Investigation of the role of antagonism of the Interleukin-7 receptor in the treatment of multiple sclerosis in humans and in vitro differences between genetically stratified subjects based on Interleukin-7 receptor genotype

Dr Onajite Kousin-Ezewu

Department of Clinical Neurosciences
University of Cambridge
Homerton College

July 2019

This dissertation is submitted for the degree of Doctor of Philosophy

DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant degree committee.

ABSTRACT

Dr Onajite Kousin-Ezewu

Investigation of the role of antagonism of the Interleukin-7 receptor in the treatment of multiple sclerosis in humans and in vitro differences between genetically stratified subjects based on Interleukin-7 receptor genotype

Multiple Sclerosis is an autoimmune disease mediated by activated lymphocytes entering the central nervous system. Treatments with the greatest efficacy either prevent the entry of activated lymphocytes, or deplete the lymphocyte population, before allowing lymphocyte reconstitution. IL-7Rα was identified by genetic studies in MS pathogenesis and is involved in the homeostasis and proliferation of lymphocytes.

This thesis investigates the role of antagonism of IL-7Rα in the treatment of MS in humans and *in vitro* differences between genetically stratified subjects based on IL-7Rα genotype. It also explores the role for biomarkers during reconstitution of lymphocytes after Alemtuzumab treatment in MS, in which IL-7Rα plays a major role.

Chapter 3 describes the prematurely aborted clinical trial of subjects with an IL-7Rα antagonist. This first-time-in-human trial demonstrated the drug was safe and well tolerated in this limited cohort of subjects.

Chapter 4 investigated the differences between individuals based on IL-7Rα genotype with *in vitro* IL-7Rα antagonism and stimulation. It demonstrated greater activation through IL-7Rα in individuals with the protective genotype. Differences in negative feedback mechanisms of IL-7Rα were explored.

Chapter 5 investigated the tolerability of palifermin, a keratinocyte growth factor, with alemtuzumab, which was well tolerated as part of a dose escalation sub-study of the CAMTHY trial. The main CAMTHY trial investigated if palifermin could cause increased thymic lymphopoiesis, offsetting the IL-7 driven homeostatic proliferation of lymphocytes and secondary autoimmunity associated with alemtuzumab.

Chapter 6 investigated the use of CD4+ lymphocytes as a biomarker for relapses after Alemtuzumab treatment. This contradicted the findings of a previously published paper, using a much larger cohort of patients in Cambridge.

This work underlines the importance of IL-7 in the pathogenesis and treatment of MS. It points towards the IL-7Rα pathway as a future avenue for biomarkers and novel treatments for MS.

PREFACE

The overall aim at the start of my PhD was to train in translational medicine in order to become a clinical trials specialist. Therefore the funding from the Wellcome Trust for the translational medicine and therapeutics PhD incorporated funding from the Wellcome Trust and GlaxoSmithKline (GSK).

The aim was to take a translational project at GSK. This was a first time in human trial of an IL-7Rα antagonist. During the first two parts of the trial I was due to work as a sub-investigator on the trial, learning about clinical trials in healthy volunteers. The final part of the trial was a novel approach to Phase 1 trials with the introduction of Multiple Sclerosis (MS) patients early in the clinical trial process. It was intended that I would be the chief investigator leading this part of the trial.

Unfortunately after dosing 16 healthy volunteers my PhD had to change course due to the trial being prematurely terminated by GSK due to data fraud in the pre-clinical scientific work. My experiences of this is described in more detail in chapter 3, where I also describe the observations of the healthy volunteers that were dosed prior to termination of the trial.

After termination of the IL-7Rα trial, GSK stopped all work on the IL-7Rα pathway within the organization. Therefore I had to change course during my research period. However once work could re-start on the IL-7Rα pathway after an investigation into what had led up to the data fraud in China, I moved my focus away from the clinical aspect of IL-7Rα and focused on signaling through IL-7Rα. This was due to the fact that there was continuing uncertainty about how blocking IL-7Rα would affect patients. This led to a genetic study, with the genetic stratification by IL-7Rα genotype drawn up by geneticists at GSK before I began my period of research. This investigated if IL-7Rα antagonism affected subjects differently according to IL-7Rα genotype. The data from this first project led to further questions about if the differences seen between the genetic groups in the first project was due to negative feedback mechanisms such as downregulation of IL-7Rα after stimulation of the receptor. Therefore the second project in chapter 4 investigated this further with an IL-7 stimulation in vitro of blood from subjects stratified according to IL-7Rα genotype.

During the period when work on the IL-7Rα pathway had been stopped by GSK, I wanted to continue my interest in translational medicine, the original purpose of my PhD. Therefore I became a sub-investigator on the CAMTHY trial, a study investigating lymphocyte reconstitution post alemtuzumab (in which IL-7 plays a major role), a drug known to deplete lymphocytes, which is a highly effective treatment for MS. The aim of the study was to use palifermin, a keratinocyte growth factor, in order to drive thymic reconstitution of lymphocytes and increase the diversity of the lymphocyte population, rather than peripheral reconstitution of lymphocytes that were not depleted after alemtuzumab. Prior to this trial commencing a safety sub-study was performed to investigate if palifermin was tolerable as this was a dose that had never before been used in humans. I was heavily involved in the practical administration of this study whilst also investigating and collecting the clinical data on the patients that were dosed. I have described my experiences of this study in chapter 5. I have also included in the appendix a paper, which has been published, of the main CAMTHY trial, which followed this sub-study (Coles et al., 2019).

During the period when I was unable to work on the IL-7Rα pathway at GSK I also did further work investigating the reconstitution of lymphocytes post alemtuzumab. I investigated if there was a relationship between the level of reconstitution of lymphocytes and the clinical outcome in MS patients such as disability, relapses and MRI imaging. This work followed a controversial paper, which claimed increased CD4+ counts post alemtuzumab was associated with increased MS disease activity. I have summarized this work in chapter 6, which led to a publication in the journal Neurology (Kousin-Ezewu et al., 2014).

In summary this thesis explores the role of the IL-7Rα pathway in MS, first through antagonism of IL-7Rα *in vitro*, but also in a first time in human trial. It also explores the effects *in vitro* of stimulation of the IL-7Rα pathway and how this differs between genetic groups stratified according to IL-7Rα genotype. Whilst working with alemtuzumab, a potent lymphocyte depleting monoclonal antibody, I was able to explore reconstitution of lymphocytes, a process in which IL-7 and its receptor play a very important role. I also explored if this reconstitution could be altered in humans by attempting to stimulate increased thymic reconstitution, ultimately attempting to reduce the autoimmune clinical side effects of alemtuzumab, which could improve the suitability of this highly effective drug for a wider range of MS patients.

ACKNOWLEDGEMENTS

I would like to thank my mother and brothers for continuing to encourage my study through the years and for helping to keep me grounded.

I would like to thank the Therapeutic Immunology Group who have guided me through the PhD process.

Finally I would like to give my love and thanks to Louisa and Dorothy. Louisa has tirelessly listened when things have not gone according to plan and has always suggested sensible ways forward. She uniquely understands the difficulty of doing a PhD in our family and I will forever be in her debt. I look forward to enjoying family life with her, Dorothy and Urias.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

Kousin-Ezewu, O. & Coles, A. 2013. Alemtuzumab in multiple sclerosis: latest evidence and clinical prospects. *Ther Adv Chronic Dis* 4(3):97-103.
This review was published in the journal Therapeutic Advances in Chronic Disease.

Kousin-Ezewu, O., Azzopardi, L., Parker, R. A., Tuohy, O., Compston, A., Coles, A. & Jones, J. 2014. Accelerated lymphocyte recovery after alemtuzumab does not predict multiple sclerosis activity**.** *Neurology,* 82**,**2158-64.
This paper was published in the Neurology Journal.

Joanne Ellis, Andre van Maurik, Lea Fortunato, Sophie Gisbert, Keguan Chen, Ann Schwartz, Simon McHugh, Andrew Want, Sara Santos Franco, Joao-Joaquim Oliveira, Jeffrey Price, Kim Brown, Donna Su, Jenny Craigen, Jiansong Yang, Sara Brett, Bill Davis, Joseph Cheriyan, **Onajite Kousin-Ezewu**, Frank Gray, Paul Thompson, Alasdair Coles and Disala Fernando**.** 2019. Anti-IL-7 receptor α monoclonal antibody (GSK2618960) in healthy subjects – a randomised, double-blind placebo-controlled study. *Br J Clin Pharmacol* 85(2):304-315.
This paper was published in the British Journal of Clinical Pharmacology.

Tuohy, O., Costelloe, L., Hill-Cawthorne, G., Bjornson, I., Harding, K., Robertson, N., May, K., Button, T., Azzopardi, L., **Kousin-Ezewu, O**., Jones, J., Compston, D. A. & Coles, A. 2015. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy**.** *JNNP* 86(2):208-215.
This paper was published in the Journal of Neurology, Neurosurgery and Psychiatry.

C. J. S. Nye, A. Wagner, **O. Kousin-Ezewu**, J. L. Jones, A. J. Coles. 2019.
A Case of Anaphylaxis to Alemtuzumab. J Neurol 266(3):780-781.
This case report was published in the Journal of Neurology.

Alasdair Coles, Laura Azzopardi, **Onajite Kousin-Ezewu**, Harpreet Kaur Mullay, Sara Thompson, Lorna Jarvis, Jessica Davies, Sarah Howlett, Daniel Rainbow, Judith Babar, Timothy Sadler, William Brown, Edward Needham, Zoya Georgieva, Adam Handel, Stefano Maio, Mary Deadman, Ioanna Rota, Georg Hollander, Sarah Dawson, David Jayne, Ruth Seggewiss, Daniel Douek, John Isaacs and Joanne

Jones. 2019. Keratinocyte growth factor impairs human thymic recovery from lymphopenia. JCI Insight 7;5.
This paper was published in the Journal of Clinical Investigation Insight.

Abrahamsson, S. V., Angelini, D. F., Dubinsky, A. N., Morel, E., OH, U., Jones, J. L., Carassiti, D., Reynolds, R., Salvetti, M., Calabresi, P. A., Coles, A. J., Battistini, L., Martin, R., Burt, R. K. & Muraro, P. A. 2013. Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis. *Brain* 136(Pt 9) 2888-2903.
I contributed to this paper (acknowledged within the paper) that was published in the journal Brain.

C.L. McCarthy, J.W.L. Brown, O. Tuohy, L. Azzopardi, **O. Kousin-Ezewu**, J.L. Jones, A. Compston, A.J. Coles. 2015. Long-term safety of alemtuzumab in relapsing-remitting multiple sclerosis: pregnancy and infection data from a cohort of patients on open label studies in Cambridge, UK.
This was a poster presentation at ECTRIMS 2015.

Onajite Kousin-Ezewu, Laura Azzopardi, Orla Tuohy, Richard Parker, Alasdair Coles, Joanne Jones. 2013. Differential Lymphocyte Reconstitution after treatment of Relapsing-Remitting Multiple Sclerosis with Alemtuzumab is not linked to disease stability.
This was a poster presentation at ECTRIMS 2013.

J.W.L. Brown, O. Tuohy, O**. Kousin-Ezewu**, L. Azzopardi, T. Button, C. Mccarthy, T. Alli, C. Callan, M. Folks, S. Heller, H. Laidley, B. Nourallah, C. O'Neill, D. Obute, R. Piper, O. Prankerd Smith, H. Wickham, X. Zheng, K. May, A. Gerritz, D.A.S. Compston, J.L. Jones, A.J. Coles. 2015. Alemtuzumab in multiple sclerosis: long-term follow-up of the early cohorts.
This was a poster presentation at ECTRIMS 2015.

Onajite Kousin-Ezewu, Joao Oliveira, Bill Davis, Joanne Jones, Alasdair Coles, Simon McHugh. 2015. IL-7Rα haplotype dependent differences in signaling through the IL-7Rα pathway with *ex vivo* dosing with an IL-7Rα antagonist.
This was an oral presentation at the GlaxoSmithKline IL-7 Research Day.

Onajite Kousin-Ezewu, Laura Azzopardi, Orla Tuohy, Richard Parker, Alasdair Coles, Joanne Jones. 2014. Differential Lymphocyte Reconstitution after treatment of Relapsing-Remitting Multiple Sclerosis with Alemtuzumab is not linked to disease stability.

This was a poster presentation at the Translational Medicine and Therapeutics (TMAT) Research Day.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADCC - antibody-dependent cell-mediated cytotoxicity
ANOVA – Analysis of Variance
APC – Allophycocyanin
APP – amyloid precursor protein
BCL-2 – B cell lymphoma 2
BSA – Bovine Serum Albumin
CD – cluster of differentiation
EAE – experimental autoimmune encephalomyelitis
EBV – Epstein-Barr virus
EC50 – half-maximal effective concentration
EDSS – expanded disability status scale
ELISA – Enzyme-linked Immunosorbent Assay
ETS – E26 Transformation-specific
FACS – fluorescence-activated cell sorting
FCS – foetal calf serum
FOXp3 – Forkhead Box P3
FS – forward scatter
GABPα – GA-binding protein alpha chain
Gfi-1 – Growth factor independence-1
GSK – GlaxoSmithKline
GWAS – Genome-wide Association Study
HLA – Human Leukocyte Antigen
IFN-γ – Interferon gamma
IL-2R – Interleukin-2 receptor
IL-7 – Interleukin-7
IL-7Rα – Interleukin-7 receptor alpha. Also known as CD127.
IPEX - immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IV – intravenous
JAK – Janus kinase
KO – knockout
LD – linkage disequilibrium
LLN – lower limit of normal
Log10 – the logarithm to base 10
MFI – median/mean fluorescence intensity

MHC – Major Histocompatibility Complex

MRI – magnetic resonance imaging

mRNA – messenger ribonucleic acid

MS – Multiple Sclerosis

NF-κB - nuclear-kappa-light-chain-enhancer of activated B cells

NOAEL – no-observed-adverse-effect-level

PBS – Phosphate Buffered Saline

PCR – polymerase chain reaction

PD - pharmacodynamics

PE – Phycoerythrin

PK – pharmacokinetics

PPMS – primary progressive multiple sclerosis

pSTAT5 – phosphorylation of STAT5

R-CHOP – chemotherapy treatment consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone

Rh – recombinant human

RO – Receptor Occupancy

RPMI – Roswell Park Memorial Institute

RRMS – relapsing remitting multiple sclerosis

SCID – Severe Combined Immunodeficiency

SNP – Single Nucleotide Polymorphism

SPMS – secondary progressive multiple sclerosis

SS – side scatter

STAT – signal transducer of activation

T2 – T2 weighted MRI imaging due to timing of the radiofrequency pulse sequences

TCR – T cell receptor

TNF – Tumour Necrosis Factor

TNFR1 – Tumour Necrosis Factor Receptor 1

TSLPR – Thymic Stromal Lymphopoietin receptor

LIST OF FIGURES AND TABLES

FIGURES

TABLES

CHAPTER 1 - INTRODUCTION

1.1 THE NATURAL HISTORY OF MULTIPLE SCLEROSIS

The clinical presentation and course of multiple sclerosis can be variable and unpredictable in nature although four broad clinical categories are generally accepted: relapsing-remitting, secondary progressive, primary progressive and progressive relapsing multiple sclerosis (Lublin and Reingold, 1996).

1.1.1 RELAPSING REMITTING MULTIPLE SCLEROSIS

85% of patients with multiple sclerosis present with relapses. Relapses are defined as acute or subacute neurological dysfunction attributable to demyelinating disease, in the absence of a fever, which persists for at least 24 hours (Poser et al., 1983). It normally evolves over days to weeks; plateaus and then the symptoms slowly improve. Recovery varies from minimal to complete. The average relapse rate is higher early in the disease (one per year) and diminishes over time (Alastair Compston, 2008) (Clarke, 2016).

1.1.2 SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS

This is defined as when patients move from relapsing-remitting disease to accumulating disability progressively, outside of relapses. The proportion of people who develop secondary progressive multiple sclerosis increases with follow-up. One Canadian study found 41% of people with relapsing remitting multiple sclerosis entered the progressive phase 6-10 years after disease onset. After 11-15 years it was 58%. After 20 years it was 80% of people who entered the secondary progressive phase of the disease (Weinshenker et al., 1989). It is still possible that some relapses may occur in this phase of the illness but it is less frequent than in the relapsing remitting phase of the disease (Lublin and Reingold, 1996).

1.1.3 PRIMARY PROGRESSIVE MULTIPLE SCLEROSIS

This is defined when there is progression of disability from the onset of the disease, in the absence of relapses and accounts for 11-18% of the cases seen with multiple

sclerosis (Runmarker and Andersen, 1993) (Weinshenker et al., 1989) (Thompson et al., 1997). The onset is slightly older at approximately 40 years of age (compared to approximately 30 years of age in relapsing remitting disease) (Thompson et al., 1997). There is also an equal frequency between males and females (in relapsing remitting disease there is a 3:1 difference in favour of females) (Thompson et al., 1997) (Orton et al., 2006).

1.1.4 PROGRESSIVE RELAPSING MULTIPLE SCLEROSIS

Although clinically this term is not used frequently, it refers to patients that have progressive disease with superimposed relapses (Lublin and Reingold, 1996). The relapses are normally mild and the insidious progression is normally the most dominant feature, in a similar way to that seen in primary progressive multiple sclerosis (Clarke, 2016).

These descriptive terms were based on consensus data amongst neurologists in the 1990s (Lublin and Reingold, 1996). There has more recently been a move to keep the descriptions of relapsing and progressive, but also to acknowledge that assessment of disease activity is now not just based on clinical assessment but also with the use of imaging of the central nervous system (Lublin et al., 2014).

1.1.5 PROGNOSIS

The clinically isolated syndrome is the first clinical event suggestive of acute demyelination. Those who also have an abnormal MRI scan have also shown consistently in studies to have a higher risk of converting to clinically definite multiple sclerosis. In long-term follow-up studies ranging from 7-20 years those with an abnormal MRI scan, with at least three typical demyelinating lesions, develop multiple sclerosis in 56-88% of people and those with a normal scan develop multiple sclerosis in 8-22% of those affected with an isolated demyelinating event (Brownlee and Miller, 2014) (Fisniku et al., 2008).

Natural history studies have shown that the median time to develop the need for assistance to walking is between 15 and 30 years. Although the accumulation of disability is slower in the relapsing remitting disease group of patients, once the progressive phase starts the initial course of the disease does not seem to affect the future prognosis (Weinshenker et al., 1989) (Confavreux et al., 2000).

It is difficult at this stage to comment on the role of relapses on the course of long-term disability. Ideally there would be 30 year follow-up data with a drug that has potent efficacy in reducing relapses and relapse associated short-term disability such as natalizumab and alemtuzumab, in order to confidently assert a view on how reducing the number of relapses may affect long-term disability.

However, work by George Ebers from a Canadian cohort of patients studying the natural history of multiple sclerosis, has shown that early clinical features can predict disability outcomes. An increased relapse rate in the first two years of the disease and a shortened first inter-attack interval decreases the time to onset of progressive disease. It can also increase the latency of the progressive phase of the disease (Scalfari et al., 2010).

