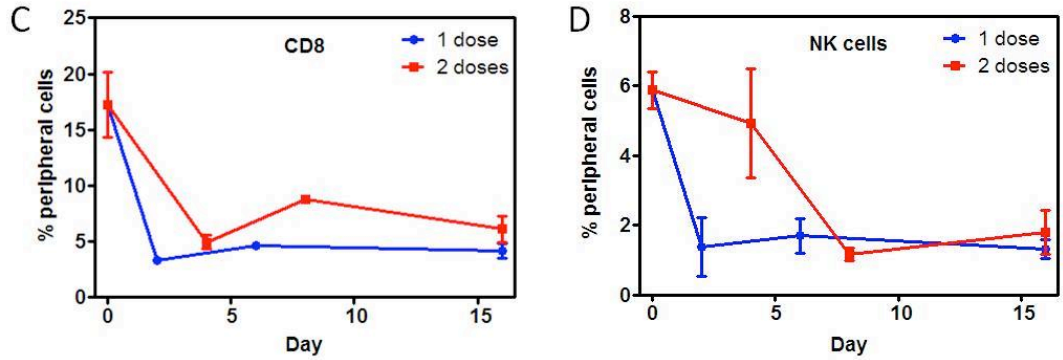

Figure 19. Partial depletion of CD8 or NK cells does not affect disease initiation or severity.

Administration of depleting antibodies to L7Fox mice alongside depletion of Treg populations. Mice were observed and scored daily and disease scores averaged within each group to generate mean progression shown with SEM at each day (A,B). Scale of

cellular depletion and recovery of cell populations tracked (C,D) (n=3 per point). Peripheral lymphocytes were extracted from the spleen of L7Fox mice administered either one or two doses of either αCD8 or αNK1.1 depleting antibody. Cells were stained to determine level of depletion, $CD8^+$ cells measured using an antibody for a different epitope, NK cells measured as $CD3^-$ $NKp46^+$. Stained cells were read using a BD FACS Aria II. Number of mice affected in each group and progressing to score 4 or above shown in E.

In this experiment, the dose regime of DT was the largest correlate with disease outcome, such that as seen in previous experiments, mice receiving two doses of DT progressed through to full disease at a higher frequency than those only receiving one dose (Figure 19E). The small sample size of each group limits the significance of these frequencies, and larger groups would be required to gain statistical power in this observation. Some mice receiving two doses of DT managed to resist progression to full disease as previously seen and in this case two mice receiving the isotype control and only one dose of DT also progressed fully (Figure 19E).

Because of the failure of differential doses of depleting antibody to provide different rates of repletion, and the limited survival of mice receiving two doses of DT, meaningful conclusions are only going to be found through comparison of groups receiving one dose of DT. Through comparison of the disease course of these groups, it appears that the depletion of CD8 cells may cause later onset and milder signs of disease whereas the depletion of NK cells did not show any difference to isotype control (Figure 20A). This

effect is very marginal and would need to be confirmed by complete depletion experiments or much larger sample sizes. No statistical significance can be determined but development of disease signs appear to be delayed by two days in the absence of $CD8^+$ cells and scores fail to achieve an average greater than 2.

The depleting antibodies used in this study had previously shown reliable efficacy at depleting their respective populations in previous work done in the lab and in other published studies so the only partial depletions were not expected. YTS169, developed in the laboratory of Herman Waldmann, has been used successfully by many other groups to deplete CD8 T cells (Cobbold 1990) and the αNK1.1 antibody used in this study is commercially available and has been used widely to deplete NK cells in previous studies (Kitaichi 2002).

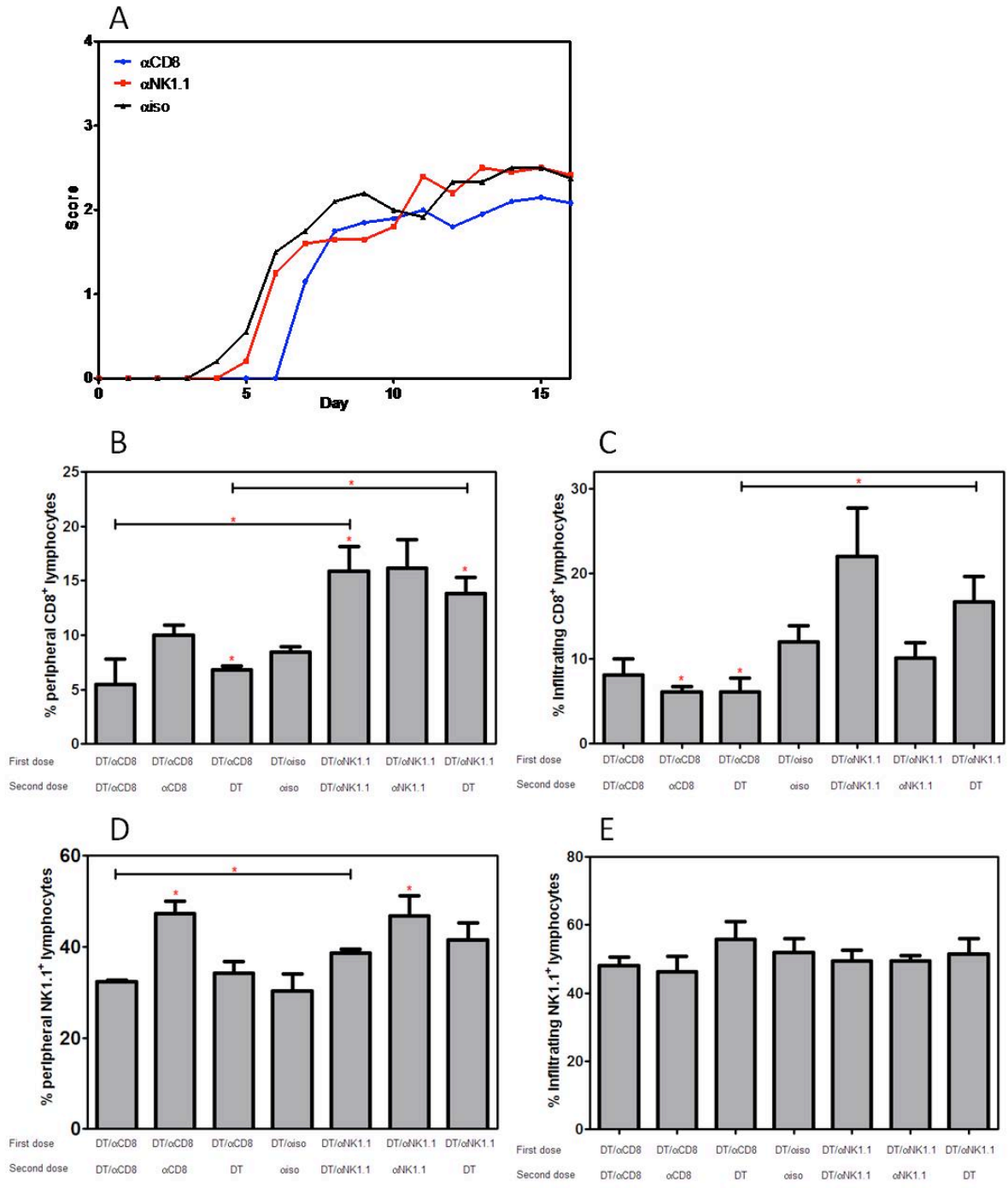

Figure 20. Variations in lymphocyte cell proportions after administration of depleting antibodies.

A - Average disease progression shown for each group receiving only one dose of DT to compare dynamics (n=5 per group) Proportions of CD8 (B,C) and NK (D,E) cell populations

*in the periphery and infiltrating the CNS as measured in the whole lymphocyte population at that location (n=29 total). Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. Dosing regime outlined beneath figure. Asterisks denote statistical significance between administration protocols. An asterisk above individual bars denotes statistical significance with regard to the isotype contol group (centre). Asterisks with associated lines between two groups denotes statistical significance between the two groups; * p<0.05, ** p<0.01 (two-tailed T test).*

Reflecting the poor depletions, there are still substantial populations of cells targeted for depletion present in the periphery and infiltrating the CNS from both groups (Figure 20B,C,D,E). The lower numbers of CD8 cells illustrate that the depletion had some effect on the CNS infiltrate, with the two groups showing significant reductions relative to the isotype control (Figure 20C). However $CD8^+$ cells still make up over 5% of infiltrating cells even in the mice that progressed very fast through to complete disease (Figure 20C). In the periphery only one group showed a significant reduction in size of the CD8 population, and this was the group receiving only one dose of depleting antibody rather than the expected groups receiving two doses (Figure 20B). No change was observed in the size of the infiltrating NK cell population after CD8 depletion (Figure 20E) although the administration of two doses of αCD8 did lead to a significant increase in the peripheral NK cell population (Figure 20D).

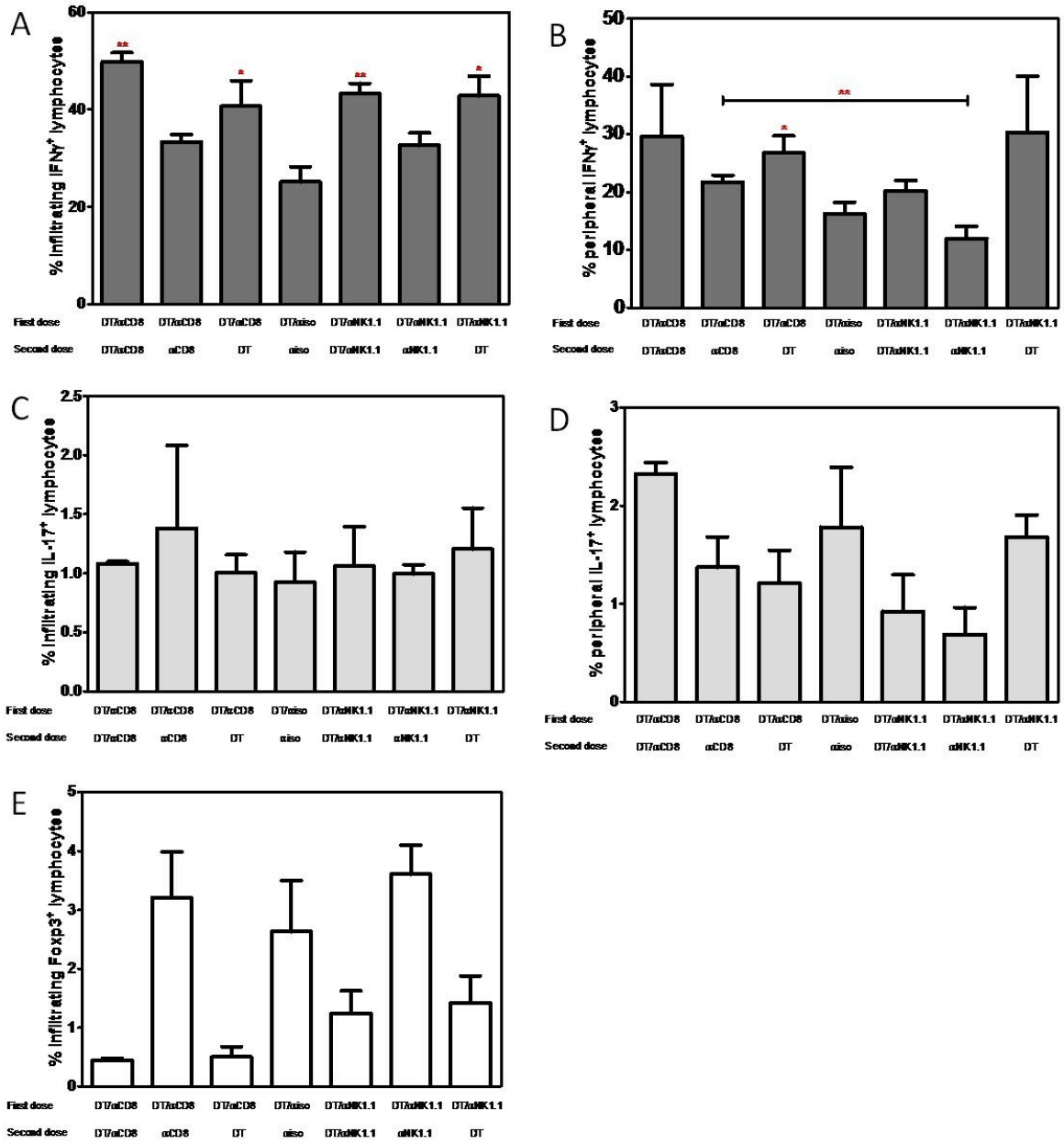

Figure 21. Changes in cytokine dynamics of peripheral and CNS infiltrating cells after administration of depleting antibodies.