The late Christian Confavreux showed that relapses occurring once the progressive phase had commenced do not alter the long-term prognosis for disability. However his viewpoint that MS relapses have only a marginal effect on the overall accumulation of disability in the long-term, contrasted with the work of George Ebers mentioned above. He felt the predictors of early milestones of irreversible disability in multiple sclerosis lose their predictive value once a certain level of disability has been

reached, a process he referred to as the ‘amnesia’ of multiple sclerosis. He felt that the attainment of irreversible disability outcomes was largely determined by the patient’s age, regardless of age of onset (Confavreux and Vukusic, 2006).

Factors which confer a better prognosis include, a complete recovery from the first attack of demyelination; a long period between the first and the second relapse; a low relapse frequency in early disease; no disability after 5 years and a normal MRI scan (Miller et al., 2005). Others point towards the existence of a monosymptomatic relapse and also sensory relapses (e.g. optic neuritis and paraesthesiae) (Confavreux et al., 2003). It is important to point out that some of these historical associations are weak and although important at a population level it is more difficult to attribute this reliably to predict the prognosis for a particular individual (Clarke, 2016).

Mortality in multiple sclerosis is increased. In one Canadian study, lifespan was reduced by 7 years compared to a control population (Weinshenker et al., 1989). Causes of death in at least half of patients were due to complications directly attributable to multiple sclerosis. There have also been higher rates of suicide in the multiple sclerosis population (7.5 times in the Canadian study). In a Danish study, lifespan was 10 years less than the control population with suicide rates more than twice that of the general population (Brønnum-Hansen et al., 2004).

1.2 THE PATHOLOGY OF MULTIPLE SCLEROSIS

The pathological hallmark of multiple sclerosis is multiple areas of myelin loss called 'plaques' in the central nervous system. Associated with these areas of demyelination is gliosis and inflammation with relative sparing of the axons (Popescu and Lucchinetti, 2012b). Although these lesions are spread throughout the central nervous system there is a predilection for certain areas such as the optic nerves, spinal cord, brainstem, the juxtacortical and periventricular white matter. There has also been recent interest in the presence of cortical plaques that has demonstrated demyelination within cortical gray matter (Calabrese et al., 2010) (Pirko et al., 2007).

The pathology in multiple sclerosis changes dependent on the stage of the disease process (Popescu et al., 2013). Within each stage of the disease the plaques undergo pathological changes, which are described below.

1.2.1 ACUTE ACTIVE PLAQUES

Acute active MS lesions are infiltrated with macrophages that contain myelin degradation products, the analysis of which can denote the stage of acute demyelination (Brück et al., 1995). Degradation of minor myelin proteins such as 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG) occur rapidly, indicating early active demyelination. The larger major myelin proteins such as proteolipid protein and myelin basic protein (MBP) are digested more slowly by macrophages and can persist within them for up to 10 days. The presence of these major myelin proteins in the absence of minor proteins denotes a late active demyelinating lesion. Inactive demyelinating lesions still contain macrophages but they lack myelin debris and may contain empty vacuoles and periodic acid Schiff positive degradation products (Popescu et al., 2013).

Demyelinating lesions also contain inflammatory infiltrates suggesting that the demyelination is inflammatory in nature (Popescu and Lucchinetti, 2012b). However, it is still not clear from pathology that inflammation leads to secondary axonal degeneration.

Alongside the activated macrophages there are lymphocytes, the majority of which are CD8+ T cells, with fewer CD4+ T cells, B cells and plasma cells. There is a damaged blood brain barrier as evidenced by gadolinium enhancement in MRI scans of active multiple sclerosis lesions. B cells and plasma cells tend to accumulate in the perivascular spaces (Frischer et al., 2009). There is also proliferation of astrocytes within active lesions, which help to form a matrix from which other cells are suspended.

In addition to demyelination the progressive overall loss of oligodendrocytes, which precludes effective remyelination of remaining axons is another hallmark of multiple sclerosis pathology. Oligodendrocyte progenitors have been demonstrated in the multiple sclerosis brain and are responsible for the partial remyelination seen even within early lesions (Halfpenny et al., 2002). There is only partial remyelination, although this is more extensive and widespread than originally thought (Patani et al., 2007), as these oligodendrocyte precursors do not get the signal to differentiate. One of the mechanisms preventing the differentiation of the oligodendrocyte precursors is myelin debris (Fancy et al., 2010).

There is conflicting evidence on the role of oligodendrocytes in active multiple sclerosis lesions. Some authors suggest that they are preferentially destroyed in early lesions (Prineas JW, 1997), whilst another study suggests the injury to oligodendrocytes is more variable with several oligodendrocytes present in some active lesions, possibly denoting concurrent early remyelination (Brück et al., 1995).

1.2.2 CLASSIFICATION OF EARLY WHITE MATTER LESIONS

Despite the pathologic heterogeneity found in early demyelinating white matter lesions, Luchinetti and colleagues have demonstrated that these active demyelinating lesions can be classified into four categories depending on specific myelin protein loss, plaque extent and topography, oligodendrocyte destruction, immunoglobulin deposition, complement activation and the presence or absence of remyelination (Lucchinetti et al., 2000).

Pattern 1 lesions are found in 15% of patients that have sharply demarcated lesions with equal involvement of all myelin components. There is a variable loss of oligodendrocytes at the active lesional border with numerous oligodendrocytes

reappearing at the more inactive plaque center. There is a high incidence of plaque remyelination. In pattern 1 lesions there is an activated macrophage and T lymphocyte background but a lack of immunoglobulin deposition and complement activation. Therefore the pattern of damage seen is mediated by toxic factors produced by activated macrophages.

Pattern 2 lesions are found in 58% of multiple sclerosis lesions that are biopsied. There is equal loss of all myelin protein components and the lesions are sharply demarcated. There is a high incidence of remyelinated shadow plaques, with a variable loss of oligodendrocytes at the active border of the lesion but a reappearance of the oligodendrocytes at the inactive plaque center. In pattern 2 lesions there is immunoglobulin and complement deposition on myelin as well as phagocytosis by opsonized macrophages of myelin that has been previously targeted by complement. This is on an inflammatory background of T cell inflammation. These findings suggest that the demyelination found in pattern 2 lesions may be induced by antibody mediated and complement mediated mechanisms. It has been known for sometime that elevated immunoglobulins are present in the cerebrospinal fluid of multiple sclerosis patients in the form of oligoclonal bands. However the specific target of these antibodies has not yet been identified.

Pattern 3 lesions are found in 26% of biopsied MS patients and show a preferential loss of periaxonal myelin components (minor myelin proteins such as MAG and CNPase). The lesions are more ill defined but do show active demyelination. There is oligodendrocyte apoptosis on the plaque border, which extends into the normal appearing white matter. There is an absence of remyelination with the center of the plaque devoid of oligodendrocytes. There is no evidence of immunoglobulin or complement activation. These changes occur on an inflammatory background of mainly a CD8+ T cell infiltrate. The type of pathological appearances seen in pattern 3 lesions are reflected in inflammatory demyelination induced by viruses and damage of oligodendrocytes induced by toxicity such as cuprizone.

Pattern 4 lesions are found in 1% of multiple sclerosis biopsies. They demonstrate non-apoptotic cell death of oligodendrocytes in the peri-plaque white matter, without inflammation. Some authors suggest this demonstrates a potential primary metabolic oligodendrocyte disturbance that makes oligodendrocytes particularly vulnerable to the toxic effects of inflammatory mediators (Prineas JW, 1997).

One of the pathological hallmarks of multiple sclerosis is demyelination with relative axonal sparing. However axonal injury does occur, evidenced by axonal swellings with a beaded appearance. There is also accumulation of amyloid beta precursor protein, which acts as a marker for focal accumulations of proteins that are normally moved along axons by axon transport. Mild axonal loss is also seen (Bjartmar et al., 2003). The axonal injury is most pronounced during active inflammatory demyelination and contributes to the relapse associated disability seen in multiple sclerosis (Filippi et al., 2012) (Popescu et al., 2013). The extent of axonal damage correlated with the number of lymphocytes and activated microglia. The axonal damage is caused by the release of toxic mediators by the inflammatory cells in close apposition with the axons, which leads to increased mitochondrial damage, oxidative stress and energy deficiency (Dutta and Trapp, 2011) (Fischer et al., 2012).

1.2.3 CHRONIC PLAQUES

Chronic active plaques are seen in those patients with progressive multiple sclerosis. The lesions are sharply demarcated with myelin rich macrophages expanding around the edge of the plaque with an ever-decreasing number of myelin-laden macrophages as you get towards the plaque's more inactive center. Some 'smouldering' chronic active plaques contribute to progression and are characterized by an increase in the number of activated microglia, which contain little in the way of myelin degradation products, surrounding the inactive center (Prineas et al., 2001).

Chronic inactive plaques are sharply demarcated and are completely demyelinated. There is substantial loss of axons and oligodendrocytes. There is astrogliosis, with some infiltration by microglia and lymphocytes. As the plaques progress from a chronically active to inactive state, astrocytes produce glial fibers and eventually a glial scar fills the demyelinated plaque (Popescu and Lucchinetti, 2012b) (Fawcett and Asher, 1999).

Perivascular inflammatory infiltrates are seen in chronic lesions but the blood brain barrier remains intact (Frischer et al., 2009). Hans Lassman has described the idea of inflammation 'trapped' behind the blood brain barrier in chronic multiple sclerosis. One mechanism for this could be the formation of lymphoid follicular structures in perivascular spaces, which have been found post mortem in patients with secondary progressive multiple sclerosis (Magliozzi et al., 2007). Plasma cells are hypothesized

to have formed even in the chronic disease stage and persist when the initial inflammation has cleared. Thus the inflammation seen in chronic multiple sclerosis is trapped behind the blood brain barrier (Frischer et al., 2009).

1.2.4 NEURODEGENERATION

Axonal damage and axon loss is one of the features of chronic multiple sclerosis. In chronic inactive plaques axonal density is reduced up to 80% within the plaque (Kutzelnigg et al., 2005).

Neurodegeneration within demyelinated lesions is associated with inflammation. However in older patients with chronic inactive lesions the levels of inflammation are similar to those seen in controls, so the inflammatory process dies out over time (Frischer et al., 2009).

There are a number of mechanisms purported to account for the chronic axonal damage and neurodegeneration seen in multiple sclerosis:

1. **Repeated demyelination and oxidative stress caused by inflammation.**

   Early axonal transection is thought to occur due to the vulnerability of demyelinated axons to inflammation as evidenced by SMI32 staining denoting axonal spheroids (Dutta and Trapp, 2011). Higher levels of axonal spheroids are seen in acute active lesions than chronic lesions. There is also evidence of inflammation in acute lesions by the accumulation of amyloid precursor protein (APP) on the edge of acute active lesions, but not in their center. Chronic lesions showed minimal APP staining (Ferguson et al., 1997). Activated immune and glial cells release many substances including proteolytic enzymes, matrix metalloproteinases, cytokines, oxidative products and free radicals that can damage axons (Hohlfeld, 1997) (Nave and Trapp, 2008).

   It is more controversial to say if there is direct and specific immune attack of axons. This can be suggested by the correlation suggested above between inflammation and axonal transection. There is direct immune attack of axons in the peripheral nervous system in acute motor axonal neuropathy (AMAN),

a variant of Guillan Barre syndrome (Ho et al., 1998). Also pathologically in multiple sclerosis the terminal axonal ovoids are surrounded by macrophages and activated microglia. There is uncertainty about the role of the inflammatory cells surrounding the ovoids. Do they play a role in directly attacking the axon, or do they help to protect the axon and remove debris? Although there is not a large amount of evidence supporting direct and specific immune attack of axons it is important to acknowledge that cell-mediated mechanisms of axon loss is still a possibility. However the overriding point that should be made is most axons survive the demyelination process, therefore it is still unlikely that there is a specific immunological attack of axons (Dutta and Trapp, 2011).

The attempt to correlate inflammation with axonal transection and axon loss may be too simplistic. Other studies have suggested that the correlation between plaque load and axon loss is poor (Kutzelnigg et al., 2005) (DeLuca et al., 2006).

DeLuca et al demonstrate the poor correlation between plaque load and axonal loss in a population with multiple sclerosis with disease duration of 17 years (DeLuca et al., 2006). Therefore this demonstrated poor correlation in patients who have already acquired disability and would fit with the epidemiological data by Confavreux (Confavreux and Vukusic, 2006). The amount of APP and inflammation correlate in early stages of multiple sclerosis but this correlation falls away as the disease progresses. Therefore early axonal damage is more likely to be linked to inflammation but it is more difficult to draw this conclusion with later axonal loss (Wilkins and Scolding, 2008).

2. **Axonal degeneration due to lack of trophic support from myelin and oligodendrocytes.**

Studies involving mice that lack myelin proteins such as MAG, CNPase and PLP have shown that these proteins can be removed from oligodendrocytes without too much effect on myelination (Nave, 2010) (Nave and Trapp, 2008) (Nave, 1996). All three lines of mice develop a late onset slowly progressive axonal degeneration, which in itself shows that alterations in single myelin

proteins can cause axonal degeneration (Nave, 2010) (Nave and Trapp, 2008).

It has been shown that cortical oligodendrocyte precursor cells increase cortical neuronal survival via direct cell contact with neurons and also through the secretion of soluble growth factors such as IGF-1 (Wilkins et al., 2001). Other studies also show that in addition to insulaton of axons, oligodendrocytes provide trophic support to axons (Byravan et al., 1994) (Dai et al., 2001) (Dougherty et al., 2000).

In MAG-null mice there is a reduction in axonal caliber quite prominent in the paranodal regions, in part due to reduced phosphorylation of neurofilaments (Yin et al., 1998). In CNP and PLP-null mice there is axonal swelling at the distal paranodes (Griffiths et al., 1998) (Klugmann et al., 1997). This suggests there is a defect in retrograde axonal transport at the Nodes of Ranvier.

3. **Accumulation of mitochondria in a setting of increased energy demands and mitochondrial oxidative stress.**

The central hypothesis of degeneration of chronically demyelinated axons is an imbalance between energy demand and energy supply. The Na+/K+ ATPases which are necessary for the maintenance of the ionic gradients necessary for neurotransmission are the largest consumers of ATP in the central nervous system (Ames, 2000). Therefore normal myelination should not just be seen as a way of promoting rapid nerve conduction but it should also be seen as a way of conserving energy.

Demyelination renders axons far more vulnerable to physiological stress and degeneration, by increasing the energy requirements for nerve conduction. Due to the redistribution of sodium channels along demyelinated axons, the neuron is loaded with greater intracellular sodium and their extrusion through the Na+/K+ exchanger leads to increased ATP demand (Peterson et al., 2005). This consistent feature of demyelinated axons may allow the continuation of action potentials and in the context of multiple sclerosis, allows some recovery of clinical function.

In demyelinated axons there is an increase in activity of complex IV, the terminal subunit in the electron transport chain, which consumes 90% of cellular oxygen. In all models where there has been demyelination with a resultant increase in complex IV activity, an increase in mitochondrial content has been observed.

There has been some debate as to the effects over time of increased mitochondria in the axon. In the short term increased mitochondria lead to increased survival of the axon. There is some more recent evidence that in the long term there is a detrimental outcome of increased mitochondria in the axon. When the axon specific mitochondrial docking protein syntaphilin (which is normally increased in demyelinated axons) is knocked out in the Shiverer dysmelinated mouse model (when the gene for myelin basic protein is also knocked out), an improved clinical outcome was noted with less axon degeneration. This lead to the conclusion that the degradation of unhealthy mitochondria, which over a prolonged period can produce harmful reactive oxygen species, is important in the survival of demyelinating axons (Campbell and Mahad, 2018).

Neurodegeneration is a fundamental aspect of multiple sclerosis pathogenesis as a loss of axons, dendrites and neurons is a major cause of permanent disability in multiple sclerosis patients. Axon loss does occur early in the course of the disease, which then progresses slowly. The transition from relapsing remitting multiple sclerosis to secondary progressive multiple sclerosis is thought to occur when the compensatory mechanisms of the central nervous system (repair, plasticity and remyelination) is reached, leading to the steady progression of permanent neurological symptoms (Dutta and Trapp, 2011).

1.2.5 REMYELINATION

Remyelinated plaques are characterized by thinly myelinated axons with short inter-nodal distances. When remyelination is more extensive it is characterized by new myelin sheaths and the presence of oligodendrocyte precursor cells. Oligodendrocyte precursor cells are frequently found in the active plaques of multiple sclerosis (Wilson et al., 2006) (Popescu and Lucchinetti, 2012b).

Remyelination is seen in Pattern 1 and 2 lesions. Oligodendrocytes are frequently lost at the expanding peripheral edge of the plaque, with oligodendrocyte precursor cells found in the center of the plaque, which is less active than the periphery. In Pattern 3 and 4 lesions there is loss of oligodendrocytes without oligodendrocyte precursor cell recruitment and remyelination. This points towards the fact that oligodendrocyte precursor cells are key to remyelination (Lucchinetti et al., 2000).

Remyelinated plaques are seen macroscopically as ‘shadow’ plaques, with reduced myelin density and thin myelin sheaths. Shadow plaques are extensive in progressive multiple sclerosis. Evidence for remyelination can be found in almost half of chronic multiple sclerosis lesions in people with relapsing-remitting multiple sclerosis (Barkhof et al., 2003).

Older remyelinated plaques show a near normal thickness of myelin and are sometimes difficult to pathologically distinguish from normal appearing white matter. Interestingly remyelinated plaques are more likely to be struck again with a second inflammatory episode than normal appearing white matter (Bramow et al., 2010).

It is important to note that remyelination tends to progressively fail in multiple sclerosis. This failure of remyelination may be due to age dependent loss of trophic support from microglia. It may also be due to oligodendrocyte precursor cell exhaustion by repeated demyelinating insults. Also the dense glial scar created by astrocytes may act as a barrier to oligodendrocyte precursor cells migrating into lesions (Popescu et al., 2013).

1.2.6 CORTICAL LESIONS IN EARLY MULTIPLE SCLEROSIS

In the cortex, multiple sclerosis leads not only to axon loss and cortical atrophy but also to cortical demyelinating lesions.

Three different types of cortical lesions have been described. The first is the subpial lesion, which can extend throughout the entire width of the cortex and may involve many gyri. The second are intracortical lesions, which are perivascular in nature and are small demyelinating lesions. These lesions spare both the superficial cortex and the adjacent white matter. The third type of lesion are leukocortical lesions, which involve the gray-white matter junction with sparing of superficial cortical layers (Popescu et al., 2013) (Peterson et al., 2001).

It has been noted that patients with early cortical involvement may have a worse prognosis. Early cortical lesions may be linked to early cognitive impairment and epilepsy. The accumulation of cortical lesions has been linked with disease progression and disability. The cortical lesion load also positively correlates with white matter MRI T2 lesion load and brain atrophy. Minimal cortical lesion load has also been linked with a more benign multiple sclerosis course (Calabrese et al., 2010) (Geurts and Barkhof, 2008) (Popescu and Lucchinetti, 2012a).

The majority of cortical lesion types will show demyelination as evidenced by myelin-laden macrophages. Perivascular and parenchymal inflammatory infiltrates of macrophages, T cells, B cells and plasma cells, with breakdown of the blood brain barrier, are also present (Lucchinetti et al., 2011).