Intracellular cytokine staining of infiltrating and peripheral lymphocytes extracted when each mouse was removed from the study. Stimulated with PMA/ionomycin for 4h with BFA. Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were

*removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. IFNγ (A,B), IL-17 (C,D) secreting cells, and Foxp3^{+} (gfp^{+}) cells (E) measured as proportion of total lymphocyte population. Dosing regime outlined beneath figure (n=29 total). Asterisks denote statistical significance between administration protocols. An asterisk above individual bars denotes statistical significance with regard to the isotype contol group (centre). Asterisks with associated lines between two groups denotes statistical significance between the two groups; * p<0.05, ** p<0.01 (two-tailed T test).*

The administration of αNK1.1 antibody significantly increased the proportion of CD8 cells in the periphery relative to the isotype control as would be expected with a reduction in the NK cell population (Figure 20B). However, significant increases could also be observed in the peripheral NK cell population after administration of 2 doses of αNK1.1 (Figure 20D) making the derivation of this result unclear. No significant changes in either infiltrating CD8 or NK cell populations were observed after attempted NK cell depletion (Figure 20C,E). Both the detection and depleting αNK1.1 antibodies react to the same epitope of NK1.1. This further emphasizes the poor efficacy of depletion as any depleting antibody remaining in the mouse would be expected to block binding of the detection antibody and so yield lower proportions. The ability to detect NK1.1^{+} populations therefore demonstrates a failure of the depleting antibody to mediate NK cell depletion.

The severity of symptoms, measured by the number of mice still being alive at the end of the run was correlated with higher Treg infiltrates (Figure 21E). This was seen irrespective of which depleting antibody had been administered and demonstrated the overriding importance of Tregs on controlling disease initiation and progression. In groups receiving αCD8 and two doses of DT there were significantly lower Treg infiltrates in the CNS of mice compared to the isotype control group. In these groups, all but one mouse progresses through to full disease (Figure 19E). This pattern was recapitulated in the corresponding groups receiving αNK1.1 although it was not statistically significant (Figure 21E). Interestingly a trend towards a different proportion of infiltrating Tregs were observed in mice receiving the second dose of DT alone with groups receiving αCD8 showing lower Treg infiltration than those receiving αNK1.1 (Figure 21E).

Irrespective of which depleting antibody had been administered, higher levels of $IFN\gamma^+$ cells infiltrating the CNS were seen in groups with low Treg infiltration, all significantly different to the isotype control (Figure 21A). Both groups receiving depleting antibodies but only one dose of DT showed lower levels of $IFN\gamma^+$ cell infiltration, more comparable to that seen in the isotype control, although it was still significantly higher in the group receiving αCD8 (Figure 21A).

There was however a noticeable increase in peripheral levels of $IFN\gamma^+$ cells from mice administered αCD8 compared to those receiving isotype control in contrast to a consistent proportion in those mice receiving αNK1.1. This increase was significant in the group administered αCD8, apart from those receiving two doses of DT which only showed a trend towards an increase, likely due to the high variation within these groups (Figure 21B).

No variation in infiltration or peripheral presence of IL-17$^+$ cells was observed (Figure 21C,D) after administration of any depleting antibody and DT combination. There was no change in IL-17$^+$ cell infiltration replicating results from earlier Treg depletion experiments. In the periphery lower proportions of IL-17$^+$ cells were observed in mice receiving two doses of αNK1.1 and those within groups receiving αCD8 and DT followed by either DT or αCD8 alone although none of these differences showed statistical significance when compared to the isotype control group. This was likely due to the high variation of the isotype control group but as IL-17$^+$ cells only represented 2% of the total lymphocytes at their maximum, the sample sizes are relatively small.

Despite the incomplete depletion of CD8 or NK cells in this protocol, the partial removal of CD8 or NK cells highlighted some properties of these cells that can help to shed light on their role in autoimmunity. Consideration of groups receiving only one dose of DT and two doses of either depleting or isotype control antibody, allows comparisons to be made between mice without the added variable of DT dose and therefore Treg dynamics. Under partial depletion of CD8 cells, IFNγ production showed a trend towards an increase in the spleen in contrast to IL-17 which did not vary (Figure 22A,B). The release of IFNγ from the CD4$^+$ population was also increased relative to the isotype control (Figure 22D), despite the overall CD4 population remaining stable (Figure 22D). The proportion of IFNγ and IL-17 coming from the CD8 population was significantly decreased in both the peripheral (Figure 22F,H) and infiltrating cell populations Figure 22E,G) reflecting the partial depletion but as no major difference in symptoms had been observed it is not possible to say that this was a major factor. These results suggest a potential regulatory role for CD8 cells in suppressing cytokine release from CD4 cells.

This is limited to IFNγ and not IL-17 although the importance of this result is questionable as the same increases were not observed in the infiltrating cell populations (data not shown).

The partial depletion of NK cells increased the proportion of $CD8^+$ cells in the periphery (Figure 23E) and as there were consistent proportions of CD4 cells (Figure 23D) and a significant increase in NK cells despite the depleting antibody (Figure 23F), this is unlikely to be just an artifact of measuring percentages but actually an increase in number of cells. There is also an increase in infiltrating CD8 cells that produce $IFN\gamma^+$ (Figure 23B) but not a similar pattern for $IL\text{-}17^+$ cells (Figure 23C). This is not simply due to an increase in CD8 cell population (Figure 23A) but likely reflects increased activation. Therefore these results would lead to there being a regulatory role for NK cells. They may act to suppress CD8 proliferation and cytokine release, which could, if completely removed, lead to more severe symptoms derived from increased pro-inflammatory cytokine release.

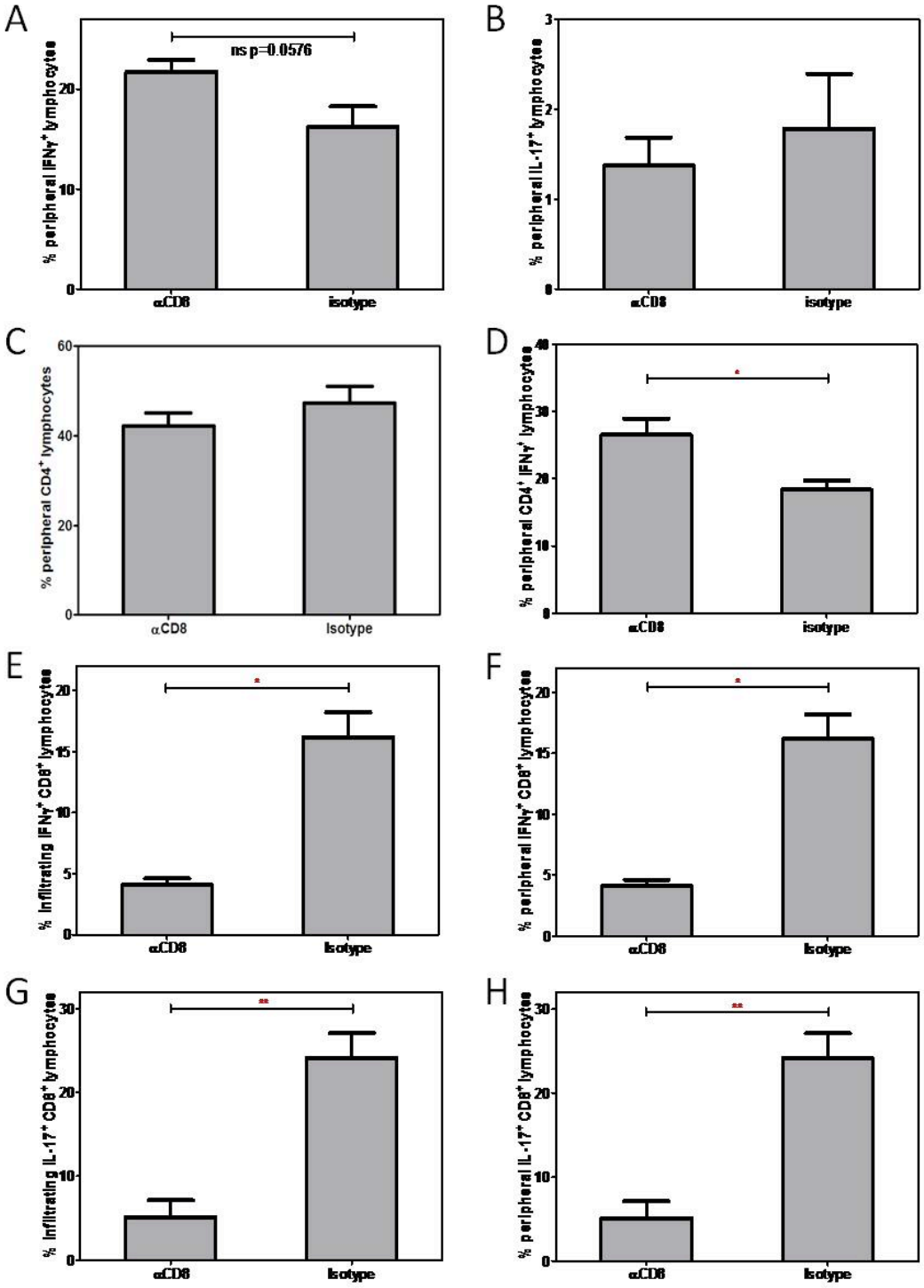

Figure 22. Cellular dynamics after administration of depleting antibodies.

Intracellular cytokine staining of peripheral and infiltrating lymphocytes from groups receiving one dose DT and two doses either αCD8 or isotype control antibodies after

*stimulation with PMA/ionomycin and BFA for 4h. Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. Peripheral IFNγ (A), IL-17 (B) and CD4 (C) positive cells as measured as proportion of total lymphocyte population and IFNγ positive cells as a proportion of peripheral CD4 positive cells (D). The proportion of cytokine positive cells that are $CD8^+$ is measured in both the periphery and cellular infiltrate for $IFN\gamma^+$ cells (E,F) and IL-17 (G,H). (n=4 per group). Asterisks denotes statistical significance in the size of cell population between the two groups; * p<0.05, ** p<0.01 (two-tailed T test).*

It was noticeable that in the isotype control group, 2 of the mice progressed fully through disease compared to 3 that did not. Observation of the cellular infiltrate showed that whether a mouse progressed to full disease was determined by the success of Treg infiltration into the CNS and peripheral recovery after DT administration (Figure 24A,B). A significantly higher Treg infiltrate was seen in the mice resisting disease progression compared to those which developed full hind limb paralysis (Figure 24A) but only a trend towards an increase in peripheral Treg numbers was seen as this was not shown to be statistically significant.

With larger sample sizes it should be possible to determine whether this is a true relationship or not. Taken with previous results however, these further illustrate the importance of Tregs in halting progression of disease in this model.