Focal perivascular and diffuse meningeal inflammation are strongly associated with the cortical lesions of early multiple sclerosis. The production and release of inflammatory cytokines in the subarachnoid space in early multiple sclerosis may drive cortical demyelination and promote inflammation and demyelination of the underlying subcortical white matter as a consequence (Lucchinetti et al., 2011). Some EAE models have demonstrated the importance of trafficking of T cells via CCL20 into the central nervous system with subsequent antigen presented to T cells and then release of cytokines leading to a second wave of T cell infiltration across pia vessels, with an upregulation of vascular cell adhesion molecules in the deeper

brain vasculature with subsequent parenchymal invasion and onset of disease (Reboldi et al., 2009) (Bartholomäus et al., 2009).

1.2.7 CORTICAL LESIONS IN CHRONIC MULTIPLE SCLEROSIS

Cortical demyelinated lesions in progressive multiple sclerosis may represent the substrate for irreversible disability, progression and cognitive decline. In progressive multiple sclerosis extensive demyelination can be seen throughout the cortex (Kutzelnigg and Lassmann, 2006).

In progressive multiple sclerosis cortical lesions lack the breakdown of the blood brain barrier and so are less easily visualized with gadolinium enhanced scans, although may be seen with triple dose gadolinium (Filippi et al., 1998). They also lack extensive inflammatory cell infiltration and complement deposition. They do have activated microglia, which is associated with atrophy and apoptosis of neurons with damage to oligodendrocytes (Peterson et al., 2001) (Wegner et al., 2006).

There are also meningeal inflammatory infiltrates. These are composed of T cells, B cells and macrophages and are found in patients with both primary and secondary progressive multiple sclerosis. The extent of the meningeal inflammation correlates with microglial activation and the degree of demyelination and neurodegeneration in the underlying cortex (Magliozzi et al., 2007) (Choi et al., 2012) (Howell et al., 2011). Meningeal inflammation with ectopic B cell follicles have been described in secondary progressive multiple sclerosis. These are located in the deep sulci of the temporal, cingulate, insular, and frontal cortices and are associated with subpial lesions (Howell et al., 2011). This meningeal inflammation drives cortical injury with soluble cytokines produced by activated lymphocytes diffusing into local cortical tissue, causing demyelination and neurodegeneration either directly or indirectly through activation of microglia (Popescu et al., 2013).

1.3 THE GENETICS OF MULTIPLE SCLEROSIS

Epidemiological studies have revealed that you have an increased risk of developing multiple sclerosis if you are related to someone with the disease. The background population risk for MS is 0.3% in northern white European populations. With identical monozygotic twins the risk is 25%. For dizygotic twins the rate is 5% (Compston et al., 2008).

The risk for first-degree relatives remains at approximately 3% with siblings remaining at 5% and parents and children at 2%. For second and third degree relatives the risk is approximately 1% (Compston et al., 2008) (Oksenberg et al., 2008).

With all of these studies taken together, it points towards a significant but complex genetic burden to the underlying risk for multiple sclerosis. This is not to undermine the potential role of environmental factors in the aetiology and pathogenesis of multiple sclerosis, which is discussed elsewhere in the introduction.

1.3.1 GENE LINKAGE STUDIES AND CANDIDATE GENE STUDIES

Up until ten years ago the dominant techniques of studying genetics within multiple sclerosis was by two means. The first was the analysis of multiple case families to determine linkage to broad chromosomal regions. The second was to collect a modest amount of cases and controls and investigate candidate genes (Oksenberg et al., 2008).

Linkage studies would involve analysis of the data from families that are affected by the disease. More than one family member would need to be affected and discrete chromosomal segments that co-segregated with those who suffered from multiple sclerosis could potentially be identified. Once several different families have been analysed, regions of the genome that co-segregate with the disease with each generation in families are identified.

The success of monogenic genetic disorders with linkage analysis and also the initial success in some more complicated polygenic disorders (e.g. ApoE gene and

Alzheimer's disease) (Corder et al., 1993) (Ogura et al., 2001) drove the application of this technique to multiple family data sets in multiple sclerosis. Many different loci were identified as potential candidates for driving susceptibility in multiple sclerosis (Fernald et al., 2005), which was consistent with the consensus view of multiple sclerosis being a polygenic disorder. However only one locus at 6p21, coding for HLA Class II reached the threshold for statistical significance

The Human Leukocyte Antigen (HLA) is a cluster of genes on 6p21 that encode for proteins that function in the immune system. More specifically within this cluster of genes is HLA Class II, which is comprised of proteins that participate in the recognition and presentation of antigen to T lymphocytes. HLA Class II molecules have been associated with several autoimmune disorders and it is due to the fact that they are able to recognize and present self-antigen to T lymphocytes. There are three HLA Class II molecules DP, DQ and DR. They are a combination of two proteins, an alpha and beta chain. These combine inside the antigen-presenting cell, bind to a peptide and travel to the cell surface for presentation of the peptide to T cells.

The first discovery of the HLA locus in MS was the HLA-DRB1 gene (DRB1*1501) (Compston et al., 1976) (Terasaki and Mickey, 1976). Although the discovery of the HLA association with MS was made in the 1970s this HLA-DRB1 gene has only been consistently found in Northern European Caucasian populations. One review looked at 72 studies (Schmidt et al., 2007), which examined the differences between cases of MS and controls and again the DRB1 gene came through as the predominant marker of the major histocompatibility complex in multiple sclerosis. This DRB1 gene has also been linked with other conditions apart from MS such as narcolepsy and systemic lupus erythematosus. The HLA-DRB1*1501 haplotype has also been associated with disease severity, as females with a younger age of onset are associated with this haplotype (Hensiek et al., 2002). This points towards genetics not just influencing susceptibility to multiple sclerosis but also disease course and severity.

The HLA-DRB1*1401 gene has been found to be protective for MS, overcoming the effect of HLA-DRB1*1501 in those carrying this gene (Barcellos et al., 2006). The mechanism of action of this protection is unknown but various mechanisms have been proposed including engagement of MHC-promiscuous, auto-reactive

thymocytes with resultant T regulatory cell formation as an explanation (Tsai and Santamaria, 2013) (Hollenbach and Oksenberg, 2015).

1.3.2 GENOME WIDE ASSOCIATION STUDIES

The study of the genetics of multiple sclerosis really developed with the advent of genome wide association studies. It had become clear that linkage studies were not powered to detect the small effects seen from the differences between genes in complex disorders. It was also apparent that these differences were not necessarily inherited in a Mendelian fashion. Alleles that are associated with complex diseases of multifactorial aetiology like multiple sclerosis are often common traits, which make up most of the genetic differences between individuals. These alleles tend to occur with a minor allele frequency of greater than 1%.

In the human genome there are approximately ten million variants of genes that are considered 'common' with a minor allele frequency of greater than 1%. This is among all genetic variants, which are thought to run into the billions. However these common genetic variants are said to account for >90% of the genetic differences between any two individuals (Wang et al., 2005) (Oksenberg et al., 2008).

Genome Wide Association Studies (GWAS) were conducted assessing multiple variations in genes called single nucleotide polymorphisms (SNPs), comparing the likely odds of those variations being associated with the cases rather than the controls. The principle of Linkage Disequilibrium whereby groups of genes are inherited together in 'linkage disequilibrium (LD) bins', meant that analysis of tagging SNPs using commercially available assays of approximately 500000 of such 'LD bins' meant it was possible to survey the whole genome.

Due to the nature of genetics in complex diseases, the Wellcome Trust Case Consortium proposed GWAS needed to include at least 2000 cases and controls, to ensure adequately powered studies (Oksenberg et al., 2008). They also suggested a newer level of significance of $p < 5 \times 10^{-8}$ was adopted in GWAS, to ensure results were more likely to be true than false. The very large numbers of cases and controls that were needed (approximately 10000 each) was finally achieved in 2011 with a worldwide collaboration with the International Multiple Sclerosis Genetics Consortium (IMSGC) (International Multiple Sclerosis Genetics et al., 2011).

Prior to this, a number of GWAS were conducted which helped to identify 26 loci in multiple sclerosis.

GWAS	**Number of cases and controls**
A genome screen in multiple sclerosis reveals susceptibilty loci on chromosome 6p21 and 17q22 (Sawcer et al., 1996)	466 cases; 303 controls
Risk alleles for multiple sclerosis identified by a genomewide study. New England Journal of Medicine (Hafler et al., 2007)	2322 cases; 789 controls
Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. Nature Genetics (Newport et al., 2007)	1000 cases; 1500 controls
Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. Human Molecular Genetics (Baranzini et al., 2009)	978 cases; 883 controls
Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. Nature Genetics (Bahlo et al., 2009)	1618 cases; 3413 controls
Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nature Genetics (De Jager et al., 2009)	Meta-analysis of 2624 cases; 7220 controls Replication in 2215 cases 2116 controls
Variants within the immunoregulatory CBLB gene are associated with multiple sclerosis. Nature Genetics (Sanna et al., 2010)	882 cases; 872 controls Replication in 1775 cases; 2005 controls
Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature (International Multiple Sclerosis Genetics et al., 2011)	9772 cases; 17376 controls

Table 1. 1: GWAS conducted which helped to identify loci in multiple sclerosis

The significance of the GWAS conducted was not only deciphering the genetic basis for multiple sclerosis susceptibility, as when combined with the loci identified in the GWAS taken alongside candidate gene studies, this explains up to 30% of the

heritability of multiple sclerosis. This is equivalent to 5% of the causation of multiple sclerosis. However identification of the genes associated with the identified loci showed that they were mainly associated with the immune system. This helped to emphasize the immune pathogenesis behind multiple sclerosis (International Multiple Sclerosis Genetics et al., 2011).

Further work will need to be done to explore the functional role of each of the genes identified within multiple sclerosis pathogenesis. The identification of these pathways may lead to the eventual identification of new therapeutic agents for multiple sclerosis.

1.3.3 FINE MAPPING OF THE GENOME – IMMUNOCHIP

Following the extensive contribution of the GWAS to both our understanding of the aetiology of multiple sclerosis and identification of potential pathways to target with immunotherapies, it was still clear that a large component of the heritability of a number of complex autoimmune conditions could still not be explained.

We may have been starting to see the limitations of the GWAS as it depended on the phenomenon of linkage disequilibrium. Therefore reliance on tagging SNPs may have missed some of the more rare variations in the genome. Some of the missing heritability may also have been explained by gene-gene interactions and gene-environment interactions.

A number of the loci found in the GWAS from 2011 have subsequently been mapped in greater detail. This is by use of the 'Immunochip' which was a collaboration between groups working on a range of autoimmune diseases (Cortes and Brown, 2011). The 'Immunochip' is a genotyping chip containing 196,524 polymorphisms. This collaboration determined 184 regions for fine mapping. From the 57 loci that were identified in the 2011 GWAS, 38 of these loci were contained on the Immunochip and were mapped in greater detail.

The immunochip was able to cover in finer detail the top-ranking SNPs from the original GWAS. It was also able to identify genes, which are applicable to a number of different autoimmune diseases.

As a result of the Immunochip, where 14,498 cases and 24,091 controls were analysed, the number of SNPs associated with multiple sclerosis rose from the 57 SNPs identified in 2011 (International Multiple Sclerosis Genetics et al., 2011) to 110 SNPs (International Multiple Sclerosis Genetics et al., 2013).

1.3.4 FUNCTIONAL STUDIES OF CANDIDATE GENES

We are still in the infancy of understanding the pathways involving the loci identified in the multiple sclerosis GWAS. There has been some work that has underlined the importance of understanding the underlying nature of the pathogenesis of the disease. This may lead to increased understanding of the clinical relevance of these associations and also eventually to manipulation of these immune pathways with pharmacological therapies.

There have been three SNPs identified in the GWAS that have led to increased levels of soluble protein receptors associated with the at risk variant. The first was the discovery that the SNP rs1800693 was associated with increased levels of the soluble form of TNFR1, which blocks TNF. This SNP was associated with multiple sclerosis but not with other autoimmune disorders in which there has been successful use of anti-TNF drugs, whereas in multiple sclerosis use of anti-TNF drugs exacerbates the disease (Gregory et al., 2012). It is not clear how TNF blockade leads to multiple sclerosis.

The second was the SNP rs6897932, which was associated with increased levels of soluble IL-7Rα. This will be discussed in more detail in the section on the Interleukin 7 receptor. However the at risk SNP resides in the trans membrane domain of IL-7Rα and is associated with skipping of the exon 6 part of the protein. This results in increased levels of soluble IL-7Rα (Gregory et al., 2007). I will discuss in more detail in later chapters the pursuit of the role of IL-7 receptor antagonism in multiple sclerosis.

The third was the SNP rs2104286, which is associated with increased levels of soluble IL-2R. This soluble form of the protein is associated with inhibition of signaling through the Interleukin 2 receptor (Maier et al., 2009). The effects on further

downstream events are not yet clear. Knowledge and analysis of the IL-2R pathway has led to the use of Daclizumab in multiple sclerosis (Kappos et al., 2015).

Other mechanisms apart from the role of soluble receptors have been identified. The at risk SNP rs6677309 resulted in reduced expression of CD58 which is a co-stimulatory cell adhesion molecule involved in strengthening the T cell and antigen presenting cell interaction. Reduced expression of CD58 resulted in reduced function of T regulatory cells due to concomitant reduced expression of FoxP3 (De Jager et al.).

In another study the protective SNP rs34536443 was associated with a reduction in tyrosine kinase 2 activity, which led to a reduced inflammatory environment with the enhancement of T helper 2 lymphocyte cytokine profiles (Couturier et al., 2011).

The functional work on the variants found in the GWAS will continue and this will help to build the body of knowledge about multiple sclerosis pathogenesis. Hopefully in the future a consensus will build about the pathways that will become the most amenable to drug treatment.

1.4 ENVIRONMENTAL RISKS IN MULTIPLE SCLEROSIS

Multiple Sclerosis is triggered by a combination of environmental risk factors and an individual's genetics. The closer to the equator one is born, the lower the risk of developing multiple sclerosis. However this risk is decreased twofold if migrants move from high risk areas to low risk areas (Kurtzke et al., 1985). This has led to different hypotheses on the environmental factors involved in the triggering of multiple sclerosis. The most notable environmental risk factors associated with multiple sclerosis are – Epstein Barr Virus (odds ratio 3.6), Vitamin D (levels below 50nM odds ratio 1.4), smoking (odds ratio 1.6), adolescent obesity (odds ratio 2), nighttime working (odds ratio 1.7) and organic solvent exposure (odds ratio 1.5) (Olsson et al., 2017). In this chapter I will focus on some of the more prominent environmental risk factors postulated including Epstein Barr Virus, Vitamin D levels and smoking.

1.4.1 EPSTEIN BARR VIRUS

Epstein Barr Virus (EBV) is a B lymphotrophic human DNA herpes virus that can cause glandular fever in children and young adults but more commonly is carried asymptomatically in adults.

Many studies have shown a higher rate of seropositivity with EBV in multiple sclerosis patients than controls (Haahr and Hollsberg, 2006) (Ascherio and Munger, 2007) (Sumaya et al., 1985). If you are infected with EBV at a young age you have a subsequently increased risk of developing multiple sclerosis (Martyn et al., 1993). This was reviewed in a meta-analysis and those who have had clinically overt glandular fever (clinical syndrome secondary to EBV infection), have a two fold increase in risk of developing subsequent multiple sclerosis (Handel et al., 2010).

There have also been several studies implicating abnormal immune activation to EBV in multiple sclerosis patients, with increased CD4+ and CD8+ T lymphocyte immune responses in both the cerebrospinal fluid (Holmoy and Vartdal, 2004) and blood (Cepok et al., 2005) (Lunemann et al., 2006).

One study also suggested that B lymphocytes are infected with EBV in B cell follicles in the meninges in the brains of patients with secondary progressive multiple

sclerosis (Serafini et al., 2007). However this finding has only been partially replicated in other studies (Olsson et al., 2017) and concerns still remain about the sensitivity and specificity of the techniques for detection of EBV in multiple sclerosis lesions (Lassmann et al., 2011).

There seems to be a remarkable difference in the seropositivity to EBV of individuals who go on to develop multiple sclerosis. There are much higher levels (at least four-fold) of antibodies to EBV nuclear antigen 1 (EBVNA1). In one case-control study all individuals previously negative for EBVNA1 converted to being positive for EBVNA1 prior to developing multiple sclerosis (Levin et al., 2010). Other diseases with an established causal role of EBV such as Burkitt's lymphoma, nasopharyngeal carcinoma and EBV related Hodgkin's disease also have antibodies to EBV increased several years prior to diagnosis (Ascherio and Munger, 2007).

One of the arguments against a causal role for EBV is that many individuals do not develop multiple sclerosis following infection with EBV, as it is a common infection affecting 95% of the adult population. This goes against the fact that a virus that is commonly contracted can then go on to cause a relatively rare disease such as multiple sclerosis. This would lead others to argue against the actual association of EBV with multiple sclerosis. Those in favour of the EBV hypothesis would point towards the example of polio, with infection being endemic in certain countries during the last century with only a small proportion going on to develop clinical poliomyelitis.

The increased antibody titers to EBV in people with multiple sclerosis may be a consequence of the immune-genetic environment found in those patients, with subsequent poor clearance of the virus as opposed to a causal role for the virus (Olsson et al., 2017). Finally some have pointed out that belief in the association of EBV with multiple sclerosis is paradoxical as this would go against the 'hygiene hypothesis' that those who tend to get infections with viruses at a younger age have a smaller chance of going on to develop autoimmune disorders (Ascherio and Munger, 2007). However others who support the EBV association with MS would point out that children who later develop multiple sclerosis would still share the same clean hygienic environment as those who develop other autoimmune disorders, but the key to the development of multiple sclerosis is the infection with EBV in later adolescence as opposed to their 'cleaner' childhood environment.

EBV stands out as the main infectious agent associated with multiple sclerosis. However some features of MS epidemiology, particularly the reduction in risk with migration from high to low risk areas for multiple sclerosis, are not explained by EBV and point towards the involvement of other factors in multiple sclerosis pathogenesis (Ascherio and Munger, 2007).

1.4.2 VITAMIN D

Low sun exposure has also been associated with multiple sclerosis. This is primarily from the observation that there is increasing MS prevalence with increasing worldwide latitude. Therefore as you get further away from the equator, more people suffer from multiple sclerosis. These results are slightly confounded by the distribution of the HLA-DRB1*1501, which is also more commonly found within these populations.

One of the strongest correlates of latitude is the duration and intensity of sunlight. Several studies have found similar results finding that the annual hours of sunshine is inversely correlated with the prevalence of multiple sclerosis (Acheson et al., 1960) (van der Mei et al., 2001) (Leibowitz et al., 1967). An interesting study in Switzerland also found an inverse correlation between MS prevalence and altitude, which is also a marker of sunlight intensity (Kurtzke, 1967). Although subject to recall bias a discordant twin study also looked at sunlight exposure. Twins with MS reported on average lower sun exposure during childhood (Islam et al., 2007).

Unsurprisingly significant inverse correlations have been found between risk of multiple sclerosis, sun exposure and Vitamin D levels. For the majority, vitamin D is generated from Ultraviolet B radiation (290-320 nm), which converts 7-dehydrocholesterol in the skin to pre-vitamin D3 that then spontaneously converts to vitamin D3. Vitamin D3 then undergoes a series of hydroxylations before it becomes the active form 1,25 dihydroxy vitamin D3.

Some of this information has come from longitudinal studies from the US army whereby bloods were taken prospectively and 257 subjects who subsequently developed multiple sclerosis had their 25 hydroxy vitamin D levels checked which showed significantly lower levels (Munger et al., 2006). These results as with all observational studies may be subject to unknown confounders with particularly

sunlight itself potentially being a confounder as it is known to have immunosuppressant effects and ultraviolet light has been seen to suppress EAE (Hauser et al., 1984) and enhance T regulatory cell function (Aubin, 2003).

Some groups have pointed towards the fact that a month of birth effect (Bayes et al., 2010) was previously attributed to lower levels of Vitamin D and less sun exposure at the time of conception and during the first trimester of pregnancy as a potential reason for an increased incidence of multiple sclerosis births in the spring as opposed to the autumn (Willer et al., 2005) (Dobson et al., 2013). The month of birth effect has been disputed due to previously unidentified heterogeneity in the numbers of births at different times of the year in populations at higher latitudes (Fiddes et al., 2013).

Single Nucleotide Polymorphisms (SNPs) in CYP27B1, which predisposes patients homozygous for the variant to Vitamin D deficient rickets, has shown an increased predisposition to multiple sclerosis in heterozygote carriers (Ramagopalan et al., 2011). The variation in CYP27B1 was thought to be a plausible candidate as the cytochrome p450 system is involved in synthesizing the active ingredient of Vitamin D - 1,25 hydroxy Vitamin D3. However these findings have not been replicated in other studies so some doubt remains about the validity of these earlier claims (Ban et al., 2013) (Barizzone et al., 2013).

Studies with experimental autoimmune encephalomyelitis (EAE) mice has shown that injection of 1,25 dihydroxy vitamin D3 has been shown to prevent clinical and pathological signs of EAE (Lemire and Archer, 1991). Supplementing mice with vitamin D prior to the induction of EAE reduces the severity of the disease in mice (Pedersen et al., 2007) (Spanier et al., 2012). The onset of EAE has also been shown to be accelerated in vitamin D deficient mice (Cantorna et al., 1996). EAE has also been attenuated and delayed by providing vitamin D supplements (Spach and Hayes, 2005).

In humans, vitamin D supplementation in multiple sclerosis patients has reduced CSF neurofilament levels (Sandberg et al., 2016). It has also been negatively correlated with increasing disease progression as evidenced by clinical measures and MRI imaging (Ascherio et al., 2014) (Fitzgerald et al., 2015).

How vitamin D exerts its effects are unknown, although it is thought to mediate its immunological effects via the vitamin D receptor which has increased the effect of regulatory T cells (Smolders et al., 2008), regulates cytokine secretion by antigen presenting cells and modulates the Th17 cell response (Joshi et al., 2011), which is thought to play a key role in autoimmune diseases (Peelen et al., 2011).

Despite the evidence on vitamin D discussed above, some neurologists are yet to be convinced that there is clear-cut evidence that vitamin D supplementation affects clinical relapse rate or expanded disability status scale (EDSS). Despite these reservations, replacement of vitamin D among the multiple sclerosis population has become common practice, mainly due to the fact that a large trial has shown that large doses of vitamin D can be given safely (Wingerchuk et al., 2005).

1.4.3 SMOKING

Smoking associated with multiple sclerosis was suggested after a pooled analysis on a number of smaller studies (Hawkes, 2007) (Handel et al., 2011). This was then replicated in a much larger case-control study (Hedström et al., 2009). According to some authors there is a dose-dependent relationship between smoking and multiple sclerosis (Hedström et al., 2009) (Ghadirian et al., 2001).

The mechanism for the smoking association with multiple sclerosis is purported to be due to the fact that smoking is irritant to the lungs and causes inflammation (Hedström et al., 2011a) (Olsson et al., 2017). Other autoimmune diseases such as rheumatoid arthritis have been associated with smoking (Klareskog et al., 2009). It has also been shown in EAE that smoking can activate encephalitogenic cells present in the lungs to become ‘migratory’ in their behaviour, causing more disease in the central nervous system (Odoardi et al., 2012).

The hypothesis of inflammation in the lungs rather than a nicotinic effect is supported by studies from Sweden, where a large amount of oral tobacco is consumed. A dose dependent protective effect of oral tobacco consumption was seen in this study (Hedström et al., 2009).

Smoking as a risk factor in multiple sclerosis displays some interaction with the HLA genetic risk factor. The HLA Class II genetic variant (HLA-DRB1*1501 with lack of

HLA-A*02) gives an odds ratio of 5 and when dividing the population into smokers gives an odds ratio of 14 (Hedström et al., 2011b). Therefore the effect of smoking impacting on the subsequent risk of multiple sclerosis is dependent on the individual's HLA genotype. This data suggests smoking interacts with antigen presentation to T lymphocytes.

With this knowledge about smoking as a risk factor for multiple sclerosis it would be possible to reduce MS incidence with public health measures, which reduce tobacco smoking and passive smoking.

Smoking is associated with a worse prognosis in multiple sclerosis (Sundström and Nyström, 2008) and an aggravated disease course (Manouchehrinia et al., 2013) (Zivadinov et al., 2009). It has also been associated with an increase in the neutralizing antibodies following treatment with natalizumab (Hedström et al., 2014a) and interferon-beta (Hedström et al., 2014b). Physicians caring for patients with multiple sclerosis should strongly encourage them to stop smoking.

1.5 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) – STRENGTHS AND WEAKNESSES OF THIS ANIMAL MODEL

Multiple Sclerosis is a disease involving many different facets of the immune system. In order for an animal model to be useful in depicting the disease it should be able to demonstrate the different facets of multiple sclerosis, mainly a period of inflammation, initiated by CD4+ T cells, then subsequently driven by CD8 T cells and B cells. There would also have to be a subsequent period of neurodegeneration.

The EAE model evolved from when a small number of patients receiving the live rabies virus vaccine (a live attenuated strain grown in a rabbit's central nervous system) developed encephalomyelitis. The realization was that the encephalomyelitis was not a result of the rabies vaccine itself but a hypersensitivity reaction to the rabbit central nervous system constituents contaminating the vaccine. This initiated the development of EAE as a model for multiple sclerosis (Lovett-Racke et al., 2011).

There are many different types of EAE models. I will go on to describe the different types of EAE and their particular strengths, but also how they may not resemble the pathology seen in multiple sclerosis.

1.5.1 MODELS WITH CD4+ T CELL INFLAMMATION

Transfer of encephalitogenic T cells as a model for EAE was first shown in work by Philip Paterson (Paterson, 1960). Once animals were sensitized to brain tissue, lymphocytes were drawn from the peripheral blood and then transferred to a naïve recipient; this stimulated a neuroinflammatory disease. This principal was demonstrated when T cells specific for myelin basic protein were intravenously transferred to animals which triggered an encephalomyelitis (Ben-Nun et al., 2017).

This passive transfer of T cells is a good model for studying the mechanisms of inflammation by T cells and macrophages with limited microglial activation. In this model there is little axonal loss or demyelination. This model tends to be used to analyse the molecular mechanisms involved in brain inflammation related to CD4+ cells and may be useful for in vivo testing of anti-inflammatory treatments (Lassmann and Bradl, 2017).

Some of the limitations of this model is it only tends to involve inflammation mediated solely by CD4+ cells which is unlike the pathology seen in multiple sclerosis, where many different types of cells are involved such as microglia, CD8+ cells and B cells, with relatively few CD4+ cells found in CNS lesions. It also tends to cause inflammatory disease of the central nervous system with variable axonal injury and secondary demyelination, but without the more widespread primary demyelination seen in multiple sclerosis. Therefore the relevance this type of inflammation has for multiple sclerosis patients is currently unclear.

There are also active sensitization models of EAE whereby active immunization with an antigen from the central nervous system together with a strong adjuvant (e.g. CFA or complete Freund's adjuvant) (Baxter and Hodgkin, 2002) and administration of pertussis toxin is another mechanism by which to induce EAE (Bernard, 1976). The most frequently used method is induction in mice with myelin oligodendrocyte glycoprotein$_{33-55}$ (MOG) peptides in CFA (Mendel et al., 1995).

Some of the strengths of this model are that it is relatively easy to induce an acute or chronic inflammatory disease. However some of the drawbacks of this model are that it tends to only affect the spinal cord, with larger lesions due to axonal degeneration with secondary demyelination, with not much in the way of primary demyelination, which is normally seen in multiple sclerosis. The limitations to this model make it difficult to predict what will happen in MS patients, as the type of inflammation seen in humans is very different (Lassmann and Bradl, 2017).

Another model using active sensitization that more closely resembles multiple sclerosis is sensitization of either rats (Storch et al., 1998), guinea pigs (Lassmann and Wisniewski, 1979) or primates (Jagessar et al., 2015) with the recombinant extracellular domain (amino acids 1-125) of MOG with either myelin or brain tissue in CFA. This model induces not only a CD4+ T cell encephalitogenic response but also encompasses a demyelinating autoantibody response against MOG (Linington et al., 1988). This model produces large confluent plaques of demyelination with areas of axonal sparing similar to what is seen in multiple sclerosis. The distribution of the lesions depends upon how severe the disease is in the affected animal but also the genetic background of the animals used (Weissert et al., 1998). In this model the disease is not just restricted to the spinal cord and in certain rat strains large cortical demyelinated lesions are seen, related to chronic inflammation in the meninges (Pomeroy et al., 2005).

Although one of the positives of this model is that it closely resembles the pathology seen in multiple sclerosis, it has to be said that the use of MOG antibodies means that it will always be a questionable about how representative of multiple sclerosis this model can be. MOG antibodies are on the whole not found in patients with multiple sclerosis and when present the clinical presentation differs from typical multiple sclerosis and is more likely to resemble aquaporin-4 antibody negative neuromyelitis optica or acute disseminated encephalomyelitis. Therefore this model may be good for these other clinical syndromes, which are distinct to multiple sclerosis (Sepúlveda et al., 2016) (Kim et al., 2015).

1.5.2 MODELS WITH CD8+ T CELL INFLAMMATION

Passive transfer of T cells can also occur with CD8+ T cells (Saxena et al., 2008). With this model inflammation is associated with destruction of antigen contained within oligodendrocytes by cytotoxic T cells resulting in demyelinating plaques.

Another method used to induce CD8+ T cell inflammation was to actively sensitize mice with myelin basic protein and then withdraw their CD8+ T cells and subsequently transfer the CD8+ T cells into naïve mice (Huseby et al., 2001).

With CD8+ T cell mediated inflammation you get low macrophage recruitment with significant microglial activation (Lassmann and Bradl, 2017). The direct tissue injury is mediated by CD8+ cytotoxic T cells. The studies above show that CD8 T cells alone can induce brain inflammation without the need for recruitment of other T cell populations.

One of the advantages of the CD8+ T cell EAE models are that it presents a good opportunity to analyze molecular mechanisms involved in inflammation and tissue injury induced by Class I MHC restricted T cells. There are also a lot of CD8+ T cells within MS lesions, which suggests that CD8+ T cells play an important role in MS pathogenesis.

Some of the disadvantages of these models are that they have high intra-experimental variation. Also more importantly the evidence for direct CD8+ T cell cytotoxicity in the pathogenesis of MS is limited. It has proved very difficult to induce

active CD8+ T cell autoimmunity by active immunization (Lassmann and Bradl, 2017).

1.5.3 VIRUS MODELS OF INFLAMMATORY DEYMELINATION

Virus infections in animals can give rise to a central nervous system inflammatory disease (Denic et al., 2011).

The Theiler's virus model is induced by direct intracerebral infection of animals with a virus. The disease course and mortality depend upon the virulence of the virus strain and the genetic background of the host animal to mount a specific T cell response (Lassmann and Bradl, 2017). Popular virus strains include BeAn and Daniel's strains, with mice MHC haplotypes H-2^{qrsvfp} which causes an acute encephalitic phase followed by chronic demyelination which mostly affects the spinal cord (Denic et al., 2011). These spinal cord lesions include the formation of confluent plaques of primary demyelination with a variable extent of axonal injury and remyelination depending on the particular host animal used. Many of the pathological features seen in the model share the pathological features seen in multiple sclerosis.

The Mouse Hepatitis Virus (MHV) model was first discovered after MHV virus was isolated from a paralysed mouse that had disseminated encephalomyelitis with demyelination (Bailey et al., 1949). Neurological disease develops after nasal or intracranial infection with MHV. The neurological disease has two phases. The first is a pan-encephalitis. A second phase occurs four weeks later, with inflammatory demyelinating lesions, which cause paralysis. This secondary phase is pathologically characterized by the formation of confluent plaques of demyelination followed by variable degrees of acute axonal injury. The viral antigen is seen in the acute phase of the disease and is seen in many different types of immune cells (Bender and Weiss, 2010). Once there has been recovery of the acute phase the virus is cleared, but viral DNA persists throughout the chronic demyelination phase of the disease.

Findings using the drug fingolimod to treat mice from the MHV model tend to suggest that the inflammatory mechanisms involved in virus clearance and the induction of demyelination are different. This is because fingolimod tends to increase the severity of the first stage of the illness and decrease the demyelination phase of the illness (Blanc et al., 2014).

There are some drawbacks to these models. The first is complexity. Virus models have a very complex pathogenesis, involving direct virus effects, anti-viral immunity with additional autoimmune mechanisms most likely through antigen/epitope spreading. This makes it extremely difficult to dissect the effects of these different components (Denic et al., 2011).

1.5.4 TOXIC MODELS OF DEMYELINATION AND REMYELINATION

The toxic models used such as with cuprizone (Gudi et al., 2014) (Praet et al., 2014), Lysolecitin (Hall, 1972) (Jeffery and Blakemore, 1995) and ethidium bromide (Jeffery and Blakemore, 1995) are very good models to study the biology of demyelination and remyelination.

These models have the advantage of being highly reproducible, producing well-defined pathophysiological mechanisms for demyelination. Another advantage of these models is that they are not complicated by the changes in the central nervous system due to the inflammatory processes driven by the adaptive immune system (Lassmann and Bradl, 2017).

Cuprizone is a copper chelating drug which induces apoptosis in oligodendrocytes and induces demyelination by oxidative injury (Gudi et al., 2014) (Praet et al., 2014). Most of the cuprizone models are with C57BL/6 mice, that have cuprizone applied for 4 weeks, which induces demyelination and is followed by extensive remyelination (Hiremath et al., 1998). Demyelination is predictably seen in the corpus callosum and the relatively predictable time course of lesions with subsequent remyelination provides an excellent template for understanding mechanisms involved in demyelination and remyelination.

The rapid and extensive remyelination seen is a limitation when using this model in order to predict what occurs within multiple sclerosis as it does not show the progressive permanent remyelination failure which is particularly characteristic of progressive multiple sclerosis (Lassmann and Bradl, 2017). It is worth noting that despite the extensive remyelination, progressive motor decline does occur within cuprizone treated animals with ongoing axonal injury occurring within remyelinated axons (Manrique-Hoyos et al., 2012).

Therefore a different model was developed to overcome the problem of rapid remyelination. This involved the exposure of animals to cuprizone for 12 weeks rather than 4 (Matsushima and Morell, 2001). This difference lead to chronic demyelinated lesions with little remyelination. The impaired remyelination is due to a reduction in the oligodendrocyte progenitor cell population and changes in the local cytokine environment (Praet et al., 2014). Similar factors have been implicated in the failure of remyelination seen in multiple sclerosis and also in the age related decline in remyelination (Doucette et al., 2010) (Shen et al., 2008).

When lysolecitin is injected into the white matter tracts of the central nervous system, it induces focal plaques of demyelination due to a direct action of the toxin, which damages the myelin sheath (Jeffery and Blakemore, 1995). This model has the advantage of being highly reproducible, however similar to the cuprizone model it is followed by rapid and extensive remyelination. However the speed and degree of remyelination is age-dependent (Franklin et al., 2002). This shares features with the remyelination failure seen in multiple sclerosis (Lassmann and Bradl, 2017).

Another model for focal demyelination is when ethidium bromide is injected into the white matter tracts (Blakemore, 1982). This leads to the degeneration of astrocytes as well as oligodendrocytes. This model was key to demonstrating that oligodendrocyte remyelination required the presence of astrocytes. Without the presence of astrocytes, Schwann cells were responsible for the remyelination that occurred. Extensive Schwann cell remyelination is seen in Neuromyelitis optica, when there is involvement of astrocytes within the pathophysiology with secondary demyelination (Ikota et al., 2010). There is also a small subset of fulminant multiple sclerosis cases with severe astrocyte injury.

We know that the EAE mouse model is not completely analogous with multiple sclerosis as has been demonstrated with other drugs in the past that have been shown to ameliorate EAE, but have also been shown to worsen multiple sclerosis. Other drugs to have caused suppression of EAE but subsequently shown to activate multiple sclerosis include anti-TNF (The Lenercept Multiple Sclerosis Study Group, 1999) (Mohan et al., 2001), altered peptide ligands of myelin basic protein (Bielekova et al., 2000), interferon-gamma (Panitch et al., 1987), and Atacicept which binds to cytokines BLyS (B Lymphocyte Stimulator) and APRIL, which are involved in B cell stimulation, maturation and survival (Kappos et al., 2014).

Overall the use of these animal models has provided significant insights into mechanisms underlying the pathology of multiple sclerosis. However many of these animal models fail to represent the complicated nature of all aspects of the immune response in multiple sclerosis, particularly the primary demyelination seen with the disease. Due to a number of different factors, it has also been difficult to develop animal models that accurately represent the progressive phase of multiple sclerosis. Therefore in order to more appropriately understand how a particular molecule will affect multiple sclerosis, in my view; it has to be tested in humans affected by the disease.

1.6 THE TH1/TH2 PARADIGM IN MULTIPLE SCLEROSIS

Once T cells come into contact with antigen, they expand and become activated and form into many different phenotypes. MS susceptibility genes play a role in this process of activation and expansion, particularly IL-7Rα.

In 1986 Mossman and Kaufer (Mosmann et al., 1986) developed the theory of reciprocal inhibition for subsets of helper T cells. Th1 cells are derived from naïve T helper cells when these naïve T helper cells are stimulated with interleukin-12 and interleukin-18. The Th1 lymphocytes then go on to secrete the cytokine interferon gamma (please refer to figure 1.1). Interferon gamma causes the upregulation of MHC class II on a variety of immune cells and activates macrophages.

Interleukin-4 is crucial for the development and maintenance of Th2 responses. Th2 cells also produced interleukin-4. Dysregulated Th2 responses play an important role in allergic responses mediated by eosinophils (please refer to figure 1.1).

Th1 cells were thought to drive the inflammation seen in multiple sclerosis. This comes from experiments (Ando et al., 1989) (Wildbaum et al., 1998) that demonstrated encephalitogenic T cells in EAE produced interferon gamma. Also myelin basic protein stimulated T cells from multiple sclerosis patients produced more interferon gamma then healthy controls (Voskuhl et al., 1993).

EAE resistant mice produce Th2 responses. Preventing the functioning of IL-4 removes this resistance to EAE. Also by blocking the p40 subunit of interleukin-12 with neutralizing antibodies, EAE was ameliorated (Constantinescu et al., 2001). These studies served to underline the interplay between Th1 and Th2 pathways in activation and abrogation of EAE and other organ specific autoimmune diseases.