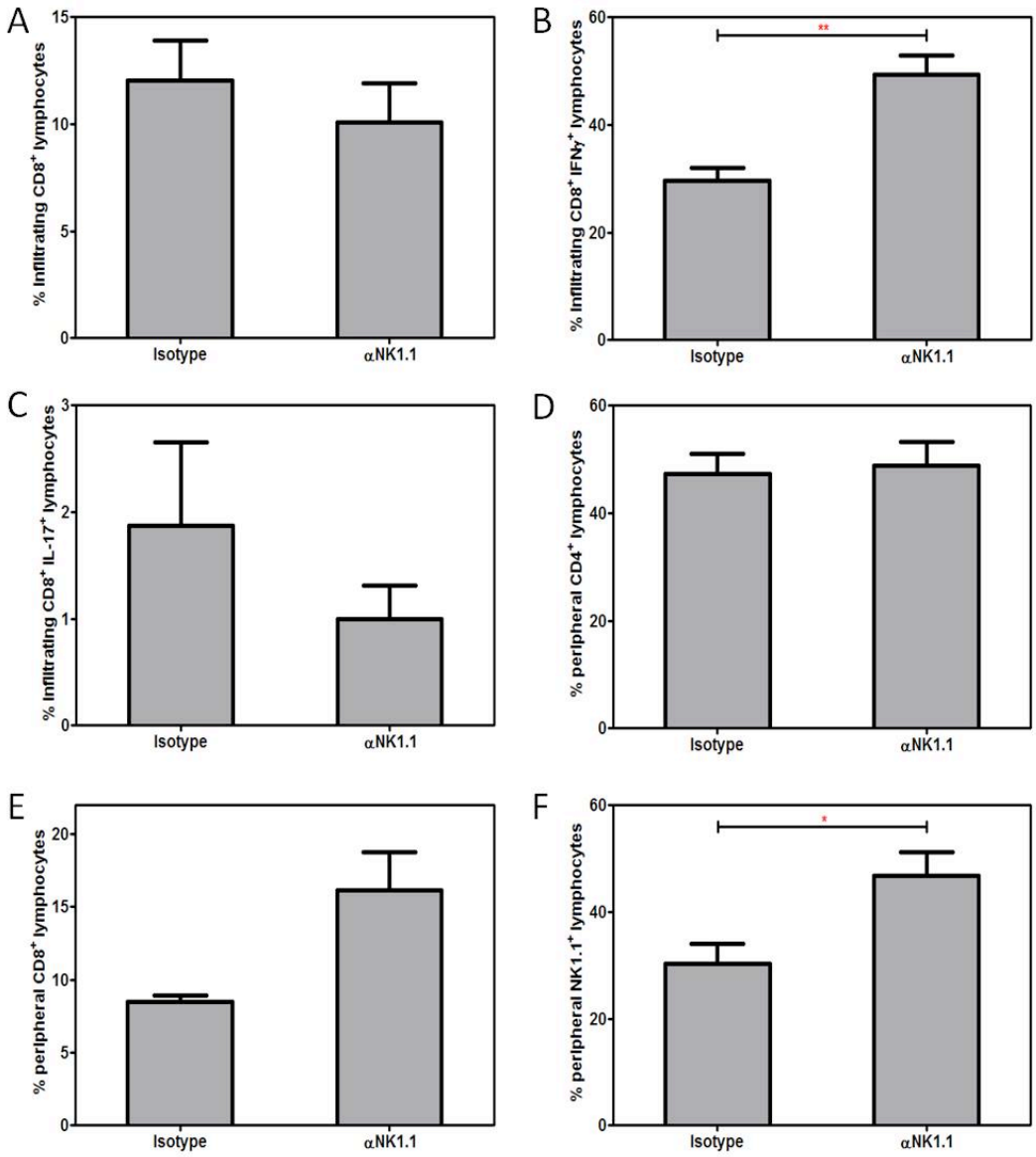

Figure 23. Comparison of cellular dynamics groups receiving one dose DT and two doses of isotype control or αNK1.1 antibodies.

Surface and intracellular staining after PMA/ionomycin stimulation with BFA for 4h. Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. Infiltrating CD8$^+$ cell population recorded as proportion of total

*lymphocyte population (A) and percentage of these that were also secreting IFNγ (B) or IL-17 (C). Lymphocytes were first gated on CD8 and positive cells interrogated to determine the size of the cytokine positive population. Peripheral CD4 (D), CD8 (E), and NK (F) cell populations measured as proportion of total lymphocytes. (n=4 per group). Asterisks denotes statistical significance in the size of cell population between the two groups; * p<0.05, ** p<0.01 (two-tailed T test).*

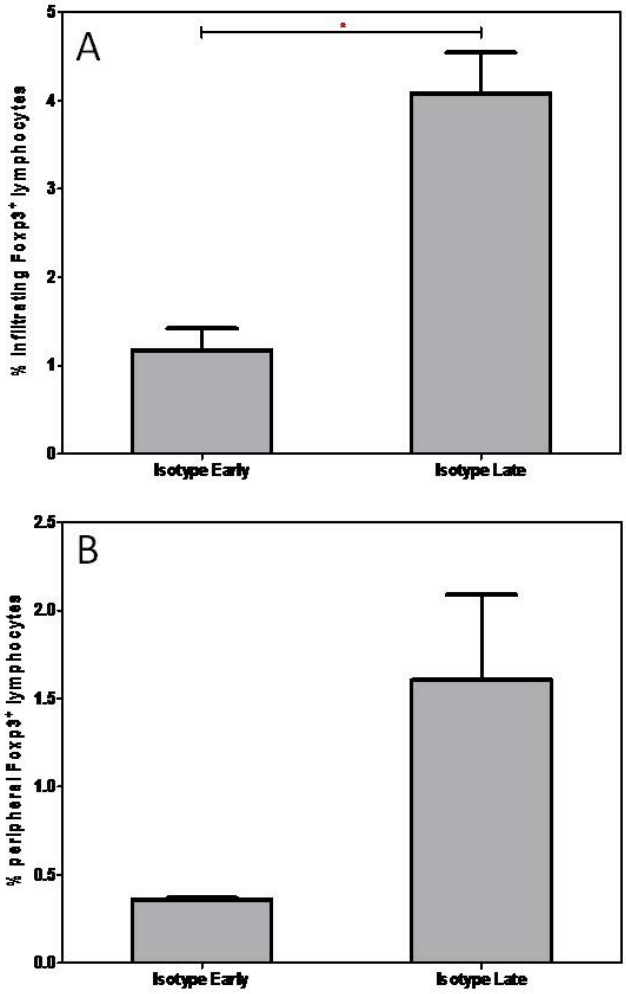

Figure 24. Higher Treg numbers in mice from isotype control that did not progress through to full disease.

*Infiltrating lymphocytes were extracted from the CNS of perfused animals by enzymatic digestion followed by separation on percoll density gradients when mice were removed from the study. Peripheral lymphocytes were extracted from the spleen of mice removed from the study. GFP^+ cells shown as proportion of total lymphocytes in the infiltrating (A) and peripheral (B) cells. (n=2 per group). Asterisks denotes statistical significance in the size of cell population between the two groups; * $p<0.05$, ** $p<0.01$ (two-tailed T test).*

11.3 Discussion

Investigations into the functionality of Tregs in MS have been hampered by both the source if using human tissue samples, and the nature of the EAE model if concerned with mouse studies. Human Tregs are not derived from inflammation and the use of immune adjuvants in EAE may artificially impair Treg function to generate disease (Chen 2006). Previous studies depleting Tregs have relied on αCD25 antibodies, but the degree of success of these depletions is debatable, impacting on the relevance of any conclusions drawn (Kohm 2006, Stephens 2006, Zelenay 2006). In the L7Fox model, it is possible to completely deplete mice of Tregs, without affecting any other cell type by targeting Foxp3 (Kim 2007), and under those circumstances autoimmunity progresses unabated. Disease develops in all mice at a very rapid rate at an age where development of symptoms is not expected for at least another 2 months. Mass infiltration is associated with the removal of Tregs, upon DT administration, suggesting an additional role in limiting cell migration and supported by Treg control of infiltration observed in adoptive transfer experiments (Kohm 2002). There has been debate as to the role DT plays in the mouse, and whether the fatal autoimmunity observed in Foxp3 knockout mice is attributable to breakdown of epithelial barriers (Chen 2008, Kim 2009). In this study, DT was administered to Line7 mice and did not lead to breakdown of the BBB. Depletion of the Treg population could be observed however, demonstrating the effectiveness of this treatment. No extra cellular infiltration was observed illustrating that the BBB was still intact, preventing the infiltration of T cells, and paralysis to develop, confirming that the paralysis in the L7Fox line were mediated solely by a depletion of Tregs.

A significant advantage in using the L7Fox mice is that Tregs can be allowed to return in the face of an autoimmune attack without requiring adoptive transfer of *in vitro* purified Treg populations (Kim 2009). There is no sudden burst of Tregs into the system, rather they return at a rate determined by each mouse's physiology, but what is clear is that upon return they are able to prevent any further development of autoimmunity.

Even one dose of DT, with Tregs depleted for 2 days was sufficient for autoimmunity to initiate and reach peak severities. Such an observation demonstrates the cliff-edge at which the mice live, only requiring a small lapse in regulation to fall off the edge. Disease develops at an alarming rate without regulation such that if Tregs have not returned by day 6 then it is too late. This further underlines their importance but also demonstrates their effectiveness under normal circumstances in preventing disease progression in the Line7 base-strain. A competent Treg population is able to prevent progression to full signs of disease for near a year, and in some cases completely. They limit inflammation in a mouse line primed for development of autoimmunity, both in only allowing a slow accumulation of cells into the CNS but also limiting their activation once they infiltrate.

Removal of the Treg population allows recognition of which cell populations were under Treg control. It could be seen that only peripheral activation of cells was released, Tregs usually acting to limit both IFNγ and IL-17 production. It has been thought that Tregs are unable to control Th17 activation and only act on Th1 (O'Connor 2007) and although statistical significance and as large an increase as from $IFN\gamma^+$ cells were not achieved, there was a noticeable increase in $IL\text{-}17^+$ cells from $CD4^+$ spleen cells in this study. No peripheral cell populations varied demonstrating that aside from the removal of the Treg population, the cellular dynamics were unchanged.

Tregs appear to exhibit control over CD4 and CD8 infiltration as these cells increased in much higher proportions and lead to disease development. Such control does not seem to be present for NK cells or γδ cells as neither differed in infiltrating proportion after Treg depletion. The high proportion of the infiltrate that was NK1.1^{+} remained constant suggesting that their infiltration was not linked to Tregs. Their activation however was released and greater levels of IFNγ were observed from the CNS infiltrating populations, mirrored also by CD4 and CD8 cells, demonstrating a level of control of Tregs over NK cells, an observation seen in other circumstances (Ghiringhelli 2005).

Most noticeable was that the severe disease was initiated and developed in the absence of IL-17 in the CNS. Cytokine positive cells were almost exclusively IFNγ^{+} and this proportion increased greatly upon removal of Tregs. Any doubts that IFNγ^{+} cells were most important in disease initiation were dispelled, and it suggests that IL-17^{+} cells are not required to move to full blown disease but rather differentiate after inflammation has been established for a prolonged period of time in contrast to the many studies insistent on Th17 cells mediating autoimmunity.

These results highlight two roles that Tregs play, both the inhibition of activated effector T cells and the control of infiltration into the CNS. Most noticeable in the temporary depletion experiment was the correlation between cellular infiltration and period of time without Tregs. It appears therefore that Tregs can act as the gatekeepers of the BBB, preventing infiltration of autoreactive cells into the CNS.