On the other hand, challenging the hypothesis of the Th1/Th2 paradigm and EAE was the observation that interferon gamma knockout mice can develop EAE (Ferber et al., 1996). Therefore some authors concluded that interferon gamma was not crucial to the development of EAE. This led to the search for other mechanisms involved in the induction and clinical course of EAE, which are described below.

1.7 THE TH1/TH17 PARADIGM IN MULTIPLE SCLEROSIS

It has been known for some time that innate cells of the immune system have produced IL-17 (Cua and Tato, 2010). However in 1995 CD4+ T cells were discovered to secrete IL-17 (Yao et al., 1995). A few years later a distinct subset of CD4+ T cells were found to secrete IL-17 (Park et al., 2005) (Langrish et al., 2005). This subset of CD4+ T cells was distinct from Th1 cells that secreted IFN-γ and Th2 cells which secreted IL-4. In fact during the discovery of Th-17 cells it was found that the cytokines IFN-γ and IL-4 inhibited the secretion of IL-17.

Experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis, has been used to investigate T cell driven organ specific autoimmune diseases (Lassmann and Bradl, 2017). T cells that secrete IFN-γ can induce EAE. It has also been shown that EAE can be induced in mice deficient in the Th1 effector cytokine IFN-γ (Ferber et al., 1996). Therefore this indicates that Th1 cells are not the only type of T helper cells vital for the induction of EAE. It was shown in 2003 that IL-23p19 mice have complete resistance to EAE. This implicated Th17 cells as the major T helper subset in inducing autoimmunity, as without the support of IL-23 the Th17 cell secretion of IL-17 cannot be sustained. Without the continued secretion of IL-17, the IL-23p19 mice did not develop EAE (Cua et al., 2003). Please refer to section 1.5 for more information about EAE. IL-23 is not only responsible for stabilizing IL-17. It is responsible for a host of other reactions within T cells including secretion of IL-10 (Stumhofer et al., 2007) and induction of IL-7Rα (McGeachy et al., 2009) (Arbelaez et al., 2015).

However it is clear that T-bet KO mice are resistant to EAE (Bettelli et al., 2004) and the clear role for T-bet in the development of Th1 cells is well established (Szabo et al., 2000) (Mullen et al., 2001). Therefore both Th1 and Th17 with its associated cytokines and transcription factors are essential for the generation of pathogenic T cells and the development of EAE.

We now recognize IL-17 as IL-17A, as there is a whole host of cytokines within the IL-17 family (IL-17A – IL-17F). IL-17A is proinflammatory and has been linked with defense against microbial infections and with cell-mediated autoimmune disease. Although we know that IL-17A is the signature cytokine of Th17 cells, it has been shown that IL-17A is not solely secreted by Th17 cells and is also secreted by cells of the innate immune system such as γδ T cells (Martin et al., 2009) (Shibata et al.,

2007), lymphoid tissue inducer-like (LTi) cells (Takatori et al., 2009) and Tc17 cells (Huber et al., 2013) (reviewed in Gu et al., 2013).

The discovery of Th-17 cells further illuminated the discoveries mentioned above by Mossman et al in 1986. This highlighted the development of particular T effector CD4+ cells for particular types of inflammation. For example Th1 cells induced by IL-12, with activation of transcription factor T-bet (STAT 1 dependent) and secreting IFN-γ, upregulate MHC class II on many immune cells which helps to activate macrophages and induce them to destroy intracellular pathogens (e.g. Listeria). Th2 cells induced by IL-4, with activation of the transcription factor Gata-3 (STAT 6 dependent) and secretion of IL-4; help to induce eosinophils in the fight against parasites. Th17 cells are induced by TGF-β and IL-6 (subsequent exposure to IL-23 helps to continue the stimulation and maintenance of Th17 cells from naïve CD4+ cells), with activation of the transcription factor Retinoic Acid Receptor related orphan receptor gamma t (RORγt) and secretion of IL-17 (also IL-21, IL-22 and granulocyte macrophage colony stimulating factor). Please refer to figure 1.1.

Th17 cells in MS have been identified in tissue from acute brain and spinal cord lesions in MS patients (Montes et al., 2009). Prior to this finding there had been interest in IL-17 secreting lymphocytes in the blood and CSF of patients (Matusevicius et al., 1999), and that there was over-representation of IL-17 expressing immune cells in chronic MS lesions (Lock et al., 2002) (Tzartos et al., 2008).

In terms of treatment of MS, fingolimod has been purported to have a preferential effect on IL-17 producing T helper cells (Mehling et al., 2010). On the one hand this has directly linked Th17 cells with promoting inflammation in MS. On the other hand neutralizing antibodies to the common p40 subunit of IL-12 and IL-23 did not demonstrate efficacy in reducing inflammation in MS patients (Segal et al., 2008), however some authors have pointed towards the fact that these patients had advanced disease as a reason for the lack of efficacy of ustekinumab with MS patients (Longbrake and Racke, 2009).

In summary, the evidence points towards the involvement of Th17 cells in MS. These cells however are plastic and commonly co-produce IFN-γ (Kebir et al., 2009), particularly when they enter the central nervous system. However, they have distinct properties, which are separate from Th1 cells.

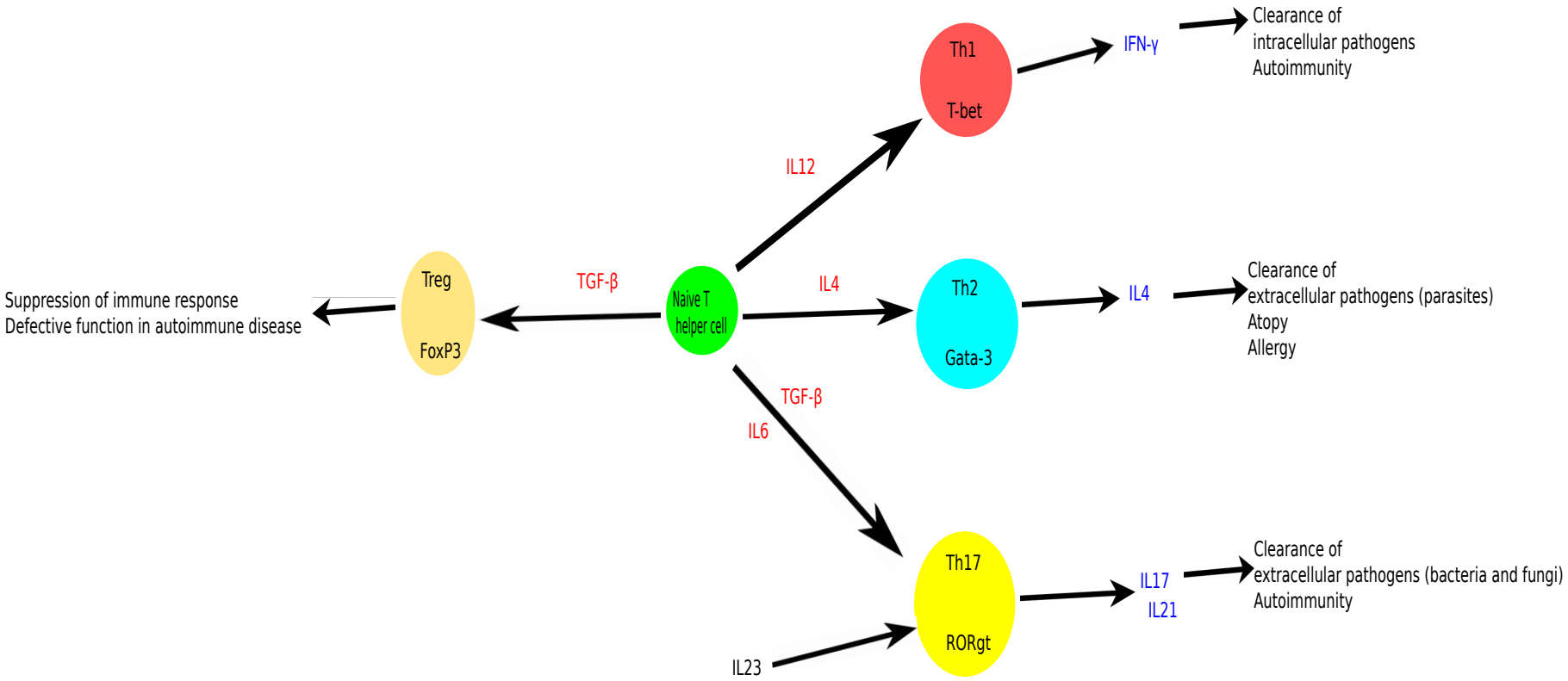

Figure 1. 1: Paradigms of CD4+ subsets involved in Multiple Sclerosis Pathogenesis.

Naïve T helper cells are stimulated with cytokines (red) to differentiate into the CD4+ cells Th1, Th2, Th17 and naturally occurring T regulatory cells. These differentiated CD4+ cells then secrete cytokines (blue) that have specific effects on the immune response as outlined. Naturally occurring T regulatory cells have defective function in autoimmune disease.

1.8 T REGULATORY CELLS IN MULTIPLE SCLEROSIS

Experiments in mice in 1985 demonstrated the existence of a T cell subset that if depleted led to the development of autoimmune disease. This T cell subset was thought to be involved in maintaining self-tolerance (Sakaguchi et al., 1985). The same group, ten years later, demonstrated how these T regulatory cells (defined in mice as CD4+ CD25+ T cells) were involved in down regulation of peripheral immune responses to self and non-self antigens. In humans they were subsequently defined as CD4+ $CD25^{high}$ T cells. In this seminal paper it was proposed that abnormalities in this T regulatory cell subset could be a cause of autoimmune disease. Before this time it was not widely believed that regulatory T cells existed (Sakaguchi et al., 1995).

T regulatory cells are split into naturally occurring T regulatory cells (Tregs) and induced T regulatory cells. Naturally occurring Tregs are a distinct T cell population optimized for suppressive function during its development in the thymus. The transcription factor Forkhead Box P3 (Foxp3) has been shown to be a specific marker for T regulatory cells. Mutations in FoxP3 in humans have been associated with the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome that manifests in humans as neonatal onset of type 1 diabetes mellitus, anaemia, eczema, thrombocytopenia, immune dysregulation and hypothyroidism.

However the characterization of the human T regulatory cell was more difficult than in mice as the population was more heterogeneous. Human T cells express CD25+ when they become activated so it is more difficult to distinguish human T regulatory cells from activated T cells. The discovery of CD4+ $CD25^{high}$ cells (only 1-2% of the total CD4+ T cell population consists of $CD25^{high}$ cells) as human T regulatory cells (Baecher-Allan et al., 2001) was an important first step. However there was always controversy as to where the boundary stood for $CD25^{high}$ and CD25 intermediate CD4+ cells. With the discovery of FoxP3 as a marker for T regulatory cells in humans, identification of these cells became easier (Roncador et al., 2005) (Hori et al., 2003).

T regulatory cells were also discovered to exhibit low levels of IL-7Rα. This was discovered in humans during investigation of lymphocyte homeostasis following

treatment with alemtuzumab (Cox et al., 2005). This discovery had earlier been made in mice (Cozzo et al., 2003).

T regulatory cells in Multiple Sclerosis were found to be defective in function in 2004. The actual number of T regulatory cells was the same as in healthy controls. A series of experiments in which varying ratios of regulatory T cells when mixed with responder cells demonstrated the lesser degree to which T regulatory cells from MS patients were able to suppress the responder cell population (Viglietta et al., 2004). These results were also replicated in 2005 (Haas et al., 2005). Another group have shown that in untreated MS patients there was reduced levels of FoxP3 mRNA and reduced levels of protein expression in the CD4+CD25+ T regulatory cell population, which would be in keeping with the reduced level of suppressive function in the cells of MS patients (Huan et al., 2005).

Haas et al in 2007 (Haas et al., 2007) went on to further explain the poor function of T regulatory cells in MS patients. It was found that the decreased suppressive function disappeared with the depletion of naïve T regulatory cells that had recently emigrated from the thymus. This was also found by another group looking into the function of memory (mature) T regulatory cells and naïve T regulatory cells. In both secondary progressive MS (SPMS) and relapsing remitting MS the naïve T regulatory cells were deficient in both number and function. There was an increase in the number of memory T regulatory cells in these patients, with an increase in the suppressive function of memory T regulatory cells in those with a long disease course (>10 years) and also in those with SPMS (Venken et al., 2008).

The same group in 2011 linked altered T regulatory cell function to expression and signaling through the IL-7Rα and TSPLR during thymic development. They were unable to demonstrate a link to IL-7Rα genotype. They thought that impaired genesis of naïve T regulatory cells via reduced signaling in IL-7Rα resulted in the defective overall function of the T regulatory cells in multiple sclerosis patients (Haas et al., 2011).

There are a number of mechanisms by which T regulatory cells suppress other T cells. The first is by secretion of regulatory cytokines TGF-β and IL-10. Another mechanism is by competing for IL-2 due to the high expression of IL-2R present on T regulatory cells. IL-2 is a key cytokine involved in activation of cells. Another mechanism is via cell-to-cell contact. There is TGF-β on the cell membrane of T

regulatory cells but there is also a host of other molecules such as CTLA-4, Fas ligand receptor, Granzyme B and LAG3. For example with T regulatory cells overexpressing CTLA-4, this successfully competes with the co-stimulatory receptors on the effector T cells reducing activation of effector T cells (Sakaguchi et al., 2010). Finally some T regulatory cells express CD39 and CD79, which are ectonucleotidases that convert ATP to adenosine. Effector T cells have A2A receptors on their cell surface and the uptake of adenosine rather than ATP initiates suppressive signaling in effector T cells (Fletcher et al., 2009).

1.9 THE ROLE OF B CELLS AND ANTIBODIES IN MULTIPLE SCLEROSIS

B lymphocytes are involved in the early stages of the formation of multiple sclerosis plaques. In pattern II lesions, found in 58% of MS biopsies, there is immunoglobulin and complement deposition on myelin. There is phagocytosis of complement-opsonized myelin debris by macrophages (Popescu et al., 2013). B cells also form packed aggregates in the leptomeningeal space covering the cortex (Serafini et al., 2004). These lymphoid follicles seem to resemble germinal centers. Leptomeningeal aggregates are a common feature of progressive multiple sclerosis but this phenomenon has also been recognised in early relapsing-remitting multiple sclerosis (Lucchinetti et al., 2011).

It was postulated that B cells mitigate their effects in multiple sclerosis by (Wekerle, 2017) (Hohlfeld and Meinl, 2017):

1. Secretion of pathogenic autoantibodies.
2. Antigen presentation to T lymphocytes.
3. Secretion of cytokines either pro-inflammatory such as GM-CSF or the failure to produce anti-inflammatory cytokines such as TGF-β or IL-10 from B regulatory cells.

Investigation into these mechanisms in vitro has been difficult due to the traditional methods of investigation in experimental autoimmune encephalomyelitis (EAE), which normally uses adjuvant immunization or passive immune cell transfer. These are artificial methods of induction of EAE and therefore spontaneous models of EAE have been introduced via the use of transgenic mice (Wekerle, 2017).

The OSE (C57BL/6) and RR (SJL/J) mice are spontaneous models of EAE with between 60 and 100% of mice eventually developing EAE. B cells play a significant role in both models. In OSE mice B cells capture soluble myelin oligodendrocyte glycoprotein present in high dilutions, concentrate and process the antigen and present it to T cells (Krishnamoorthy et al., 2006). This amplifies the autoimmune process.

In RR mice the role of B cells is the production of autoantibodies (Pollinger et al., 2009). Anti-MOG antibodies appear in the plasma from 4 weeks of age in RR mice and recognise MOG epitopes on the myelin surface. The B cells are crucial in this

mouse model as when depleted from this model EAE does not develop. When present EAE develops in 100% of mice. When these autoantibodies are transferred to recipient mice they develop large confluent demyelinating lesions, which are strongly reminiscent of multiple sclerosis plaques.

1.9.1 OLIGOCLONAL BANDS IN MULTIPLE SCLEROSIS

Normally the healthy cerebrospinal fluid (CSF) does not contain immunoglobulins and other plasma proteins. In multiple sclerosis the CSF typically contains immunoglobulins detected as individual bands on gel agarose electrophoresis. The principle involves the separation of proteins (IgG) in the paired serum and CSF using agarose gel electrophoresis followed by passive transfer onto a nitrocellulose membrane. The separated immunoglobulins are then detected by horseradish peroxidase labeled anti-human antibody. If immunoglobulin bands are present in the CSF that is not present in the serum, this represents intrathecal synthesis of IgG, which is seen in multiple sclerosis.

Freedman and colleagues described in a consensus statement on CSF analysis for diagnosis of MS that oligoclonal bands (OCBs) were the ‘gold standard’ with sensitivity greater than 95% and excellent specificity (Freedman et al., 2005).

Neither the cellular origin nor the target antigens of the IgG bands seen in the CSF of multiple sclerosis patients have been identified. However one group using transcriptomics to characterize the gene repertoire of CSF B cells and proteomics in order to sequence the individual immunoglobulin bands, showed that most of the OCBs were produced by local, CSF or parenchymal B lymphocytes (Obermeier et al., 2008). Several groups have been able to clone the paired genes of CSF immunoglobulins in order to produce recombinant antibodies. However the antigen target of these antibodies have given divergent results. Some of these results have shown ubiquitous proteins (Brändle et al., 2016) as targets for the antibodies with another group showing binding of antibodies to lipids (Brennan et al., 2011). Therefore there has been a failure to demonstrate conclusively autoantibodies in multiple sclerosis binding to major myelin structures.

Despite being unable to find specific antigen targets for the autoantibodies in the CSF of multiple sclerosis patients there have been successful therapies used that

principally target B lymphocytes. This in itself underpins how fundamental B lymphocytes are to the pathogenesis of multiple sclerosis. Therapies such as rituximab and ocrelizumab target CD20+ cells. CD20+ cells comprise a substantial part of the B cell population ranging from pro-B cells in the bone marrow to short lived plasmablasts, but it excludes CD20+ negative long-lived plasma cells, which secrete antibodies of previously encountered pathogens (Hohlfeld and Meinl, 2017). Ocrelizumab has now become part of the armamentarium of treatments available to neurologists caring for multiple sclerosis patients. Plasmapheresis, which removes autoantibodies from the blood, has also been used in fulminant relapses of multiple sclerosis.

Rituximab substantially reduced MRI lesion load (relative reduction of 91%) over a 48 week period when compared to placebo. There was also a significant reduction in relapse rate over this period (Hauser et al., 2008).

Ocrelizumab has had two phase 3 trials OPERA I and II, which showed a reduction in relapse rate compared to interferon-beta of 46% and 47%. There was also a 95% lower rate of gadolinium enhancing lesions on the MRI scan when compared to interferon-beta (Hauser et al., 2017).

1.9.2 MECHANISM OF ACTION OF B LYMPHOCYTES IN MULTIPLE SCLEROSIS

These drug trials help us to determine the role of B cells in the pathogenesis of multiple sclerosis. It is clear that the number of both B and T cells reduced with anti-CD20+ therapies. This may be due to both the function of B cells as antigen presenting cells and also to the fact that B cells contribute to the autoimmune cytokine milieu. There are also a group of CD20+ T cells which contribute approximately 5% of the T cell population (Palanichamy et al., 2014). In the CSF of multiple sclerosis patients these CD20+ T cells have a similar frequency to the number of B lymphocytes and therefore it is postulated that they have a significant contribution to multiple sclerosis pathogenesis (Schuh et al., 2016).

According to some authors the intrathecal antibody response seen in multiple sclerosis is partly due to intracellular antigens released during tissue injury (Brändle et al., 2016). Although (as referred to above) in my view there is no consensus on the antigenic target of oligoclonal bands. It is unlikely that these intracellular antigens are

pathogenic in the same way as the newly described cell surface antibodies in autoimmune encephalitis (Hohlfeld and Meinl, 2017). However there may be enhanced recruitment of T lymphocytes that recognize the same antigens released in the debris following tissue injury. This mechanism for superadded recruitment of T cells into the central nervous system in multiple sclerosis may lead to renewed interest in reducing the antibodies produced in the central nervous system in multiple sclerosis (Flach et al., 2016).

1.10 THE TREATMENT SPECTRUM IN MULTIPLE SCLEROSIS

A lot of progress has been made from the 1990s after the introduction of the Interferons as the first disease modifying therapy for multiple sclerosis (IFNB Multiple Sclerosis Study Group, 1993) (Paty and Li, 1993). There are an increasing number of treatments for relapsing-remitting multiple sclerosis. However there are also no drugs that have been shown to be significantly neuroprotective in multiple sclerosis and therapies targeting remyelination of the central nervous system are still in their infancy.

Drugs such as the Interferons and Glatiramer Acetate (Johnson et al., 1995) tend to reduce relapses by approximately 30% with a very small effect on long-term disability (Palace et al., 2015) (Ebers et al., 2010). They have a relatively benign side effect profile when compared to the other disease modifying multiple sclerosis drugs. Side effects include flu-like symptoms, neutropenia, liver dysfunction and anti-drug antibodies (Interferons). Both drugs can give skin injection site reactions.

Teriflunomide (O'Connor et al., 2011) reduces the annualized relapse rate by approximately 30% when compared to placebo with a modest effect on disability in a similar way to the Interferons and Glatiramer Acetate. It interferes with pyrimidine synthesis by inhibiting the enzyme dihydroorotate dehydrogenase (DHODH) (Claussen and Korn, 2012). This effects rapidly dividing lymphocytes but spares more quiescent or homeostatically expanding lymphocytes which use a different pathway to DHODH. It has the advantage that it is given in a tablet form but patients have to undergo regular monitoring of blood counts and liver function. It is also unsafe in pregnancy with a risk of teratogenicity (Beart et al., 2017).

Dimethyl Fumarate (BG-12) (Gold et al., 2012) (Fox et al., 2012) reduced relapse rates by approximately 53% and MRI lesions by 90% when compared to placebo. In one phase 3 trial there was a 38% reduction in disability compared to placebo (but no effect compared to copaxone) (Gold et al., 2012). The actual mechanism of action is unknown but it is purported to have effects on oxidant pathways and it also affects nuclear-kappa-light-chain-enhancer of activated B cells (NF-κB) (Albrecht et al., 2012) (Scannevin et al., 2012). It again has the advantage of being an oral tablet and has a modest side effect profile of flushing and gastrointestinal side effects, with a drop in the lymphocyte count and elevated liver aminotransferase levels.

Daclizumab (Kappos et al., 2015) is a monoclonal antibody, which acts against CD25, the interleukin-2 receptor alpha chain (IL-2Rα), which activates T cells. Its mechanism of action also involves expansion of a natural killer (NK) cell regulatory population (Bielekova et al., 2006). When compared to placebo it reduced relapse rates by 54% with the risk of 3 month sustained disability reduced by 57% when compared to placebo (Gold et al., 2013). In a phase 2 add-on treatment trial with interferon-beta, the reduction in relapse rate was 30% when compared with interferon-beta (Wynn et al., 2010). This is a parenteral treatment (subcutaneous injections given each month) with side effects of an increase in infections, eczematous rashes and abnormalities in liver function testing. Recently the concern over 'unpredictable and potentially fatal immune-mediated' liver injury has led to restrictions on the use of daclizumab in multiple sclerosis. It has now been recommended for relapsing-remitting multiple sclerosis patients who have 'failed at least two disease modifying therapies and unable to be treated with other therapies'. These recommendations due to the risk of liver damage were made by the European Medicines Agency Pharmacovigilance Risk Assessment Committee, following a review of safety of daclizumab following the death of a patient from liver failure and the occurrence of four other cases of serious liver injury (European Medicines Agency, 2017). Daclizumab has now been withdrawn from the market due to these safety concerns.

Natalizumab (Polman et al., 2006) reduces relapses by approximately 68% with a significant reduction in disability of 42% at 2 years post treatment, when compared against placebo. It is a monthly infusion, which works by blocking the α4β1 integrin receptor (molecules found on the surface of lymphocytes and monocytes) from combining to vascular cell adhesion molecule-1 (VCAM-1), which is a mechanism that enables lymphocytes to cross the blood-brain-barrier and enter the central nervous system. The side effect profile of natalizumab includes the very rare but very serious Progressive Multifocal Leucoencephalopathy (PML) (Kleinschmidt-DeMasters and Tyler, 2005) (Langer-Gould et al., 2005). Natalizumab associated PML will be discussed in more detail later in this chapter.

Fingolimod (Kappos et al., 2010) (Cohen et al., 2010) reduces relapses by 52% when compared against placebo and is a once daily tablet medication, which acts against sphingosophine receptors. This prevents the egress of lymphocytes from lymph nodes, thus preventing the migration of lymphocytes into the central nervous system. There have been case reports of PML in patients who have received

fingolimod who had previously not received natalizumab (Food and Drug Administration, 2013). This is discussed in more detail later in this chapter. There is also a risk of opportunistic infections particularly with Herpes Zoster Virus, cardiac conduction defects and macular oedema.

Ocrelizumab is a fully humanized anti-CD20+ monoclonal antibody. It is purported to have greater antibody dependent cell cytotoxicity and less complement mediated cytotoxicity than rituximab (Beart et al., 2017). It is a chimeric anti-CD20+ monoclonal antibody, which had previously shown promise in reducing MRI activity in multiple sclerosis in phase 2 trials (Hauser et al., 2008). Ocrelizumab reduces relapses by at least 46% when compared to Interferon-β1a. It also showed a significant reduction in disability of up to 43% compared to Interferon-β1a over a period of 24 weeks. Importantly it is given as an intravenous infusion every 6 months with infusion related reactions its most common side effect. The side effect profile was benign and similar to the Interferon-β1a group (Fernandez et al., 2016). However there was a slight signal of an increased number of malignancies in the treatment versus the placebo group (2.9% vs 0.8%). Ocrelizumab also showed a 24% reduction in progression of disability and a 29% reduction in walking time in primary progressive multiple sclerosis (Montalban et al., 2016). Significant reductions in T2 brain lesions and whole brain volume loss were also observed (Montalban et al., 2017).

Alemtuzumab was first used in multiple sclerosis in 1991. It is a monoclonal antibody, which is directed against CD52, a protein of unknown function on lymphocytes. Alemtuzumab causes a lymphopenia, following which homeostatic reconstitution leads to prolonged alteration of the immune repertoire. This reduces the risk of relapse and disability accumulation in multiple sclerosis (Kousin-Ezewu and Coles, 2013). Alemtuzumab reduces relapses between 55% (Cohen et al., 2012) and 69% (Coles et al., 2012a), with significant effects on disability when compared to Interferon-β1a. It is a once yearly infusion given for two years and then as needed. There have been no reports of PML when alemtuzumab has been used alone, but it does have a significant side effect profile with the later emergence of other autoimmune diseases such as thyroid disease and immune thrombocytopenic purpura, which will be discussed later in the chapter.

Hematopoietic stem cell transplantation (HSCT) has been used in patients with aggressive forms of multiple sclerosis (Saccardi et al., 2012). The theory behind its use is similar to alemtuzumab, as the aim is to reset the immune system by first

suppressing the mature immune system through the use of a conditioning regimen (which often includes alemtuzumab) and then rebooting the immune system, hopefully free from aberrant immune responses to myelin, with the aid of autologous hematopoietic stem cells (Abrahamsson et al., 2013). The efficacy of HSCT has been good with the proportion of patients with no evidence of disease activity (NEDA) after 2 years being 78% in the HALT-MS study (Nash et al., 2015) with continuing high proportions maintained after a longer follow-up period (65-70% after 5 years) from a combination of studies (Burman et al., 2014) (Burt et al., 2014). These are remarkable figures considering the median EDSS for patients in these studies ranged between 4 and 5.5. However it has thus far been reserved for patients with either secondary progressive multiple sclerosis or aggressive relapsing-remitting multiple sclerosis as there is a mortality rate of 1.3% with HSCT (Mancardi and Saccardi, 2008). Approximately 10% of patients may also develop secondary autoimmune diseases following HSCT (Bakhuraysah et al., 2016).

1.10.1 LESSONS FROM EFFECTIVE TREATMENT

Although important pre-clinical studies have shed light and offered possible explanations for mechanisms of the pathogenesis of multiple sclerosis, testing pathways with drugs that either block or activate a particular pathway in a large number of specially selected patients is a fundamental way of testing hypotheses and learning about the mechanisms of disease.

1.10.1.1 NATALIZUMAB – FIRST ACTIVATION OF T CELLS IS IN THE PERIPHERY

For some time it was postulated that the first ‘event’ in the pathogenesis of multiple sclerosis was the activation of lymphocytes in the periphery, which then entered the central nervous system. With the efficacy shown by natalizumab in multiple sclerosis, this underlined the principle of immune cells being activated in the periphery before relocating to the central nervous system as a key event in multiple sclerosis pathogenesis, prior to the inflammatory effects on myelin in the central nervous system (Jones and Coles, 2010).

1.10.1.2 ANTI-CD20 – B CELL INVOLVEMENT

The seminal paper showing the reduction of MRI lesions in multiple sclerosis patients with the use of rituximab has underlined the importance of B cells in the pathogenesis of multiple sclerosis (Hauser et al., 2008). It is still unknown which function of B cells has the most important role. This has previously been discussed in section 1.9.2.

1.10.1.3 ALEMTUZUMAB – IMPROVED OUTCOMES FROM EARLIER TREATMENT

Induction therapies in multiple sclerosis such as Alemtuzumab and HSCT tend to have better outcomes if treatment is commenced earlier in the disease course rather than later once irreversible cerebral atrophy has begun.

This was discovered after the initial trials of Alemtuzumab in multiple sclerosis patients in 1991. There was approximately a 90% reduction in the amount of MRI lesions for at least 18 months after a single pulse of treatment (Coles et al., 1999). Unfortunately, this did not lead to a clinical improvement in the disability in these patients. In fact, their disability worsened with time at a rate of 0.02 EDSS points for each patient each year (Kousin-Ezewu and Coles, 2013).

The first use of alemtuzumab in relapsing–remitting multiple sclerosis was in an open-label pilot study of 22 patients. These patients had disease that had failed to respond to standard disease-modifying therapy or they had a high relapse rate early in the course of the disease, indicating a poor prognosis. Disease duration had a mean of 2.7 years in this patient group, with an annualized mean relapse rate of 2.21 per year (with an annualized relapse rate of 2.94 in the year prior to treatment). In the year before treatment their EDSS score had increased by a mean of 2.2 EDSS points (range 0–7.5). After alemtuzumab, there was a reduction in relapse rate by 91%. Mean EDSS scores fell by 1.4 points in this patient group, with 16/22 patients having had an improvement in their disability by 1 year (Coles et al., 2006).

From this early experience of the contrasting effects of alemtuzumab on progressive and early relapsing–remitting multiple sclerosis, it was concluded that there is a ‘window of opportunity’ early in the disease course, before there is fixed disability or

secondary progression, when inflammation is the dominant process driving multiple sclerosis.

1.10.2 PROBLEMS WITH EFFECTIVE TREATMENTS – NATALIZUMAB AND ALEMTUZUMAB

There have been major developments over the last 20 years in the armamentarium of drugs that physicians can use to treat multiple sclerosis. Unfortunately there has been a trend that those drugs with the greatest efficacy tend to have the greatest risk of harmful effects. Detailed below are some of the major harmful effects of treatments used in multiple sclerosis.

1.10.2.1 PROGRESSIVE MULTIFOCAL LEUCOENCEPHALOPATHY

Although natalizumab leads to infusion reactions, it was otherwise thought to have a benign side effect profile. However there began to be cases of PML associated with the drug which led to the withdrawal of the drug for multiple sclerosis in 2004, before it was reinstated in 2006 with a safety management program (Traynor, 2006).

Subsequent risk factors for PML associated with natalizumab were identified such as previous immunosuppressant use, JC virus seropositivity and use of natalizumab for greater than 24 months.

The risk of individuals who were not previously exposed to prior immunosuppressant use was clarified further with those seropositive for JC virus but with titres < 0.9 carrying a small risk for PML of 1 in 10000 compared to those with titres >1.5 who appear to be at a much higher risk of developing PML with a risk of 1 in 1000, within the first 24 months of treatment. The cumulative risk of PML increases over time and one group was keen to stress from month 24 to month 25 the risk does not dramatically change (Mowry and McArthur, 2017). However with a titre >1.5 after 60 months of treatment with natalizumab the risk is 1 in 100 (Plavina et al., 2014).

	MONTHS OF TREATMENT					
JCV STATUS	1-12	13-24	25-36	37-48	49-60	>60
NEGATIVE	1 in 10000	1 in 10000	1 in 10000	1 in 10000	1 in 10000	1 in 10000
< 0.9	1 in 10000	1 in 10000	1 in 5000	1 in 2500	1 in 2000	1 in 1667
0.9 – 1.5	1 in 10000	1 in 3333	1 in 1250	1 in 500	1 in 500	1 in 333
> 1.5	1 in 5000	1 in 1111	1 in 333	1 in 143	1 in 125	1 in 100

Table 1. 2: the risk of PML with natalizumab and how this changes over time

Some would point to the rather benign side effect profile of ocrelizumab as a potential candidate for an effective drug that goes against the trend, but looking at rituximab, another anti-CD20+ agent, a known side effect is PML. In February 2006, 9 years after rituximab received its initial Food and Drug Administration approval, the labeling for rituximab was changed after the discovery of increased incidence of viral infections post treatment. These viruses included Hepatitis B, Cytomegalovirus, varicella zoster virus, West Nile virus and JC virus (Steurer et al., 2003). As with natalizumab, PML associated with rituximab is seen in previously immunocompromised patients. This was reviewed in 2009 in 57 patients, following a literature search covering the period of patients treated with rituximab from 1997 to December 2008. All of these patients had received concomitant or prior immunosuppression, including alkylating agents, corticosteroids, purine analogs or drugs to prevent allogeneic stem cell or solid organ graft rejection. The median time from the last rituximab dose to PML diagnosis was 5.5 months with median time to death 2.0 months after PML diagnosis. In this series of patients the case fatality rate was 90% (Carson et al., 2009).

Estimates vary about the incidence of rituximab associated PML. One group found a 1 in 4000 incidence among 8000 rituximab-treated SLE cases (Kavanaugh and Matteson, 2008). However the incidence is expected to be much lower than this with no cases of PML found in an observational retrospective study in Sweden in 822 cases (Salzer et al., 2016). Unfortunately this study had a mean follow-up time of 14

months and we know from experience with natalizumab, PML incidence increases when treatment is continued for greater than 2 years. Other authors quote a lower incidence of PML associated with rituximab with one set of authors quoting 1 in 32000 patients (Bohra et al., 2017). This figure was determined from a study in 129000 rheumatoid arthritis treated patients, of whom 4 contracted PML (Clifford et al., 2011). The cause of these fluctuations in the estimated incidence of rituximab associated PML is due to the fact that these patient populations are normally immunosuppressed with other agents which have also been associated with PML, therefore causality and true risk are difficult to determine (Castillo-Trivino et al., 2013).

Thus far there has not been a single case of rituximab associated PML in a multiple sclerosis patient. However with the more prolonged use of anti-CD20 monoclonal antibodies that will occur in the future, continued vigilance and reporting of this serious complication will be necessary.

With dimethyl fumurate there have been several cases of PML in patients taking fumaric acid esters for multiple sclerosis and psoriasis (van Oosten et al., 2013) (Ermis et al., 2013). If lymphocyte levels drop for a prolonged period (with counts below 0.5×10^9 cells/L said to be a particular risk factor) then there is an increased risk of PML. The guidelines for physicians prescribing dimethyl fumarate indicate to stop the drug if lymphocytes fall below the level of 0.5×10^9 cells/L.

Fingolimod has a risk of PML of less than 1 in 10000, following the most recent report by the drug company that are overseeing the post marketing development of this drug. There is now a periodic update from Novartis on PML associated with Fingolimod. The latest update from May 2017 showed that there had been 13 cases of PML (out of the >213000 patients receiving fingolimod) who had not previously received Natalizumab. This represents a less than 1 in 10000 risk (Giovannoni, 2017).

1.10.2.2 SECONDARY AUTOIMMUNITY WITH ALEMTUZUMAB

From early on in the development of alemtuzumab for multiple sclerosis there was an acknowledgement that a third of patients tended to develop secondary autoimmunity while their immune system was reconstituting.

20-30% of treated patients develop thyroid autoimmunity. 1% of treated patients develop immune thrombocytopenic purpura with the index case suffering a fatal brain haemorrhage. Subsequently patients were educated about the clinical signs of bleeding and regular blood counts were taken. There have been a few cases of Goodpasture's disease with a small number of cases needing renal transplantation due to renal failure. There was also a case of Castleman's disease which was successfully put into remission with R-CHOP and there was another case of a non-EBV associated Burkitt's lymphoma which resulted in the death of a patient. There have also been single cases of autoimmune neutropenia and autoimmune haemolytic anaemia (Coles, 2013).

Autoimmunity as a side effect of lymphocyte reconstitution has been recognised before in HIV and bone marrow transplantation (Gilquin et al., 1998) (Hsiao et al., 2001). Multiple sclerosis patients and physicians have to weigh up the risks of secondary autoimmunity, particularly keeping in mind some of the disastrous outcomes from some of these autoimmune events, when deciding on beginning a patient on alemtuzumab. Due to its side effect profile, it has been recommended to start alemtuzumab only in patients with active disease, although the licence it has received is actually much wider than this (Scolding et al., 2015) and is for 'active multiple sclerosis defined clinically or radiologically'. Alemtuzumab can be used as an 'induction' agent in newly diagnosed highly active patients or as an escalation strategy, with patients still experiencing active disease whilst on other disease modifying therapies.

1.10.3 FUTURE CHALLENGES FOR MULTIPLE SCLEROSIS DRUG DEVELOPMENT

Although the multiple sclerosis treatment spectrum has broadened, those drugs with the highest efficacy still present the greatest risk to patients in terms of side effects. Therefore difficult decisions are made between neurologists and multiple sclerosis patients when deciding which disease-modifying agent to use.

One response to this situation is to develop novel therapies, which are more targeted to the underlying disease pathogenesis. In the third chapter I describe the experience

of one such approach: a trial of a novel monoclonal antibody against the Interleukin 7 (IL-7) receptor.