In addition the demonstration *in vitro* that Tregs retain functionality at peak disease, reinforces the important role Tregs play in suppressing effector T cell activity. Interestingly, in response to MBP stimulation, only IFNγ was produced by effector cells.

At a disease stage when IL-17^{+} cells can be identified in the infiltrating population, it may suggest that they are responding to other stimuli, rather than direct myelin stimulation itself. The limitation of cellular proliferation and secretion of pro-inflammatory cytokines, irrespective of extracted origin, are in direct contrast to previous studies showing that Tregs extracted at peak EAE lack ability to suppress CNS derived effector T cells (Korn 2007a). There are no localised effects impairing Treg function from the inflamed organ observed that could be used to explain the progression of autoimmunity in this model. The use of pertussis toxin in the induction protocol of other studies may responsible for this inhibition of function, and also for the importance of Tregs in the recovery phase of EAE (McGeachy 2005). Once the pertussis toxin had been excreted by the mouse, Tregs would be able to function normally, and this could lead to either the recovery from symptoms, or stabilization of symptoms as seen in other EAE models. The ability of Tregs to curtail further disease progression upon return from DT-mediated depletion acts as an example of such a mechanism. Once the mouse has processed the DT, competent Treg populations begin to return and limit both cytokine release and further proliferation. Tregs readily traffic to the CNS to in this context and suppress inflammation in a similar way that they migrate in EAE. Therefore, EAE could simply be seen as an acute model utilising a forced impairment in T cell-mediated regulation to allow neuroinflammation rather than an accurate model of longer-standing disease where inflammation progresses despite a competent regulatory cell subset.

Tregs have been shown to demonstrate ability to suppress cytokine release, both by direct *in vitro* measurement after cell culture, and implied cytokine increase upon their removal from the mouse. Most noticeably, removal of Tregs released control on both

IFNγ and IL-17 secretion in the peripheral cells, but only IFNγ was observed in the CNS infiltrating populations. This provides evidence that Tregs are capable of suppressing Th17 cells, whilst reinforcing the idea that Th1 cells are most important in the initiation of disease.

In addition to observation of those cell populations under Treg control, it is also noticeable which cells do not appear to be under Treg control and are not deregulated upon removal of the Treg population. The consistent NK and γδ cell infiltration into the CNS demonstrates that their infiltration is not influenced by Tregs but could be a steady traffic across the BBB, representative of different roles in the CNS. γδ cells show no evidence of being under Treg control as neither their infiltration or activation status increases upon depletion of Tregs. The activation of NK cells as measured by IFNγ release shows Treg control but this is not representative of an increase in proportion of total IFNγ production, as the proportion of IFNγ derived from the NK cell population does not vary. The variable levels of control exhibited by Tregs over different cell types highlights a range of roles that each play in the development of autoimmunity. The large infiltration of NK cells implicates them in disease progression but the observation that it is only their release of pro-inflammatory cytokines and not their infiltration that are under Treg control offers a note of caution when assigning a role to these cell populations. The functions of NK cells in autoimmunity are likely multi-faceted, able to exhibit their own regulation without the requirement for a Treg presence. The model also highlights a potential for location dependent regulation as IL-17 production was under Treg control in the periphery but did not appear to be so in the CNS.

To move beyond Th1 and Th17 cells, an attempt was made to investigate the roles of NK and CD8 cells using this model. Incorporating observations from the base-strain, after removal of Tregs in L7Fox mice and then in more direct antibody depletion experiments, it was hoped to establish how important these cells were to disease establishment and subsequent progression. Upon removal of Tregs, NK cells rapidly infiltrate the CNS but the lack of a change in proportion compared to controls suggests that these cells are more likely to be bystanders rather than directly involved in mediating inflammation. They do however increase their production of IFNγ upon Treg depletion as a pathogenic cell would, and also upon partial depletion, IFNγ$^+$ cell infiltration is reduced. However, under the partial depletion, control on IFNγ production from infiltrating CD8$^+$ cells appears to be released, indicative of removal of regulation.

In response to Treg depletion, an increased infiltration of CD8 cells and most of the increase in IFNγ production is indicative of the release of control of their activation. It would appear that CD8 cells might have an important role in mediating neuroinflammation and after partial depletion of CD8 cells, lower IFNγ$^+$ cell infiltration was observed to support this. However, it also led to increased IFNγ production from peripheral cells and specifically within the CD4$^+$ population, more suggestive of a regulatory role and supportive of previous studies using CD8-deficient mice which showed CD8 cells act to limit CD4-mediated inflammation (Jiang 1992, Koh 1992).

Direct investigation of the effector cell dynamics in the model was not completely successful and so limits what could be learnt. Evidence could be taken for both protective and pathogenic roles for CD8 and NK cells in this autoimmune context, as has been suggested already by other studies (Zhang 1997a, Matsumoto 1998, Huseby 2001a,

Sun 2001, Abdul-Majid 2003, Jiang 2003, Ford 2005, Xu 2005, Winkler-Pickett 2008) These results suggest that likely the use of those markers is too general and groups many cellular subtypes together (as with CD4 including Th1, Th17 and Treg cells) that should be investigated individually. It may be more informative to try and deplete simply the regulatory or pathogenic subset of CD8 cells to ascertain whether they play an important role (Najafian 2003, Rifa'i 2004, Endharti 2005). Hampered by incomplete depletions however makes it additionally hard to draw definite conclusions from this study although further investigations should be a high priority. In this vein, knockout mice would be more relevant than the use of depleting antibodies in the context of Line7 disease. Therefore a breeding strategy has been initiated between Line7 mice and recently described NK cell knockout mice (Gascoyne 2009) although it will be some time before results from this cross become clear.

In conclusion, a model of disease could therefore be proposed whereby the presence of autoreactive T cells in the periphery of a Line7 predicates it towards the development of autoimmunity. Tregs function to prevent activation of these cells and infiltration across the BBB into the CNS. In a constant battle between effector and regulatory cells, there is a slow accumulation of effector cells through the BBB leading to autoimmunity. Tregs infiltrate the CNS in reaction and act to inhibit their activity although the slow progression of symptoms demonstrates the gradual accumulation of neurodegeneration. In mice able to maintain strong regulation, minimal effector cells infiltrate the CNS and autoimmunity does not develop. The main requirement for regulation to prevent disease appears to be through their action on IFNγ release by CD4 and CD8 cells and the

maintenance of the impermeability of the BBB, to control the inflammatory cell presence in the CNS.

This could be parallel to the mechanism of human disease, whereby individual genes impact on the strength of the autoreactive or regulatory T cells. Isolated incidents of environmental stress could tip the balance temporarily in favour of effector cells leading to relapses or a failure in regulation manifesting as the progressive forms of the disease.

12 Innate activation through toll-like receptors and spontaneous autoimmunity

12.1 Introduction

A remaining question to be investigated with the L7 mice is with regard to the triggering event that leads to the development of autoimmunity. As signs of disease develop in the L7 mice spontaneously with a variable time to first signs and 40% of L7 mice do not develop any signs of disease, autoimmunity does not develop simply as a result of genetic pre-determining factors, in a similar way to human MS. There is likely an environmental influence leading to the triggering of disease and manipulation of this event by small environmental changes is possible in the L7 model where it is impossible in the EAE model as the small changes are overwhelmed by the large immune adjuvants in the induction protocol.

The uneven worldwide distribution of MS points towards an impact of the environment on the development and progression of MS. Concordance rates of only 30% demonstrate that while there is a tangible genetic component (Ebers 1986, Dyment 2004a), there is a great deal left to explain as to why the disease initiates. Whilst latitude is the most closely correlated factor with MS distribution (Skegg 1987, Fawcett 1988, Ebers 1993), such a description is used purely to group together a series of environmental factors that all vary in a similar gradient. Sunshine exposure and conversely vitamin D levels will vary (Goldberg 1986) but so will local temperatures and ecosystems, leading to different varieties of plant and animal life that people are

exposed to on a daily basis. There will also be variation in the microorganisms, some pathogenic and others not whose distribution will align similar to latitude.

In the same way that there is not one 'MS gene' responsible for disease but rather it is a polygenic condition (Kemppinen 2011), each environmental factor is likely to convey variation in susceptibility which when coupled with other genetic and environmental factors lead to the development of MS. Being able to exclude certain factors and investigate the impact of one on its own will allow dissection of any impact, however small, it may have, and is the major advantage of using animal models, where the genetics and other environmental factors can be controlled for.

Migration studies have suggested that movement from high to low risk areas conveys protection from acquiring MS (Kurtzke 1993), although at what critical age this is not appropriate remains debatable (Dean 1997, Hammond 2000). This suggests that exposure to environmental factors through maturity impact greatly to determine susceptibility with infectious or endemic organisms an attractive candidate.

Toll-like receptors have been implicated in the development of MS as increased expression has been observed in CSF and MS brain lesions and also in EAE brains (Bsibsi 2002, Hanisch 2008). MyD88 knockout mice, lacking the adaptor protein for many TLRs have been shown to be resistant to EAE (Marta 2008, Cohen 2010b) and more generally, the requirement for adjuvants in the EAE protocol suggests an influence of TLR signaling in development of disease. *M.tuberculosis* in CFA activates TLR4 and activation of TLRs by other microbial products has been shown to be capable of inducing EAE (Waldner 2004).

Previous work in the lab showed an increase in TLR2 expression which correlated with the development of disease signs in the Line7 CNS although it was unclear whether this played a causal role (Thomas Alexandris, Msc Imperial College). Therefore, it was of interest to see whether activation of this pathway could act as a triggering mechanism for development of disease in the Line7 mice. The addition of individual TLR2 agonists in regular doses was used to mimic continual environmental exposure in mice already primed to develop autoimmunity. The Line7 model is ideal to work with in this context as they are genetically susceptible to development of autoimmunity without the requirement for additional adjuvants (Ellmerich 2005), but still require an unknown triggering mechanism to initiate disease.

Responses to the introduction of TLR2 agonists would be measured not only by clinical score but also T cell proliferation and cytokine release. It was reasoned that whether or not TLR2 agonism itself may be sufficient to trigger disease, there was evidence from other studies of synergistic effects to increase the reactivity of T cells through their TCRs (Rudd 2008) which may itself impact on disease in this model. In addition, an increase in release of pro-inflammatory cytokines after TLR ligation could accelerate T cell polarization and lead to a greater likelihood of developing symptoms (Reynolds 2010).

TLRs are activated in the induction of EAE (Waldner 2004) but activation of certain TLRs may be more or less important in MS. Activation of TLRs would offer an overlap between EAE and MS as they mediate the reactivity to pathogens by the innate immune system. Environmental factors signaling through TLRs may influence the immune system and act as triggering mechanisms for the development of MS. Production of pro-inflammatory cytokines after TLR ligation may act to polarize T cell populations or upregulation of MHC

CII could both influence autoreactive T cells and aid in their activation and subsequent initial autoimmune attack (Jack 2007).

If TLR ligation has a role to play in the development of MS, there would likely be a difference in reactivity between MS patients and healthy controls. This could be either over-reaction to harmless ligands, which could lead to a disproportionate immune response and production of a large number of pro-inflammatory T cells. In contrast, a lower response to TLR ligation may be representative of tolerance.

Tolerance is an adaptive mechanism that acts to limit the extent and duration of immune activation to further challenges by an antigen. Should a TLR ligation event have been important in triggering the autoimmune attack, MS patients would be expected to be tolerised to a subsequent stimulation with the same ligand. Therefore, it is of interest to investigate whether reactivity to TLR ligands is affected in MS patients with respect to controls. Any disparity will add further support to the role of TLR ligands in the initial autoimmune attack in MS.