1.11 INTRODUCTION TO THE IL-7 PATHWAY AND ITS ROLE IN MULTIPLE SCLEROSIS – THE NORMAL BIOLOGY OF IL-7 AND IL-7Rα

1.11.1 INTERLEUKIN-7

Interleukin 7 (IL-7) is a 25 kilodalton protein cytokine. It is released mainly by stromal cells in the thymus and bone marrow, but also by platelets, intestinal epithelium, keratinocytes and dendritic cells (Sarah C. Sasson, 2006). IL-7 was first described as a murine pro-B-cell growth factor (Namen et al., 1988) and has subsequently been shown to be fundamental to the generation of new T lymphocytes and the maintenance of T lymphocytes. IL-7 is thus responsible for the homeostasis of T lymphocytes by increasing their proliferation and maturation via the thymus (Mackall et al., 2011).

In the thymus the earliest stem cells require IL-7 for proliferation, survival and T cell receptor (TCR) gene rearrangement. IL-7 is also involved in the positive selection of CD8+ cells (Schluns et al., 2000). IL-7Rα forms a heterodimer with the thymic stromal lymphopoietin receptor (TSLPR) to form a receptor, which recognizes thymic stromal lymphopoietin (TSLP), which helps to develop immature T and B cells, dendritic cells and monocytes (Soumelis et al., 2002) (Pandey et al., 2000).

TSLP has been shown to act in the thymus particularly with the positive selection of CD4+ T regulatory cells. It also stimulates CD4+ homeostatic expansion in the periphery. However mice deficient in TSLPR have normal B and T cell development, indicating that although there may be some involvement of TSLP in lymphocyte development it is not necessary for normal lymphopoiesis. In disease it has been associated with allergic inflammation in atopic dermatitis with TSLP acting on dendritic cells, which migrate to lymph nodes that prime CD4+ T cells to produceTh2 cytokines (Ziegler and Liu, 2006).

IL-7 promotes the proliferation of T cells by engaging with its receptor and activating the JAK-STAT pathway. Phosphorylated STAT5 transfers to the nucleus and acts as a transcription factor in order to promote the anti-apoptotic molecules within the B

cell Lymphoma 2 (bcl-2) family of proteins (bcl-2 and mcl-1). At the same time it inhibits the pro-apoptotic members of the bcl-2 family (BAX and BAK) (Mackall et al., 2011). The IL-7 pathway also downregulates CD95 (Fas ligand receptor), which is involved in the extrinsic pathway of programmed cell death (Lundstrom et al., 2013).

IL-7 is a limited resource in vivo (Guimond et al., 2009) (Park et al., 2004), and in states of lymphopenia IL-7 levels increase, as less IL-7 is consumed (Cox et al., 2005). There is a strong inverse correlation between IL-7 levels and CD4+ T cell numbers as the amount of IL-7 regulates the number of T lymphocytes in the periphery – if numbers of T lymphocytes are reduced, then excess IL-7 augments proliferation, if there are too many lymphocytes then there is insufficient IL-7 to support the number of T lymphocytes and they die (Jiang et al., 2005). Levels of IL-7 are normally between 2 to 8 pg/ml but in a lymphopenic setting can be as high as 60 pg/ml (Lundstrom et al., 2012). IL-7 production is actually reduced during lymphopenia due to a negative feedback loop mediated by IL-7Rα on stromal cells. Therefore elevation in IL-7 during lymphopenia is due to a lack of uptake from cells (Guimond et al., 2009).

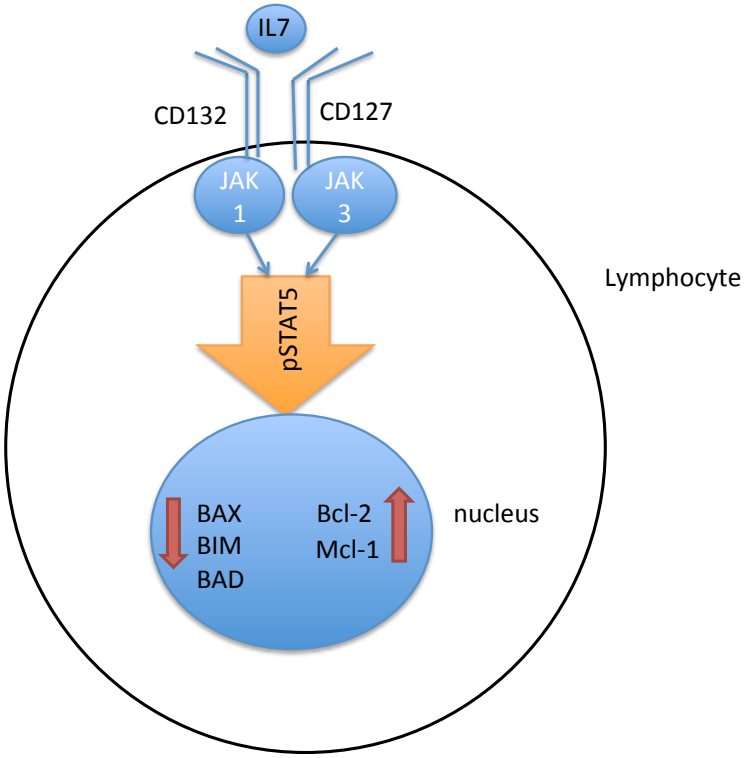

Figure 1. 2: The IL-7/IL-7R pathway

The IL-7 receptor is a heterodimer made up of IL-7Rα (CD127) and the common gamma chain receptor for cytokines (CD132). Signaling of IL-7 through its receptor activates downstream signaling mainly through the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway (particularly STAT5). Through activation of these downstream signaling pathways there is mediation of anti-apoptotic and proliferative signals through modulation of transcription factors within the Bcl-2 family.

1.11.2 INTERLEUKIN-7 RECEPTOR ALPHA

IL-7Rα is expressed abundantly on naive and memory T cells and to a much lesser extent on naturally occurring T regulatory cells and its expression is down regulated on activation (Park et al., 2004). IL-7Rα is expressed continuously on most resting T cells. This expression is different to that of the normal gamma chain family of cytokine receptors, which tend to be increased on stimulation of T cells through the T cell receptor. IL-7 is released by stromal cells continuously in picomolar concentrations therefore T cells are continuously exposed to IL-7 sufficiently to induce signaling through IL-7Rα (Lundstrom et al., 2012).

Soluble IL-7Rα is secreted by fibroblasts and activated CD4+ T lymphocytes (Badot et al., 2011). It is produced by alternative splicing of the full-length transcript, with the difference between the two isoforms being the soluble isoform is without exon 6 (Goodwin and Namen, 1989) (Pleiman et al., 1991). One study has found increased amounts of soluble IL-7Rα induced by the addition of pro-inflammatory cytokines TNF-α and IL-17 (Badot et al., 2011). The biological function of soluble IL-7Rα has been heavily debated and this is discussed in more detail below.

IL-7Rα is not expressed on mature B cells. However it is expressed on common lymphoid progenitors and on pre-B cells. There is then a down regulation on pro-B cells before a complete loss of the IL-7Rα on mature B cells. Infants with mutations in IL-7Rα with SCID tend to have circulating B cells. This has led to some authors hypothesising that IL-7 has no direct role in production of B cells. However in a phase one trial where recombinant IL-7 (rhIL-7) was given as a treatment there was an increase in immature and transitional B cells (Sportes et al., 2010). There are also increased levels of immature/transitional B cells in HIV infection in the peripheral blood (Malaspina et al., 2006). Development of B cells independent of IL-7 *ex-vivo* has been achieved using umbilical cord blood but not with progenitors from adult bone marrow, suggesting there is some dependence on IL-7 for generation of B cells as we age (Lundstrom et al., 2012).

IL-7 binding to IL-7Rα induces activation of the JAK-STAT pathway. IL-7Rα and the common gamma chains are associated with Janus Kinase 1 (JAK1) and JAK3 (Suzuki et al., 2000). Once IL-7 engages with its receptor, JAK1 and JAK3 phosphorylate each other. Once JAK3 is phosphorylated it can then bind to tyrosine

residue 449 of IL-7Rα. Tyrosine residue 449 specifically recruits the transcription factor Signal Transducer and Activator of Transcription 5 (STAT5). STAT5 is a heterodimer of STAT5a and STAT5b and once bound STAT5 is phosphorylated by JAK1 and JAK3 (Foxwell et al., 1995). As featured in figure 1.2, the phosphorylated STAT5 transfers to the nucleus where it controls the expression of anti-apoptotic target genes from the B Cell Lymphoma 2 family of proteins, particularly Bcl-2 and Mcl-1, which inhibit the mitochondrial apoptotic pathway, promoting survival and proliferation of T cells (Mackall et al., 2011).

At high concentrations of IL-7, binding its receptor also induces Phosphoinositide-3 Kinase (PI3K) activation demonstrated by phosphorylation of its downstream target AKT (Palmer et al., 2011), which may be responsible for promoting T cell proliferation (as opposed to simply survival in the JAK/STAT system) in a lymphopenic environment.

Humans can develop severe combined immunodeficiency (SCID) with a loss of function mutation in IL-7Rα. SCID can also develop if there are loss of function mutations in JAK3 and IL-2Rγ (the common gamma chain receptor - CD132). The syndrome of SCID is characterised by humans presenting in infancy with opportunistic infection, rash, diarrhoea and failure to thrive. The immunodeficiency does vary depending on the type of mutation. With IL-7Rα there is a lack of T cells but B cells and natural killer cells are still present. With mutations in JAK3 and CD132 B cells are present but there is also a deficiency in natural killer cells as well as T cells due to the lack of IL-15 with these mutations. SCID is normally a fatal condition without treatment, which consists of bone marrow replacement or gene replacement therapy (Lundstrom et al., 2012).

1.11.3 REGULATION OF IL-7Rα

Expression of IL-7Rα on T cells controls the sensitivity of the cells to IL-7. IL-7 is limited in vivo and therefore IL-7 consumption is controlled by IL-7Rα downregulation in order to maximise the number of cells that can benefit from IL-7 support (Park et al., 2004).

Downregulation of IL-7Rα is regulated at a transcriptional and a post-translational level. IL-7Rα is constantly recycled, being endocytosed into the cell via clathrin

coated pits with the majority of the IL-7Rα not being degraded via lysosomal pathways when there is little IL-7 available; however in higher IL-7 concentrations, there is greater lysosomal degradation than recycling of IL-7Rα leading to IL-7Rα downregulation. This increased degradation depends on JAK3 activity on the IL-7Rα chain of the IL-7 receptor (Henriques et al., 2010). When there is a lack of IL-7 signaling, IL-7Rα expression at the cell surface is maintained by Ephrin molecules efnb1 and efnb2, which interact directly with IL-7Rα and stabilise it on the cell membrane. Recycling of IL-7Rα is also dependent on the class 3 PI3K Vsp34. Mice with Vsp34 deficiency have decreased T cell numbers in the thymus and the periphery. Vsp34 deficient naive T cells also have a reduction in the cell surface expression of IL-7Rα, despite having normal total levels of IL-7Rα (McLeod et al., 2011).

Regulation of IL-7Rα expression also occurs at the transcriptional level. Gfi-1 acts as a transcriptional repressor of IL-7Rα and downregulates new protein synthesis in CD8+ T cells after IL-7 stimulation (Park et al., 2004). In memory CD8+ cells with high expression of IL-7Rα, GABPα, a transcription factor from the Ets family, has been shown to promote IL-7Rα expression by increasing histone acetylation on the IL-7Rα promoter. By contrast Gfi-1 acts by antagonising GABPα's binding and recruiting of histone deacetylase 1, which deacetylates the IL-7Rα promoter. Therefore reciprocal binding of Gfi-1 and GABPα provides the mechanism for IL-7Rα promoter acetylation and activity, which leads to expression of IL-7Rα on the cell surface (Chandele et al., 2008). Ets-1, belonging to the Ets family of transcription factors, has been shown to maintain IL-7Rα expression on CD4+ and CD8+ cells (Grenningloh et al., 2011). Deficiency in Ets-1 leads to reduced numbers of T cells *in vivo* (Clements et al., 2006).

1.12 THE IL-7 PATHWAY AND MULTIPLE SCLEROSIS

The evidence for the IL-7 pathway and its role in the pathogenesis of multiple sclerosis began in the late 1990s through studies investigating the effect of IL-7 on human leukocyte responses to candidate myelin auto antigens (Bielekova et al., 1999). In 2001, IL-7 enhanced the T cell proliferation response to myelin basic protein (MBP) in patients with multiple sclerosis, suggesting higher activity of myelin specific T cells in multiple sclerosis patients compared to healthy controls (Traggiai et al., 2001). These results were replicated in 2004 (Lunemann et al., 2004). Later

evidence emerged that there was increased IL-7 in multiple sclerosis lesions (Kremlev et al., 2008) (Jana et al., 2014).

Later genetic evidence for the IL-7 pathway in the pathogenesis of multiple sclerosis began to emerge, first through linkage analysis, then the discovery of single nucleotide polymorphisms in IL-7Rα, and finally the confirmation of these genetic variations in the genome wide association studies in multiple sclerosis and the Immunochip fine mapping analysis. The details of these discoveries will be covered in more detail below, particularly where controversy still exists regarding the functional consequences of the genetic variations discovered, particularly involving the role of soluble IL-7Rα.

Finally with more interest in the IL-7 pathway following the genetic association with multiple sclerosis, more evidence began to emerge in *in vivo* models with EAE mice. The first experiments on IL-7 null mice demonstrated reduced severity of EAE (Ashbaugh et al., 2013). Others showed increased IL-7 in the neuroinflammatory environment at the onset and peak of EAE (Arbelaez et al., 2015). There have also been specific effects demonstrated on differentiation of CD4+ cells with effects discovered on both Th1 and Th17 cells (Liu et al., 2010) (Lee et al., 2011) (Arbelaez et al., 2015). Amelioration of EAE was also demonstrated with the use of antibodies antagonistic to IL-7Rα (Liu et al., 2010) (Lee et al., 2011) (Ashbaugh et al., 2013).

Taken together there is a lot of evidence for a significant role of the IL-7 pathway in multiple sclerosis pathogenesis. This has encouraged pharmaceutical companies such as GlaxoSmithKline to become interested in developing therapeutics, which target this pathway.

1.12.1 IDENTIFICATION BY GENE LINKAGE OF THE REGION AT 5p12-14

Outside of HLA, early multiple sclerosis genome screens did not identify MS susceptibility loci but multiple chromosomal regions showing suggestive linkage was observed. This suggested that many genes exerting modest effects might determine MS susceptibility.

One of the multiple chromosomal regions showing suggestive linkage was 5p12-14 which was first identified in 1996 in a Canadian MS genome screen (Ebers et al., 1996), and then subsequently confirmed in a Scandinavian study of chromosome 5p markers (Oturai et al., 1999).

1.12.2 FIRST IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN IL-7Rα

An Australian group decided to investigate IL-7Rα based on its location following previous studies associating this location with linkage in multiple sclerosis. Knowledge of the function of IL-7Rα and its role in T and B cell proliferation and homeostasis also alerted the authors to the potential for IL-7Rα being a candidate gene. Thirteen single nucleotide polymorphisms associated with multiple sclerosis were identified from 728 subjects (Teutsch et al., 2003).

1.12.3 IDENTIFICATION OF THE FUNCTIONAL CONSEQUENCES OF DIFFERING GENETIC POLYMORPHISMS OF IL-7Rα

The first functional analysis of IL-7Rα SNPs was in 2007 by Gregory and colleagues (Gregory et al., 2007). This paper confirmed the association of the SNP rs6897932 with multiple sclerosis, and showed its functional effect. The high-risk allele for rs6897932 encodes for the amino acid Threonine at residue 244 on exon 6 of the extracellular domain of IL-7Rα at the border of the transmembrane region of the protein. The low risk allele encodes Isoleucine at residue 244.

By splicing analysis of IL-7Rα in a minigene construct, the authors demonstrated that the at risk allele led to an increase in frequency of skipping exon 6 during transcription, resulting in the transcript lacking the transmembrane region of IL-7Rα and thus increased concentrations of the soluble isoform of the IL-7Rα protein. Later studies confirmed greater amounts of the soluble form of IL-7Rα being secreted due to this SNP (Hoe et al., 2010) (Lundstrom et al., 2013).

The SNP rs6897932 was confirmed as the candidate SNP in the multiple sclerosis Genome Wide Association Study (GWAS) in 2011 (International Multiple Sclerosis Genetics et al., 2011). In the subsequent Immunochip paper when these SNPs underwent more detailed fine mapping, a new intergenic SNP (rs6881706) was

identified that lies next to the gene for IL-7Rα. Although this new SNP had not been previously described it remains in complete linkage disequilibrium (r2=0.99) with the previous SNP rs6897932, which was acting as the signal to this new SNP (International Multiple Sclerosis Genetics et al., 2013).

1.12.4 SOLUBLE IL-7Rα COMPETITIVELY INHIBITS ACTIVATION THROUGH IL-7Rα – CRAWLEY ET AL 2010

The work in this paper is the first to demonstrate the potential bioactivity of soluble IL-7Rα in humans. It did this in a variety of ways focusing on short-term culture experiments. There were no *in vivo* studies in this paper and the authors acknowledged the role of soluble IL-7Rα *in vivo* was still yet to be established.

The authors showed that native (through collecting supernatant from cells cultured from a bronchial cell line known to secrete IL-7Rα) and to a lesser extent, recombinant sources of soluble IL-7Rα inhibited signaling through IL-7Rα. IL-7 mediated proliferation and bcl-2 expression was also reduced by soluble IL-7Rα. The authors were able to directly pinpoint soluble IL-7Rα by depleting the molecule from a culture containing IL-7 and T cells. 10/13 samples showed anti-IL7 activity. This effect was reversed after depletion. However in the samples that did not show anti-IL-7 activity there was no effect on pSTAT5 after depleting soluble IL-7Rα.

1.12.5 SOLUBLE IL-7Rα POTENTIATES IL-7 BIOACTIVITY - LUNDSTROM ET AL 2013

The work in this paper (Lundstrom et al., 2013) demonstrated the increased bioactivity through the IL-7Rα pathway in individuals with the predisposing IL-7Rα genotype (those individuals with greater levels of soluble IL-7Rα), providing a basis for explaining the increased levels of autoimmunity seen within these individuals.

In an IL-7 dependent murine cell line (2E8) soluble IL-7Rα in combination with IL-7, enhanced IL-7 induced survival of cells and also diminished IL-7 consumption, evidenced by greater levels of IL-7 in the culture.

In vitro experiments on human T cells showed potentiation of the signaling through membrane-bound IL-7Rα in cultures containing soluble IL-7Rα and IL-7. This was

evidenced by increased levels of pSTAT5. There were also effects on negative regulators of cell proliferation evidenced by diminished upregulation of CD95 (Fas ligand receptor) and SOCS-1 (suppressor of cytokine signaling 1). Increased IL-7 in the cultures with soluble IL-7Rα again emphasised the increased bioavailability and decreased consumption of IL-7 in the presence of soluble IL-7Rα.

In vivo potentiation of IL-7 bioactivity was demonstrated first in IL-7 null mice when recombinant IL-7 and soluble IL-7Rα were injected and greater homeostatic expansion of lymphocytes was observed when compared to injecting with either IL-7 or soluble IL-7Rα alone. Second, C57/BL6 EAE mice showed significant worsening of their disease when injected with both soluble IL-7Rα and IL-7 than when compared to injection with IL-7 alone (which itself has been shown to overcome immune tolerance and has been shown to worsen EAE). Taken together, this was the first *in vivo* evidence that soluble IL-7Rα potentiated IL-7 mediated autoimmune disease.