As an attempt to identify a difference, a panel of TLR agonists was used to stimulate whole blood derived from patients and matched healthy controls. The release of pro-inflammatory cytokine TNFα was measured by ELISA to determine any differences in stimulation in response to each of the stimulants in the panel.

It has been shown that systemic challenge to TLR ligands can lead to cross-tolerance to signaling through other TLRs (de Vos 2009) which should mean that where a person is tolerised to a specific TLR ligand, changes in cytokine production would be detectable even if that specific TLR ligand is missing from the panel.

Whilst not being a comprehensive study into environmental factors in the development of MS, these experiments offer indications as to whether TLR agonism is an important factor that contributes to the triggering event leading to autoimmunity.

12.2 Results

12.2.1 Introduction of TLR2 Agonists to Line7 Mice

Previous observations in the Line7 mouse model showed that TLR2 expression, as measured by real-time PCR, correlated with disease progression. In order to try and ascertain whether this was a causative rather than just correlative effect, TLR2 agonists were introduced into groups of 4 (2 male, 2 female) Line7 mice to see whether they could act as a trigger, upregulating TLR2 expression and/or supplying evidence for a role in initiating disease in these mice. In the controlled housing of the mouse facility, the introduction of these agonists would be the only environmental factor different between highly MS-susceptible mice. PBS was administered in a separate group to control for the influence of the immunisation protocol.

3 TLR2 agonists were selected based upon their different organisms of origin. LPS derives from gram-negative bacteria, LTA from gram-positive bacteria and LM-MS from mycobacteria. LPS stimulates both TLR2 and TLR4, LM-MS induces the release of cytokines including IL-8 and TNFα in a TLR2 and MyD88 dependent but TLR4 independent manner, and LTA also stimulates the release of pro-inflammatory cytokines including TNFα. It was therefore also of interest to see whether different agonists could show different potencies for triggering autoimmunity. Immunizations were administered i.p. every Monday and Friday for three weeks so a total of 6 injections were given per mouse. Mice were culled three weeks after the commencement of the study as this was the maximum length of time permitted under the guidelines of Professor Altmann's project licence for such an experiment. The dose size of each agonist was determined after consideration of related literature, the lethal dose of each agonist in mice and relevance

to likely scale of agonist seen in the human context. Dose sizes were selected that ensured that the dosing regime would not exceed the lethal dose but as there were limited preceding studies using a continual exposure in mice as in this investigation, the doses were estimated in an attempt to try and model small non-life threatening infections similar to continual environmental exposure. A lack of availability of mice and duration of the experiment hindered detailed range-finding experiments of dose size, which would have been beneficial to do.

The CNS and a small piece of spleen tissue were extracted from all mice and homogenised in Trizol for RNA extraction, whilst cells were extracted from the remaining spleen tissue for cell culture experiments.

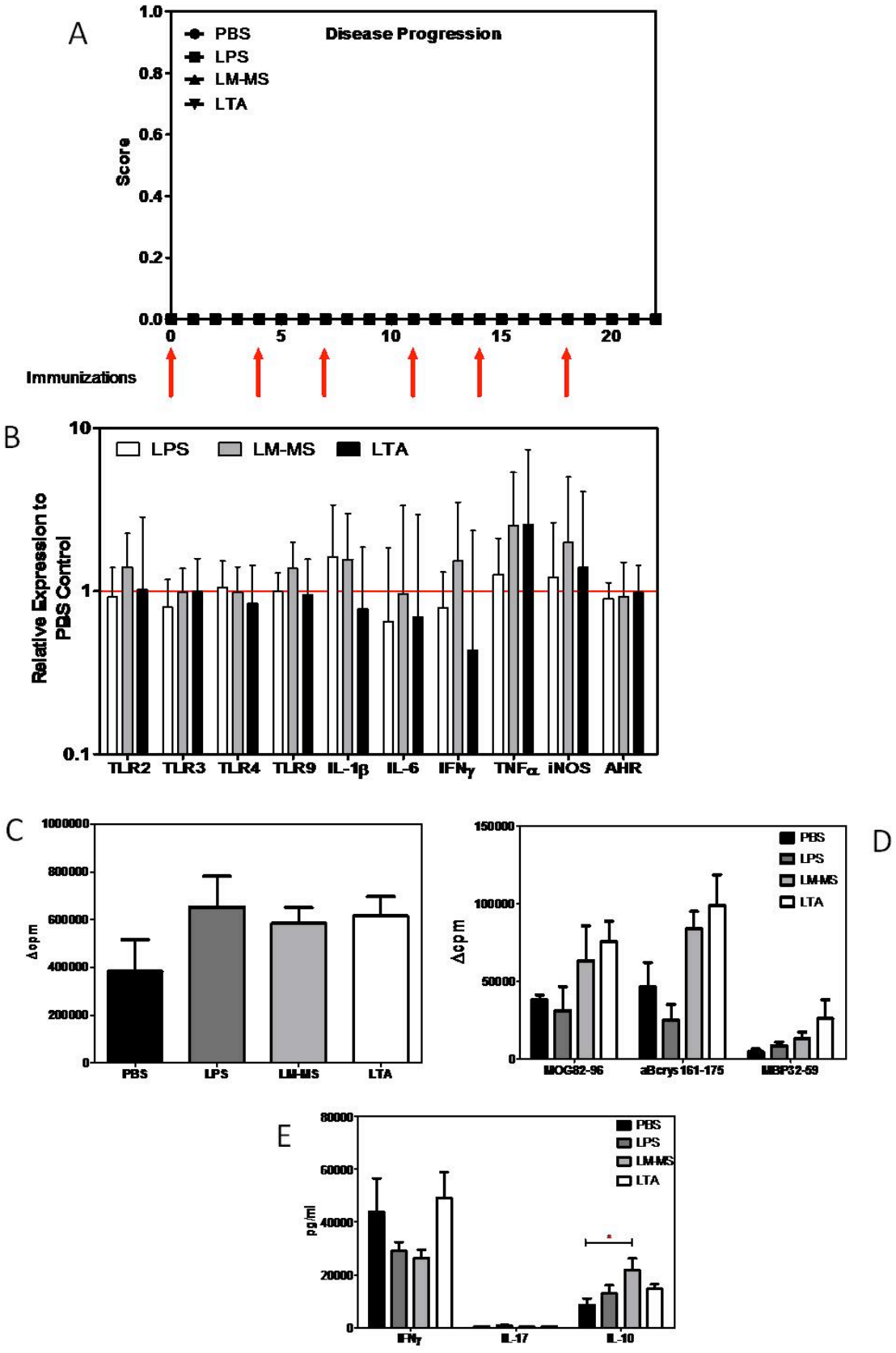

Figure 25. Administration of TLR2 agonists has no effect on disease initiation.

*TLR2 agonists were administered to mice on days indicated by arrows (A), mice scored daily and average disease scores are plotted for each group (n=4 per group). mRNA was extracted from spinal cord tissue of L7 mice receiving TLR2 agonists or PBS taken at the end of the study. Relative expression of spinal cord mRNA transcripts from each group was compared to PBS control as measured by real-time PCR using individually designed hydrolysis probe based assays and analysed using REST software (B) n=4 per group, * denotes significance compared to PBS control expression ($p<0.05$) (randomisation analysis). Mean cellular proliferation of lymphocytes extracted from the spleens of individual mice (n=4 per group) ±SEM in response to 25µg/ml MBP_{85-99} (C), 50µg/ml of other, 'myelin spread' epitopes (D) assessed after 4 day stimulation by thymidine incorporation. Cytokine secretion from these stimulated cells measured by ELISA (E). Asterisks denote statistical significance between two groups; * $p<0.05$, ** $p<0.01$ (two-tailed T test).*

The introduction of each of the 3 TLR2 agonists had no effect on disease initiation *per se* across the time frame of the study and no mice developed any symptoms (Figure 25A). To assess whether any pathways had been activated by the introduction of these agonists, the transcription profile of the spinal cord and spleen (data not shown) was examined by real-time PCR and analysed by REST software. Genes tested included a range of TLRs, and a selection of pro-inflammatory cytokines thought to be important in the development of autoimmunity. Expression levels of gene transcripts were compared to those seen in the PBS control mice and a relative expression level determined. No significant up or downregulation of any genes were detected in either the spinal cord or

spleen as a result of TLR agonist immunisation (Figure 25B), suggesting that it is unlikely that the introduction of the TLR2 agonists had much effect at this time point. Observation earlier in the study may have yielded different results but if sub-clinical autoimmunity was developing then transcript levels would still be expected to be elevated.

In order to see whether the agonists had other effects aside from stimulation of pro-inflammatory cytokine release, lymphocytes were extracted from the spleen and assessed for their proliferative potential to MBP_{85-99} and other myelin antigens to which epitope spread has previously been reported in this model (Ellmerich 2005). Line7 TCRs are specific for MBP_{85-99} and so initial autoimmunity is assumed to be mediated by recognition of this antigen *in vivo*. As disease develops, mice show reactivity to other myelin epitopes and so these were included to determine whether such a process may be accelerated by TLR stimulation. Proliferative responses of T cells by thymidine incorporation was measured after cell culture in the presence of myelin peptides for 4 days and a two-tailed t test used to determine statistical significance of differences between means. This showed that cells from those mice receiving TLR2 agonists had a trend towards being more responsive to MBP_{85-99}. Significantly higher responsiveness to spread epitope αBcrystallin$_{161-175}$ was seen from mice receiving LTA and LM-MS. Mice receiving LTA also showed a trend towards higher proliferation in response to MOG_{82-96} but no other increased responses were observed from mice receiving any TLR2 agonist to any of the spread epitopes of myelin antigens (Figure 25C,D). Addition of LM-MS and LTA lead to a trend towards upregulated proliferative responses to αBcrystallin$_{161-175}$ peptide whilst LTA also upregulated the response to MOG_{82-96} and showed a trend

towards increased proliferation in response to MBP_{38-59} (Figure 25D). LPS elicited no significant change in proliferative potential to any peptide tested.

In contrast to the proliferation data, when the cytokine profiles of responding T cells were compared between Line7 mice in the presence of absence of TLR2 agonists, clear differences were found in the nature of the autoimmune response. Cytokine release was measured by cytokine-specific ELISA on cell culture supernatants extracted from the MBP_{85-99} stimulated cell cultures.

Levels of IL-17 were barely detectable from any culture, either from those mice that had received a TLR2 agonist or the PBS control, demonstrating that this cytokine was not produced in response to MBP_{85-99} stimulation. Cells from all mice receiving a TLR2 agonist released a comparable amount of IFNγ to those receiving the PBS control, but there was a trend towards reduced IFNγ release from mice receiving LPS and LM-MS which did not achieve statistical significance (Figure 25E). This would suggest an impaired response to MBP_{85-99} which may be responsible for the lack of disease initiation observed. In support of this theory, somewhat surprisingly, higher amounts of IL-10 were released in mice that received LM-MS and LTA compared to the PBS control whereas mice receiving LPS released comparable levels of IL-10 to the control mice (Figure 25E). Taken together, the reduction in IFNγ and increase in IL-10 released may suggest that after the immunisation regime and subsequent re-stimulation, the agonists may not be stimulating the cells towards a pro-inflammatory pathway anymore. Cytokine release from cultures stimulated with spread epitopes was not detectable at levels significantly different to background and so is not shown.

Despite the failure of mice to develop signs of disease, and the limited variation generated in gene transcription, the increased proliferative responses, especially to the spread epitopes were encouraging as this is only usually seen in mice that have progressed through to full paralysis.

Working on the hypothesis that the doses of TLR agonist used had been too low to elicit sufficiently large immune responses in the mouse, the agonist showing the strongest proliferative responses, LTA, was selected and administered to 5 (2 male, 3 female) Line7 mice at a higher dose. The same dosing regime was used, so that mice received two doses per week and were culled three weeks after the first dose. PBS was administered to a matched group of mice under the same protocol to act as a control for regular immunizations.

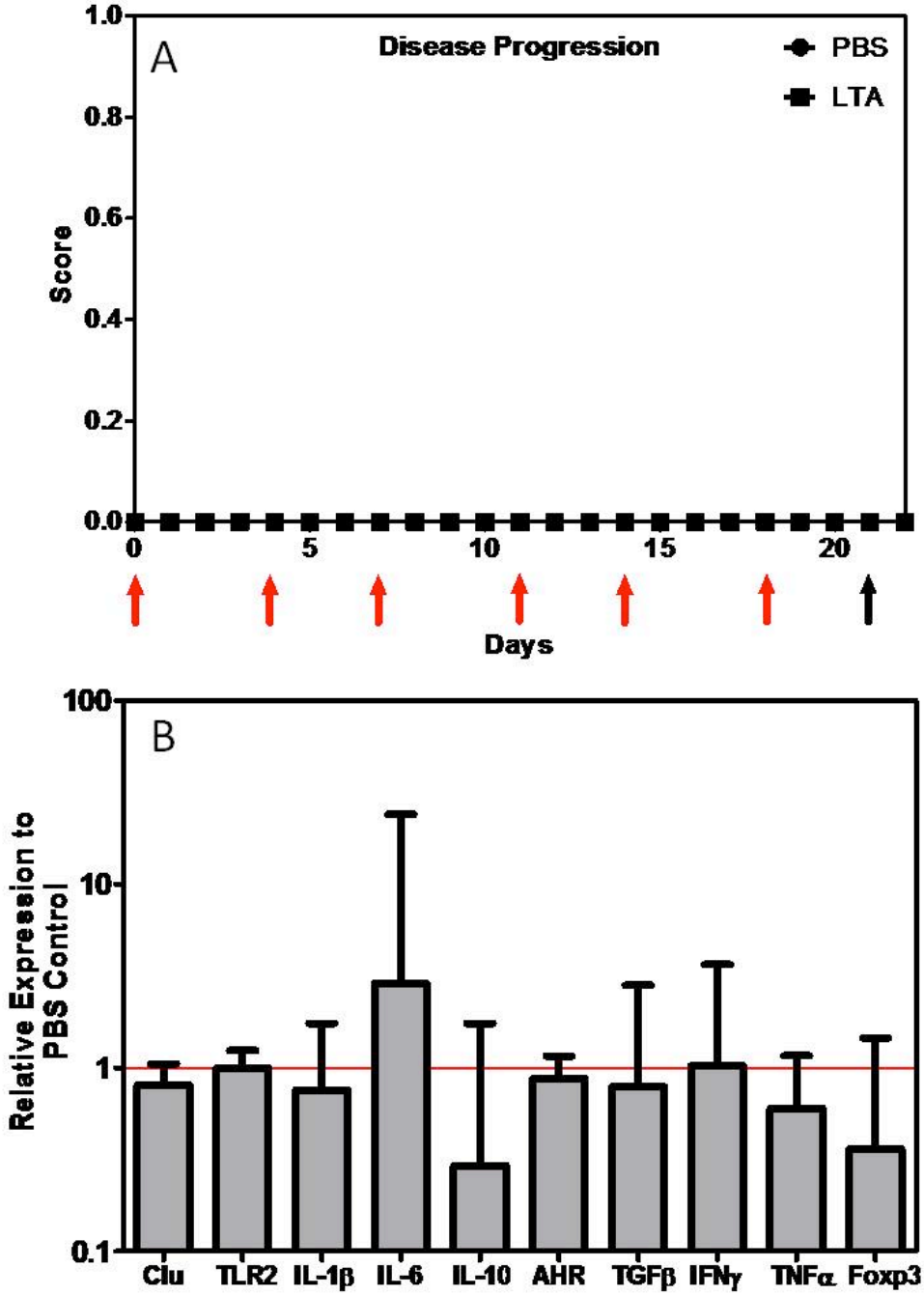

Figure 26. Administration of a higher dose of one TLR2 agonist has no effect on disease initiation.

LTA was administered to mice on days indicated by arrows (A), mice scored daily and average disease scores are plotted for each group, n=5 per group. mRNA was extracted

*from spinal cord tissue of L7 mice receiving TLR2 agonists or PBS taken at the end of the study. Relative expression of spinal cord derived mRNA transcripts from the group receiving LTA compared to PBS control as measured by real-time PCR using individually designed hydrolysis probe based assays and analysed using REST software (B) n=5 per group, * denotes significance compared to PBS control expression (p<0.05) (randomisation analysis).*

Despite the increase in dose of TLR2 agonist, no disease initiation was observed (Figure 26A). Samples from the CNS and spleen were taken as before for both analysis of mRNA production and cellular proliferation responses. Measurement of gene transcription by real-time PCR was carried out for pro and anti-inflammatory cytokines, as well as TLR2, Foxp3 and Clusterin, a protein involved in amyloid formation and clearance that has been linked to the development of multiple sclerosis as well as an association with Alzheimer's disease, another neurodegenerative condition (Stoop 2009, Thambisetty 2010). Analysis was done using REST software which also calculated statistical significance. No significant change in gene expression was observed between the mice administered LTA and those administered PBS (Figure 26B) that would suggest early stages of disease initiation. iNOS was included in this analysis, as it is known to be produced by microglia in response to MBP-specific T cells (Dasgupta 2002) but it showed no variable expression which would have been expected if autoimmunity had been triggered. Extracted spleen cells were stimulated with the same panel of myelin peptides as in the previous experiment for 4 days and supernatants collected for analysis of cytokine release. Unfortunately a technical failure of the proliferation assay means that data is missing.

The failure of any signs of disease to develop or there to be any significant changes in gene expression meant that the experiment was not repeated to generate this data. Cell culture supernatants were also not analyzed as there was no evidence that they would show results different to the previous observations.

12.2.2 Responses to TLR Ligation in MS Patients

An environmental trigger for MS is an attractive hypothesis as this might open options for therapeutic intervention or lifestyle modification interventions, and could go some way to explaining the geographical distribution of the disease observed. TLR signaling could provide mediation for the initial immune insult that could trigger the first autoimmune attack. Evidence for such an event may be detected through tolerance to TLR ligands, suggestive of previous stimulation at a level above that seen in healthy controls.

In an attempt to investigate whether previous TLR agonism could play a role as triggers for disease initiation, a panel of TLR and NBOD agonists (Table 14) was created to stimulate whole blood extracted from 10 MS patients. By comparing their responses to those of 8 age and sex matched healthy controls, it would be possible to see there are any differences in the innate immune responses that could be characteristic of MS patients and suggestive of over-reaction or tolerance to innate, environmental activation. In particular, the hypothesis was that, it may be possible to uncover a footprint of ongoing TLR agonism through the detection of functional down-modulation of particular pathways, as has been described in other diseases (Biswas 2009, De Nardo 2009, de Vos 2009). TNFα ELISA was chosen as a read-out as it has been shown to be a pro-inflammatory cytokine commonly released in response to TLR stimulation.

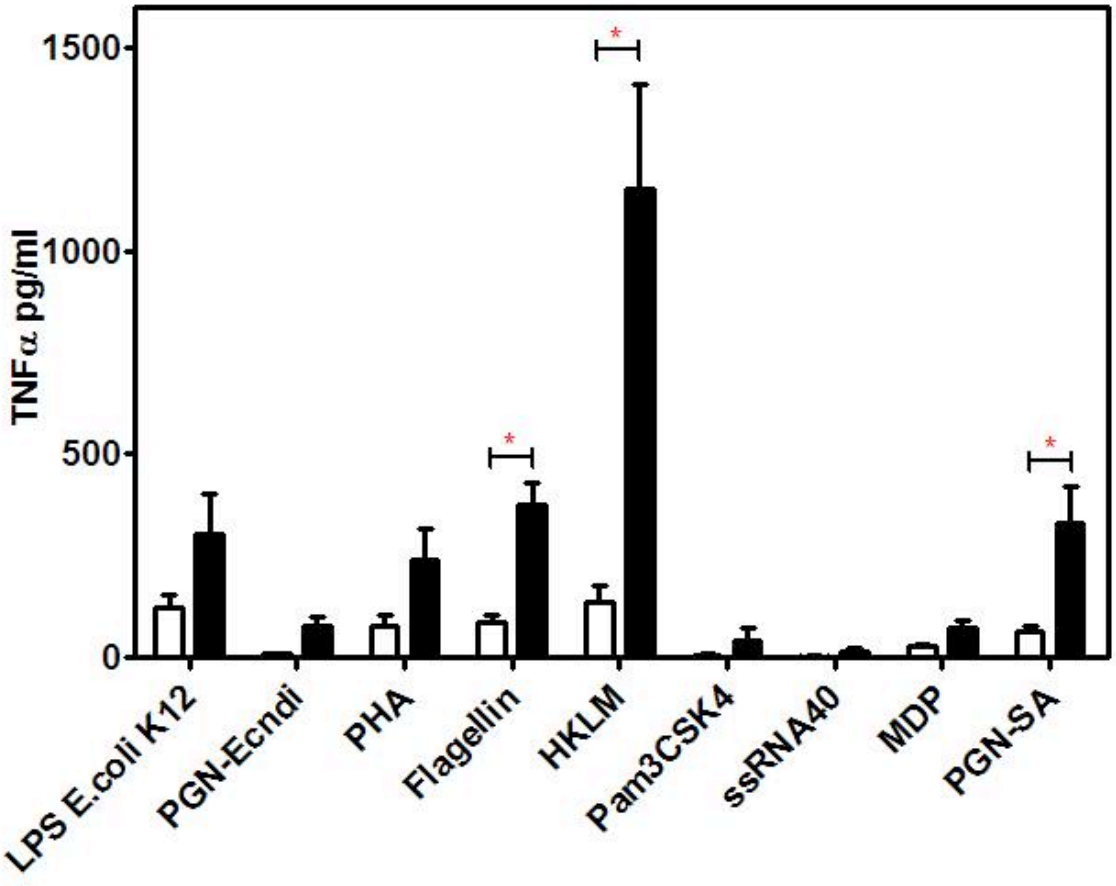

Figure 27. Lower reactivity to TLR agonists by MS patients.

*Whole blood from MS patients and healthy controls diluted 1/10 and stimulated with TLR and NBOD agonist panel. Supernatants aspirated after 24hr stimulation and TNFα secretion measured by ELISA. Differential responses recorded for MS patients (white) (n=10) and age and sex matched healthy controls (black) (n=8). Bars show average response for each group with the SEM. * shows significance between groups (p<0.05 two-tailed T test).*

When the relative responses of MS patients are compared to those seen from the healthy controls, it is clear that the MS patients' response to the TLR agonists was less than that of the healthy controls. Not all of the agonists in the panel elicited positive responses, and cytokine release could not be detected after stimulation with some agonists from both the MS patient and healthy control groups (data not shown on graph). It was possible to detect cytokine release after stimulation with 8 TLR agonists alongside the positive control and using these, compare the relative responses between MS patients and healthy controls. The only significantly different responses between MS patients and controls were to HKLM (TLR2), PGN-SA (TLR2) and Flagellin (TLR5). The MS patients' response to these agonists were lower than the healthy controls in each case. In a similar way, there were trends towards weaker responses to LPS (TLR2), PGN-EColi (TLR2), Pam3CSK4 (TLR1/2) and MDP (NBOD2) (Figure 27) in MS patients compared to healthy controls but none of these reached statistical significance. The other agonists (not shown) did not elicit responses higher than the baseline samples in either the MS patient or healthy control groups and so were not considered to have successfully stimulated the blood samples. No response from the MS patients to any TLR agonist was in excess of that by the healthy controls showing a dampened innate activation as a result of MS disease.

The absolute size of the response to each agonist can be misleading in this context however as the doses for each agonist were not uniform to each other so it is only the relative responses between patient and HC samples that are worth comparing. This data does not demonstrate an over-reactive response to TLR stimulation that could act as a trigger for autoimmune initiation although further work is required to determine

whether the lower responses are due to a systemic suppression or previous over-stimulation of TLR pathways.

12.3 Discussion

Incomplete concordance of MS between monozygotic twins among other factors illustrates an important environmental influence in the development of MS (Bulman 1991). Mirroring that, a percentage of Line7 mice that fail to develop signs of disease demonstrating that there must be a triggering point for disease initiation. Bringing together the geographical distribution of MS and the success of EAE as a model for MS, TLR ligation is an attractive target to influence the immune system and provide the metaphorical straw that broke the camel's back. It is difficult to investigate the role of TLR ligation in the development of EAE because the method of induction requires TLR ligation to break tolerance, often using *M.tuberculosis* in CFA that acts to stimulate TLR4. In support, MyD88$^{-/-}$ mice, lacking the adaptor protein for almost all TLRs have been shown to be resistant to EAE induction (Prinz 2006). Therefore, models with spontaneous disease would offer better and more relevant situations in which to assess the importance of TLR ligation without the large effect of inducing adjuvants.

Attempts using other TCR transgenic mice, that exhibit low levels of spontaneous disease, appear to have identified a role for TLR ligands in disease initiation (Waldner 2004). However, these studies have been limited as they still rely on the concept of EAE to initiate disease. In these cases, different TLR ligands were immunized alongside IFA (lacking *M. tuberculosis*) and followed with a protocol of Pertussis toxin injection. Therefore they have simply substituted one TLR ligand for another in the protocol, and rather than prove the requirement for TLR ligands in MS, have simply proved they are necessary for the EAE model.

Using the Line7 mice, which does not require any adjuvants to initiate disease in a high percentage of mice provides a more attractive and realistic experimental model. In this situation, the introduction of TLR ligands should appear as small environmental insults to which mice can respond with competent immune systems. However, initiation of disease was not seen after such an introduction of TLR ligands. Mice appeared normal with no signs of sub-clinical disease after the course of immunisations when CNS and peripheral transcriptional profiles were measured. Trends towards greater reactivity to MBP_{85-99} hinted at possible activation but with higher IL-10 release from this stimulation, it is likely not generating the pro-inflammatory responses required to initiate neuroinflammation. Indeed, the regular doses may have acted to tolerize mice to the TLR ligands and a variable dosing regime would lead to more success. A higher dose of LTA did not increase disease initiation suggesting either that it was still not high enough or that it was too high and tolerance was once again induced.

This experiment was an attempt to investigate the role played by TLR2 in Line7 disease progression. Previous observations that TLR2 expression increased in the CNS as disease severity increased did not provide a clear evidence to distinguish between a causative or simply correlative relationship. This study would lend support to the expression increase being a simple correlation of disease severity. The increased expression could be attributed to an increase in cellular infiltrate rather than upregulation of the receptor. Activation through TLR2 does not appear directly responsible for initiation of disease although a role in further development once disease is established cannot be ruled out.

The triggering mechanism itself may also be more complicated. As with the development of EAE, the requirement of both TLR ligands and immue suppressing adjuvants to initiate

disease, it would be logical to conclude that in Line7 and human disease, a number of insults would be present, and their timing relevant, to trigger autoimmunity to develop in an otherwise healthy individual.

Despite not achieving its original aim, an intriguing observation was the increased proliferation in response to myelin spread epitopes. Reactivity to these epitopes is usually associated with progression through disease (Ellmerich 2004, Davies 2005) and so it may be that TLR activation aids to widen the scope of reactivity for T cells. Extrapolating this could provide a mechanism for generation of relapses following environmental insults, although this would need to be studied much more specifically.

Data from the MS patients indicated a lower response to TLR ligands compared to healthy controls. Whether this is directly relevant to the development of the human condition is still unclear. Previous stimulation through TLRs has been shown to be sufficient to drive development of TLR tolerance, through mechanisms including down-regulation of receptors, immune cell apotopsis and alterations of signaling pathways (Biswas 2009, De Nardo 2009, de Vos 2009). Tolerance to previously encountered pathogens is of benefit to prevent disproportionate immune responses that could lead to endotoxin shock and substantial tissue damage. Such tolerance to TLR ligands may be characteristic of previous signaling through these receptors and the lower responses demonstrating greater exposure to TLR ligands in earlier life. Whether such exposure and the subsequent reaction is reflective of local environments or genetic differences between individuals can not be determined. How an individual reacts to insults early in life is likely determined by their genetics and so could offer a link between genetic susceptibility loci and the environment in which an individual lives.

Most of the patients who donated blood were on treatment or had previously been receiving treatment. This could mean that the immune dampening observed is due to immune suppression elicited by these therapies or their own body in response to a relapse. Whether the patients were currently experiencing a relapse or had recently was not consistently recorded and so unfortunately hinders use of this factor for interpretation of the results. It would be more interesting to test blood donated from a cohort of MS patients before treatment strategies are started, as these responses would be more relevant to control for the effect of the disease alone.

A lower overall innate response could be indicative of compromised immune responses, rather than induced tolerance to previous exposure. However, even both could have implications for the development of MS as general immune suppression would be a poor way to fight autoimmunity and would suggest that the function of regulatory pathways is impaired. Distinguishing between these two hypotheses would determine whether TLR ligation had a role to play in the initial development of MS. The difficulty of such experiments is that measurement is done after disease is established and so may not be directly relevant to the early stages of disease. Without relying on animal studies, it would be interesting to compare reactivity of individuals to TLR ligands over a longitudinal time frame or those suffering from initial neurological attacks (clinically isolated syndrome (CIS) patients). These samples would be more relevant to early disease when it is hypothesized that effects of TLR ligations are mediated.

13 Final Discussion

13.1 Usefulness of Animal Models

Findings in animal models hold great impact it the research community as they are much faster to do, with higher reproducibility than work in humans. Our understanding of MS pathogenesis is much greater with their use but because of the relatively small number of widely used models, the question has to be asked whether we are actually finding out relevant details for human disease or just investigating nuances in the animal models. TCR transgenic models have provided much greater specificity to disease and tolerance research, but despite the first model being described 23 years ago (Kisielow 1988a), there are still only a handful being used as pre-clinical models for any given condition. In MS research, there considerable reliance on EAE, and the adjuvants used in the protocol, to induce inflammation and autoreactivity in the CNS. The disease course is very short and synchronous, unlike that of human MS, and it is unlikely that in this short window, all of the important events in MS pathology take place. The resolution of disease signs in many EAE protocols is a weakness in terms of relevance to a chronic long-standing human disease and is more reminiscent of an acute brain infection. A more relevant model would therefore be spontaneous, having the disease initiate without the need for large immune adjuvants to overwhelm the inbuilt regulatory mechanisms and so introduce in-built biases. TMEV has been regarded in some respects to fulfill this need and be more accurate at predicting therapeutic potential than EAE (Nelson 2004) but until MS is proved to have a viral cause, the direct relevance of this model will be questioned.

The advantage of a humanized TCR transgenic mouse in this context would be that findings in each HLA-restricted epitope could be transferable to the human disease, especially if the mouse also possessed both sides of the TCR-MHC interaction. The Line7 mouse model fulfils these criteria, having a TCR from an MS-derived T cell clone (Ob1A12), seeing antigen presented by human HLA-DR15, the strongest genetic susceptibility locus implicated in caucasian disease and functionally implicated in the causality of MS. This mouse model was recently adopted by the GlaxoSmithKline Neuroscience institute in Shanghai as their key pre-clinical model for MS research. In previously published work, it has been shown that these mice are pathologically relevant to human disease and exhibit epitope spread to other myelin antigens (Ellmerich 2005). The disease course shows a slow progression of disease signs across the life-time of the mice rather than one acute attack and does not present signs until around 4 months of age. There is also a female propensity, as it is only male mice that can remain unaffected, partially reflecting the situation in humans. The disease presentation and subsequent progression of disease signs could therefore be a model for the underlying neuropathology of RR-MS patients, and in some cases show PP and SP disease courses. Relapses are not seen in the same way as in human MS and although the mice do show some variability day to day, it rarely shows up in the scoring protocol and they do not obviously resolve in between attacks. Relapses have been reported to be associated with T cell reactivity to new epitopes (McCarron 1990) and also to the presence of anti-MOG antibodies from studies with RR-EAE models (Amor 1994, Zhang 2004a, Pollinger 2009). The reactivity to new myelin epitopes offers an attractive hypothesis for development of relapses as variation has been demonstrated in the location of neuroinflammation with

the different myelin epitopes used to initiate EAE (Greer 2008). The lack of a consistent region of the CNS being attacked could be responsible for the observed relapses and would explain why relapses were not seen in Line7 mice, as the immune response is dominantly directed to MBP_{85-99} until later disease (Ellmerich 2004). The use of a combination of myelin epitopes (MOG and PLP) have been shown to be capable of inducing RR-EAE (Zhang 2004a) so it would be attractive to hypothesise that the incorporation of a second transgenic TCR into the Line7 mice, or ability to speed up epitope spread may lead to the development of relapses and transformation of the chronic progressive model into one with a relapsing-remitting profile.

The presence of MBP-specific B cells and generation of antibodies have not been investigated in the Line7 model but would offer another explanation for the lack of relapses observed. In a MOG transgenic mouse model, MOG specific B cells have been shown to be responsible for development of spontaneous RR-EAE and their removal suppressed the development of RR-EAE when started at a young age (Pollinger 2009). B cells can also work in the opposite direction, aiding Treg function and releasing IL-10 to limit inflammation (Fillatreau 2002, Mann 2007). An autoreactive polarization of the B cells in the Line7 mice would offer a simple hypothesis for the progressive disease seen although whether this is the case will only be borne out by further experiments.

These progressive signs of disease however allow a great advantage not present in other EAE models, that of the availability and consistency of samples across the development of disease. Samples can be taken before and during disease development, at all the major paralysis landmarks, to elucidate which cells are involved at each stage, rather

than simply which ones are there at the peak of disease as observed from EAE studies or post-mortem human tissue. T cells have been shown to be present in the CNS of normal mice (Brabb 2000) and they can be extracted from pre-disease presenting Line7 mice, allowing a baseline to be determined.

The Line7 model overcomes some of the limitations presented by other TCR transgenic mice and reduces the gap between animal models (EAE) and MS. Other humanized mouse models have been developed using the same TCR-MHC restriction as the Line7 (Madsen 1999) but in this case, the TCR repertoire was close to normal and very little spontaneous disease was observed. These mice more closely resemble the Line8 mice (Ellmerich 2004), a sister line of the Line7s. The higher proportion of $TCR^{+}CD4^{+}$ T cells in the Line7 allows spontaneous disease to progress without the need for adjuvants, crossing onto a $RAG^{-/-}$ background or limiting the B cell repertoire (Bettelli 2006a). Limitations in obtaining funding for large numbers of new transgenic lines and the tendency, once a model is published and accepted to share it between labs as a 'safe option' can have the effect that inferences from individual models are given disproportionate weight in extrapolations to human disease. There should be consideration of many models alongside recognition of the limitations of each when developing ideas and theories about human conditions from animal models. Only a small number of mouse and human TCRs relevant to MS or EAE have thus far been explored in transgenic models; even this small number offers clues that a wider application of the approach might help to illuminate the enormous diversity of symptoms, CNS localization, PNS involvement and severity seen in the human disease. For example, a TCR transgenic made in the Kuchroo lab, with a receptor directed against MOG_{35-55}, showed an

unpredicted optic neuritis pattern that had not been seen in other TCR transgenics (Bettelli 2003). Wider application of this approach to explore the specific pathogenic correlates of TCRs against commonly identified HLA/peptide combinations in human MS patients may thus be beneficial.

13.2 More complicated picture than Th17?

EAE has highlighted a role for Th17 in mediating autoimmune attacks. However, evidence from the Line7 model challenges that assumption and prompts questions to be asked about whether the results generated were true representations of MS, or rather artifacts of the model itself. Adjuvants (CFA and pertussis toxin) in the EAE immunisation protocol introduce biases; *Mycobacterium tuberculosis*, present in CFA, and pertussis toxin have been shown to stimulate IL-17 release (Veldhoen 2006a, Chen 2007) providing a compelling argument that the propensity of Th17 cells is a result of polarization by the immunization protocol. Whilst the intention is not to challenge the involvement of Th17 cells in EAE, the relevance of findings in EAE to MS should not be assumed. In addition, the ability to generate pure T cell polarised cultures for the adoptive transfer model of EAE is always questioned (O'Connor 2008, Jager 2009), and the presence of a residual negative cytokine-expressing population leaves the door open for arguments.

The spontaneous nature of the Line7 model allows investigation of disease without these introduced biases, with no T helper subset favoured by the method of disease induction. The TCR is *in vitro* Th0, with both IFNγ and IL-4 produced in response to MBP_{85-99} (Ellmerich 2004). The MS-derived TCR and immunogenetically human status of the mouse also provide direct relevance to the human condition. In this context, the

demonstration that Th17 cells are not important in early stages of disease should restrain unabated enthusiasm for the importance of Th17 cells in human disease. Findings regarding the encephalitogenicity of T-bet-expressing cells are reaffirmed (Gocke 2007, Yang 2009b), and noticeably, the source of pro-inflammatory cytokine is not restricted to the $CD4^+$ T cells. $CD8^+$ T cells are seen in MS lesions (Babbe 2000, Junker 2007) and their presence in Line7, despite the model being an HLA class II transgenic, underline the importance of these often overlooked cells in neuropathology. Whether these cells are protective or pathogenic is not clear from this work and would require further investigation.

Immunologists have dug themselves into a corner with their over-classification of immune cells. The largest example of this is in the division of helper T cells where the consideration of which cells are important in autoimmunity overshadows consideration of the factors that are mediating the damage observed. Experiments are interpreted with the view that T cell polarization is crucial to their function, and plasticity is a side effect, characteristic of certain helper cell commitments. An ideal immune system would be one that could react to unexpected insults and so terminally polarized T cells would not be an advantage to such a system.

The classification criteria has led to studies being considered contradictory to each other whereas the focus of each study may have been misdirected at factors unimportant in development of autoimmunity instead of those more relevant. An example of this is a recent paper by Hirota et al (Hirota 2011). In this the production of IL-17 caused T cells to express YFP for their lifespan and allowed tracking of 'ex-Th17' cells. The interpretation that as all cells infiltrating the spinal cord in EAE were yellow was less

important than the observation that IFNγ was the dominant cytokine expressed in the inflamed organ. The importance of plasticity of helper T cells was given more emphasis to the detriment of the T cell function in the autoimmune context. IL-23 was also shown to promote T-bet, a finding which in the guise of Th1 and Th17 classification adds confusion. This was interpreted as further support for the role of the Th17 phenotype in autoimmunity, but this is simply one of many interpretations. T-bet has been shown to define the encephalitogenicity of T cells in other studies and IL-23 KO mice are resistant to EAE, meaning these results go some way to explaining the seminal experiments of Cua et al in 2003 (Cua 2003) without the requirement to exclude an important role for IFNγ. It could be argued that factors assigned to Th1 and Th17 cells play a role in autoimmunity together, and such a hypothesis would be supported by observations in Line7 mice. The importance of T-bet and IFNγ in development of autoimmunity are seen in the Line7 model as well as the study by Hirota et al. Therefore whilst classification is a useful tool to compare results and further our understanding, it can be adding layers of complexity and preconceptions that hinder accurate consideration of which factors are the most important in a given context.

13.3 Controlling role for Tregs

Whilst Th1 cells appear to be involved in the initiation of neuroinflammation, the most valuable observation is with regard to the importance of Tregs. EAE studies have demonstrated autoimmunity progressing because of impairments in regulation, linked to a lower Foxp3 expression (Huan 2005, Venken 2008b) but in the Line7 model, it is shown

that Tregs are functional and autoimmunity can progress despite this regulation. This is more in keeping with the human condition, as MS patients do not suffer systemic autoimmune attacks in much the same way as patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and neurodegeneration occurs progressively over long periods of time in most cases. In fact Tregs have been shown to have normal suppressive function and Foxp3 expression in secondary progressive MS as compared to RR-MS (Venken 2006).

Origins for the results from EAE can be derived from the use of pertussis toxin in the EAE protocol. Pertussis toxin acts to inhibit Tregs (Cassan 2006, Chen 2006) alongside increasing the permeability of the BBB (Brabb 1997) although the BBB permeability could be derived from the Treg inhibition as demonstrated in the L7Fox Treg depletion experiments.

The significant role for Tregs demonstrated in this work highlights a limitation of EAE for studying regulation of autoimmunity. In EAE models, regulatory pathways are overwhelmed in the induction protocol, rendering investigation of Treg dynamics difficult. Roles for Tregs have been reserved for the recovery phase of EAE where they have been shown to be capable to suppress MOG-specific T cells and in their absence recovery is prevented (McGeachy 2005).

Tregs are present in abundance under normal circumstances and are very effective at suppressing activated T cells. This model demonstrates that autoimmunity can progress despite tight regulation and autoreactive T cells can slowly accumulate and function under such circumstances. The slow rate of progression through autoimmunity is a testament to the functional ability of Tregs rather than evidence of their inadequacy.

This is why when they are removed such large-scale devastation in the CNS is observed. Function is retained throughout the progression of autoimmunity in contrast to findings in EAE, but likely more representative of MS where the disease is not a large-scale impairment of regulation but a continuous battle to regain control over cells whose function is detrimental to the whole organism.

The chronic progression of symptoms in the Line7 allows study of competent Treg responses rather than observing autoimmunity resulting from Treg impairment. It supports the idea that development of MS is associated with a breakdown in regulation allowing the initial attack and explains why there is an observable incomplete concordance rate in monozygotic twins (Ebers 1986, Dyment 2004a). Environmental factors play an important role in the development of MS, but most likely in modulating the ability of Tregs to limit activation and infiltration of autoreactive cells. Many small factors could influence the functionality of Tregs throughout a patient's life. In this way, temporary breakdowns in regulation could lead to relapses, with remission associated with recovery of Treg functionality. Breakdowns in regulation would lead to boosts in pro-inflammatory cytokine production, and enhancement of IFNγ alone has been associated with relapses in MS patients (Frisullo 2008). In the Line7 model the lack of relapses could be explained by the consistently high level of IFNγ transcription, indicative perhaps of the presence of a constant relapse.

Breakdowns in regulation may be mediated through environmental insults that may be detected through TLR ligation. TLRs are present on many cells including Tregs and so offer a direct mechanism for environmental influence on the potential of immune

regulation. TLR ligation has been shown to both promote and suppress Treg function, for example in the presence of a TLR2 agonist, Treg numbers expand but their suppressive function is temporarily abrogated (Liu 2006a) but Tregs could be activated through the administration of lipopolysaccharide, a TLR4 ligand (Caramalho 2003). The focus of this study is not to ascertain a specific factor responsible for temporary Treg impairment but it is not beyond comprehension that such a factor or combination of factors may have influenced Treg cells in an MS patient and promoted the initial autoimmune attack.

Exposure to TLR ligands could in this way impair Tregs so that an opportunity would arise for autoreactive cells to infiltrate the CNS leading to the development of MS. Extrapolating this hypothesis, subsequent spikes in autoreactive T cell activity corresponding with temporary hindrance of Tregs could manifest themselves as relapses but further extensive research would be required to test this theory.

The percentage of mice not developing symptoms is reminiscent of non-concordant MS monozygotic twins. Studying these provides an invaluable comparison to affected animals that is lacking from EAE research. The resistance of these mice to allow CNS infiltration and development of autoimmunity despite being highly susceptible demonstrates that a breakdown in regulation is likely to be the important factor leading to the initiation of autoimmunity. Diversity of Treg function was demonstrated upon their depletion when production of pro-inflammatory cytokines was increased from CD8 cells. The increase in activation and infiltration of $CD8^+$ T cells in the absence of Tregs suggests a mechanism for their involvement and identification in MS lesions in association not contradiction to those mechanisms implicating $CD4^+$ T cells. Impairments in regulation would allow both cell populations to infiltrate lending support to relevant

regulation as the key step to prevention of autoimmunity. This idea could be further extended as NK cell activation, as measured by pro-inflammatory cytokine production was increased upon Treg depletion, although infiltration was unaffected. An additional role for Tregs at controlling inflammation in the local environment broadens the importance of these cells in managing autoimmune progression. More detailed analysis of the regulatory cells derived from these mice would demonstrate how these mice retain functional regulation over the wide range of immune cells, highlighting possible avenues for therapies. It would be an interesting experiment to see if autoimmunity could be prevented by boosting Treg cells in early age. It could be as simple as regular administration of recombinant IL-2 to boost Treg populations and allow the regulatory pathways to prevent autoreactivity rather than targeting one distinct pro-inflammatory cytokine in the myriad of redundant pathways.

13.4 Future use of Line7

The spontaneous nature of disease in Line7 mice makes it a perfect model in which to test therapeutics. The immune system of the mice is not influenced by any adjuvants and the disease is a slow progression, more in keeping with the human condition than acute EAE models. It has previously been shown to be pathologically relevant to MS (Ellmerich 2005) and dosing regimes could be tailored to mimic those expected when the therapy is trialed in patients. Therapeutic copolymers have been trialed in the sister Line8 mice previously with success (Illes 2004) and further similar studies are ongoing. Doses could be given over long periods of time and once disease is established rather than only before or during the first acute attack as in EAE. The mixed successes of EAE at

predicting therapeutic potential in MS treatment highlight the need for a better model in with to test long-term exposure to drugs (Steinman 2005b). The mechanistic differences between Line7 and EAE in initiation of disease also open the door to a new range of potential therapeutic targets for MS, and offer possible explanations for the contrasting efficacies of treatments seen between EAE and MS.

However, the limitation of the Line7 lies in its genetic specificity. Whilst DR15 shows the largest association of any gene with MS, evidence of epitope spread in MS and no consensus antigen for every clinical profile make the consideration of different myelin epitopes a high priority. The generation of similar models with other MS derived TCR clones specific for other myelin epitopes and their cognate HLA class II alleles should be explored. Comparison of the clinical profile and immunological dynamics of such models would inform whether mechanisms are TCR-dependent as this could provide one explanation for the high prevalence of Th17 cells seen in the MOG_{35-55} EAE models relative to the seemingly Th1 dominated MBP_{85-99} associated autoimmunity.

Findings in single models of disease should always be treated with caution. The tendency to overvalue findings in one model over another does not further our knowledge regarding the human conditions. Sometimes it appears as if this final aim of animal work is lost, with model selection based upon the likelihood of generating successful results and in whichever model is currently fashionable. The importance of cellular subsets can be overstated leading to wasted money and time in clinical trials. Improving the life of patients through development of effective treatments should be the ultimate aim of most disease related research and in this vein, the Line7 model and findings in it should be considered especially relevant.

14 References

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