This paper confirmed previous work of demonstrating increased levels of soluble IL-7Rα in individuals CC homozygous for the at risk genotype at rs6897932. In fact the paper demonstrated a dose allele effect with those CC homozygous (mean 64 ng/ml) demonstrating a threefold increase in soluble IL-7Rα levels over the protective homozygous TT genotype (mean 16 ng/ml), with the heterozygotes having intermediate levels of soluble IL-7Rα (mean 32 ng/ml). The authors also demonstrated increased IL-7 levels in multiple sclerosis patients with the CC homozygous at risk genotype when compared to the other genotypes. This would provide further evidence for the model of increased bioactivity over time of IL-7 due to reduced consumption in the presence of increased levels of soluble IL-7Rα. Interestingly this difference was only found in subjects with multiple sclerosis rather than healthy controls.

1.12.6 SOLUBLE IL-7Rα POTENTIATES IL-7 BIOACTIVITY – COTE ET AL 2015

The Crawley group brought out a second paper looking at the function of soluble IL-7Rα which somewhat contradicted their first paper. When human CD8+ cells were cultured over 7 days with soluble IL-7Rα and IL-7 there was an increase in the proliferation of CD8+ cells when compared to IL-7 alone.

The authors also looked at the increase in the number of IFNγ producing cells and commented that this represented an increase in function of these cells potentiated by the addition of soluble IL-7Rα to IL-7, compared to IL-7 used alone. The authors also used murine CD8+ cells to measure proliferation with IL-7 preincubated with soluble IL-7Rα compared to IL-7 without this preincubation step. They demonstrated that higher ratios of soluble IL-7Rα to IL-7 (10:1 compared to 1000:1) resulted in greater proliferation and survival of murine CD8+ cells. This excess of soluble IL-7Rα is more akin to the ratios of soluble IL-7Rα and IL-7 found in vivo.

These results contrast with the previous results by the same group in 2010 when they suggested the opposite effect from recombinant soluble IL-7Rα. This paper confirmed the finding from Lundstrom et al in 2013 about the agonistic overall effects of soluble IL-7Rα. In contrast to the Lundstrom paper they found a dose response effect. Therefore they suggested that soluble IL-7Rα could be used as an adjunct to IL-7 treatment in diseases where CD8+ cell function is impaired such as HIV and Hepatitis C virus infections.

1.12.7 HYPOTHESIS FOR ROLE OF IL-7/IL-7Rα/SOLUBLE IL-7Rα IN MS

My personal view on the role of soluble IL-7Rα within the IL-7/ IL-7Rα cascade is that it potentiates the effects of IL-7 over time. The arguments for this have been eloquently laid out by Lundstrom et al (Lundstrom et al., 2013) and importantly the group that had originally published the paper stating the opposite view (Crawley et al., 2010) have published work that contradicts their original view and advances the opinion that soluble IL-7Rα potentiates the effects of IL-7 (Cote et al., 2015).

It is not clear from Crawley's study in 2010 that the inhibitory properties of soluble IL-7Rα may be biologically relevant *in vivo* as it occurred in 62% (13/21) of individuals. In two individuals where this occurred depletion of soluble IL-7Rα led to a further

decrease in IL-7 activity suggesting that there are other factors involved in this pathway. It should also be noted from the example of IL-15 when initially it was thought that the soluble form of IL-15Rα was antagonistic to the IL-15 pathway and was later found to be agonistic through a different mechanism *in vivo* (Stoklasek et al., 2006) (Mortier et al., 2006).

Interestingly Crawley's study in 2010 suggested that perhaps soluble IL-7Rα could act as a carrier for IL-7 over time *in vivo*, unknowingly providing the hypothesis for the later work by Lundstrom, by effectively increasing its half-life in a similar way to IL-15 (Mortier et al., 2006). This was suggested, as despite giving excess soluble IL-7Rα, IL-7 activity was not completely halted. This was explained by the low affinity binding between soluble IL-7Rα and IL-7, compared to the relatively high affinity interaction between IL-7 and membrane-bound IL-7Rα. This was consistent with other work, which demonstrated enhanced IL-7 activity in vivo using an anti-IL-7 monoclonal antibody where there was greater T cell expansion than using IL-7 alone (Boyman et al., 2008).

This model also would fit with the functional studies on the outcome of the risk SNP in multiple sclerosis for IL-7Rα, which has been shown to increase soluble IL-7Rα levels (Gregory et al., 2007). It would be difficult otherwise to explain how the risk SNP causes its effects. Importantly the agonistic effects of soluble cytokine receptors have been demonstrated elsewhere with IL-15R (Stoklasek et al., 2006) (Mortier et al., 2006) and IL-6R (Peters et al., 1996). With the evidence that has accumulated IL-7Rα should be added to that list.

1.13 IL-7Rα ANTAGONISTS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

1.13.1 IL-7 AND IL-7Rα IN EAE

Following the success in discovery of the genetic variations in IL-7Rα associated with multiple sclerosis, a number of groups developed an interest in discovering the effects of manipulation of the IL-7Rα pathway on EAE.

One group compared IL-7 null mice with the C57BL/6 wild type mice. The IL-7 null mice showed a significant reduction in the inflammation and demyelination in the central nervous system. This was associated with a decrease in the Th1 and Th17 responses in the central nervous system and in peripheral lymphoid organs (Walline et al., 2011).

Another group pointed out some limitations in using IL-7 null mice as it severely restricted T cell development, which is normally driven by IL-7. As a result, IL-7 null mice lack the T cell repertoire necessary for normal EAE pathogenesis. This group demonstrated that in mice with IL-7Rα expression limited solely to the thymus, when compared with wild type mice, a less severe form of EAE was seen with a significant reduction in paralysis and myelin damage which correlated with decreased IFNγ and TNF production (Ashbaugh et al., 2013).

In this paper they also compared mice that had IL-7Rα restricted to hematopoietic and non-hematopoietic compartments and found that mice that had IL-7Rα restricted to both compartments were dramatically protected from EAE. Interestingly mice lacking IL-7Rα only on hematopoietic stem cells developed severe EAE pointing towards IL-7Rα expression in the non-hematopoietic compartment contributing significantly to the burden of the disease. They went on to demonstrate IL-7Rα expression on astrocytes and oligodendrocytes within the central nervous system (Ashbaugh et al., 2013).

1.13.2 ANTI-IL-7Rα IN EAE

There have been studies using the experimental autoimmune encephalomyelitis (EAE) mouse models and anti-IL-7Rα antibodies. In a paper in 2010 (Liu et al., 2010) an IL-7Rα antagonist was found to ameliorate experimental autoimmune encephalomyelitis (EAE) with a specific effect on Th17 cells, not only enabling their survival but also specifically helping Th17 cell expansion via the STAT5 pathway. This study was withdrawn 3 years after its original publication following claims of fraudulent data with in vitro human peripheral blood mononuclear cells (Liu et al., 2013).

In a paper by authors from Stanford University (Lee et al., 2011), attempting to replicate the results from the nature medicine paper discussed above by Liu et al, they examined the importance of the role of IL-7 in EAE, particularly in the generation of Th1 versus Th17 cells. They found IL-7 was able to promote the differentiation of naive T cells into Th1 cells rather than solely Th17 cells in contrast to Liu et al. In fact they did not find an effect of IL-7 on differentiation or expansion of Th17 cells. The effect of IL-7 on Th1 cells was only demonstrated *in vitro* in both analysis on mouse cells stimulated with myelin oligodendrocyte glycoprotein and in human healthy control naïve T cells. They were unable to replicate these results in the *in vivo* EAE model, where IL-7 had no effect on Th1 and Th17 differentiation.

They also showed through the use of another IL-7Rα antagonist (28G9), rather than the GSK IL-7Rα antagonist (SB/14), that several different types of EAE could be ameliorated even after the onset of paralysis at day 14. The SB/14 antibody did not significantly reduce disease severity of EAE with three injections as the 28G9 antibody had. The authors were initially confused at the difference between the two antibodies, as *in vitro* studies showed both antibodies binding to IL-7Rα inhibited phosphorylation of STAT5 in a similar dose dependent manner. However there was a difference in the antibodies' immune effector function via binding of the IgG Fc receptors. The 28G9 (rat IgG1) antibody bound more effectively to mouse Fc gamma receptors then the SB/14 (rat IgG2a) antibody. Therefore this emphasised that Fcγ receptor binding by the antibody could have a severe effect on the efficacy of the drug.

The authors in the paper then interestingly separated multiple sclerosis into Th1 driven disease and Th17 driven disease, with Th1 driven disease exhibiting higher serum levels of IL-7 (>150 pg/ml) and low levels of IL17F (< 46 pg/ml) more likely to respond to interferon beta treatment. They thought that IL-7 levels could act as a biomarker for patients with a Th1 driven form of multiple sclerosis as they had higher IL-7 levels in their serum.

My own view is that this aspect of this paper is controversial. Multiple sclerosis is a heterogeneous disease with many different immune cells contributing to the underlying pathogenesis. It is thought to be mediated by CD4+ cells which have had myelin associated antigens presented to it. This involves stimulation via the T cell receptor and co-stimulation with CD28 and B7. Following this the cytokine milieu in the microenvironment of the T cell contributes to the differentiation of a naïve CD4+ T cell into its different forms. Therefore in such a heterogeneous system it is difficult to ascribe disease causation to one type of CD4+ cell. However it is interesting to point out that in EAE there are differences in the clinical presentation between Th1 and Th17 phenotypes with the Th1 phenotype having inflammation based primarily in the spinal cord with ascending inflammation. In later stages when the Th17 cells increase in number there is infiltration of the brain parenchyma and there are clinical signs of ataxia (Stromnes et al., 2008) (Lovett-Racke et al., 2011). It has also been shown in one study that IL-7 may drive not only enhanced Th1 responses, but it also induces the plasticity of Th17 cells enabling them to convert into IFNγ producing Th17 cells. This adds further doubt to the theory of Th1 driven multiple sclerosis (Ashbaugh et al., 2013). It is difficult to draw parallels in this regard between EAE and multiple sclerosis in humans and I would disagree with the nomenclature of Th1 and Th17 disease.

Ashbaugh et al also used an IL-7Rα antagonist to ameliorate EAE. They demonstrated this by using the IL-7Rα antibody at the peak of EAE and found mice that recovered from the disease was similar in clinical scores to the disease burden in the thymic null mice they had used previously. This recovery in mice treated with IL-7Rα antagonist was demonstrated with reduced levels of demyelination in thoracic spinal cord sections taken 55 days after the inducement of EAE when compared to control mice (Ashbaugh et al., 2013).

1.14 IL-7Rα ANTAGONISM WITH GSK2618960

Following the functional analysis of IL-7Rα, GlaxoSmithKline (GSK) decided to target this pathway, as it was fundamental to the homeostasis and survival of T lymphocytes.

GSK developed GSK2618960, which is a humanised IgG1 monoclonal antibody acting specifically against the extracellular domain of the IL-7Rα chain with low affinity. The Fc portion of this antibody was disabled in order to reduce activation of antibody-dependent cell-mediated cytotoxicity (ADCC). This enabled GSK2618960 to inhibit IL-7 signaling via IL-7Rα without direct cell cytotoxicity for the period of time that GSK2618960 was bound to the cell expressing IL-7Rα.

It is important to note that it is conceivable that a monoclonal antibody blocking IL-7Rα could in theory potentiate autoimmunity in conditions such as multiple sclerosis. Potentiation of autoimmunity could occur as IL-7Rα antagonism could affect not just the membrane bound receptor but also the soluble IL-7Rα in the circulation. This soluble receptor would normally be binding with excess IL-7. If this soluble receptor was bound by the monoclonal antibody then there would be excess free IL-7 which could bind to membrane bound IL-7, leading to increased T lymphocyte proliferation and survival of autoreactive T cell clones.

Small changes in the level of IL-7 can induce large changes in lymphocyte homeostasis, as has been established either when exogenous IL-7 is given (Mackall et al., 2011) or if there are greater levels of IL-7 in a lymphopenic environment, (Cox et al., 2005). We know that those subjects carrying the multiple sclerosis at risk SNP for IL-7Rα rs6897932, have a greater ratio of soluble IL-7Rα to membrane IL-7Rα (Gregory et al., 2007) and some authors postulate that increased bioavailability of IL-7 from soluble IL-7Rα binding to IL-7, enables greater effects of IL-7 over time (Lundstrom et al., 2013).

This effect could potentially be exacerbated by blockade of membrane IL-7Rα with GSK2618960, enabling free IL-7 to bind with soluble IL-7Rα. This could put multiple sclerosis patients at risk of unexpected lymphocyte proliferation, particularly on stopping the drug, if GSK2618960 plasma levels lowered, enabling IL-7 once more to able to bind to membrane IL-7Rα.

Prompted by the data in the 2010 nature medicine paper GSK were keen to accelerate the drug development of an IL-7Rα antagonist for multiple sclerosis. This led to discussions with Alasdair Coles and the beginning of a Phase 1 first-in-human trial in healthy volunteers and multiple sclerosis patients in GSK's Clinical Trials Unit in Cambridge.

1.15 AIMS

The aim of my PhD (funded by a Wellcome-GSK translational medicine studentship) was to gain experience in the early human development of a novel drug, in this case a GSK anti-IL-7Rα antibody. I was the main sub-investigator in a first-in-human dose-escalation trial which first involved healthy controls and was planned to progress to involve people with multiple sclerosis. Unfortunately, as will be described, the trial was halted early for reasons outside of my control. So, I shifted my clinical trial work to ongoing trials of alemtuzumab in multiple sclerosis, from which I published a paper on the relationship between lymphocyte reconstitution and multiple sclerosis disease activity. I am also an author on the paper published for the CAMTHY trial, on increasing thymic production of T cells with palifermin, a keratinocyte growth factor, in an attempt to offset the homeostatic proliferation of lymphocytes post alemtuzumab and with that secondary autoimmunity.

The laboratory component of my PhD was to examine the effects of the anti-IL-7Rα antibody on human cells *in vitro*, particularly to establish whether its efficacy was affected by IL-7Rα genotype. I also investigated how the IL-7Rα antibody impacted on IL-7 bioavailability, and explored its partial agonistic effect. Following this I went on to investigate by stimulating IL-7Rα, negative feedback pathways associated with IL-7Rα and how this was affected by IL-7Rα genotype.

1.16 HYPOTHESES

- The antibody to IL-7Rα reduces the bioactivity of IL-7 ex vivo
- The effects of the antibody to IL-7Rα may depend on IL-7Rα genotype
- The antibody to IL-7Rα will be well tolerated in healthy volunteers when tested in a first-in-human dose escalation study
- The antibody to IL-7Rα will reduce multiple sclerosis disease activity when tested in a first-in-human trial

- Palifermin will be well tolerated in patients with MS being treated with alemtuzumab, with increased thymic lymphopoiesis and reduced secondary autoimmunity with alemtuzumab
- CD4+ lymphocyte reconstitution cannot be used as a marker for return of multiple sclerosis disease activity post alemtuzumab

CHAPTER 2 - METHODS FOR IN VITRO ANTAGONISM AND STIMULATION OF IL-7Rα

2.1 STRATIFICATION ACCORDING TO IL-7Rα GENETICS

GSK originally presented to me this project as an analysis of haplotypes derived from the Lundmark paper (Lundmark et al., 2007), which confirmed the association of IL-7Rα with multiple sclerosis. GSK included a haplotype selection as it was interested in a particular SNP rs3194051, which was later associated with ulcerative colitis (Anderson et al., 2011). This SNP was included for commercial reasons as ulcerative colitis was planned as a future indication for the IL-7Rα antagonist GSK2618960.

Despite using a haplotype analysis the Lundmark paper confirmed that all the SNPs used were in complete linkage disequilibrium and in fact the association of the haplotypes with MS was due to the SNP rs6897932 (Lundmark et al., 2007), which was later confirmed in the GWAS in 2011 (International Multiple Sclerosis Genetics et al., 2011). Further fine mapping of the region showed a new intergenic SNP rs6881706, which was in complete linkage disequilibrium with rs6897932 (D' and r^2 values of 1.0). Using rs6897932 as the at risk SNP, the Gregory paper in 2007 demonstrated increased levels of soluble IL-7Rα as a result of alternative splicing from exon 6 skipping (Gregory et al., 2007), which was confirmed by other groups (Hoe et al., 2010) (Lundstrom et al., 2013). Therefore with the focus of the investigation on differences in soluble IL-7Rα between populations, I used the SNP rs6897932 to stratify the population. The 'at-risk' SNP was homozygous for CC at rs6897932. The 'protective' SNP was homozygous for TT at rs6897932.

2.2 FLOW CYTOMETRY ASSAYS

The list of reagents used in the flow cytometry assays during chapter 4 is outlined in the tables below. The flow cytometry assays used are the receptor occupancy assay (please refer to section 2.4), the phosphorylated STAT5 assay (please refer to section 2.5) and the IL-7 titration assay (please refer to section 2.6.5).

2.2.1 TABLE OF REAGENTS

Product Description	Catalogue/ Item Number	Supplier
Diluent for IL-7	Lab stock	In-house
FACS Buffer	Lab stock	In-house
5 X Fix/lyse buffer	558049	BD
Perm Buffer III	558050	BD
Phosphate Buffered Saline	10010-056	Gibco
Rh IL-7	554608	BD
pSTAT5 PE	612567	BD
CD127 AF647	317605	Biolegend
CD3 V450	560365	BD
CD4 APC	555349	BD
PE- labeled GSK2618960	4356	GSK/Innova
Unlabeled GSK2618960	111287554	GSK
Rosette Sep Human T cell enrichment cocktail	Stemcell	15061
Foetal calf serum	In-house	Lab stock
Ficoll	17-1440-02	GE Healthcare Life Sciences
RPMI 1640	R5886	Sigma
Glutamax-I	35050-038	Gibco
Penicillin/Streptomycin	P0781	Sigma
Compensation (CST) beads	642412	BD

Table 2. 1: Reagents used in the flow cytometry assays

2.2.3 IL-7 DILUENT PREPARATION

Product Description	Catalogue/ Item Number	Supplier
Fatty acid-free Bovine Serum Albumin (BSA)	A6003-1G	Sigma
Phosphate Buffered Saline	10010-056	Gibco
Fine Balance	HR-202	Biomax

Table 2. 2: Products used to produce 'IL-7 Diluent'

0.1 g of fatty acid-free BSA was weighed out on a fine balance and dissolved in 100 ml of phosphate buffered saline in the upper reservoir of a Nalgene filtration unit. A vacuum was applied to aseptically filter the solution into the lower reservoir, which was then stored at -4 degrees Celsius.

2.2.4 FACS BUFFER PREPARATION

15 g of BSA was dissolved in a 500ml bottle of phosphate buffered saline. Under aseptic conditions, the contents were filtered into a 500 ml Nalgene unit bottle. The bottle was then capped securely and stored at -4 degrees Celsius.

2.3 IN VITRO ANTAGONISM OF IL-7Rα

2.3.1 DEMOGRAPHICS

Volunteers were drawn from the GSK volunteer panel. Subjects were selected based on their genotype for the rs6897932 polymorphism in IL-7Rα.

Initially the plan derived from GSK was to do the analysis on approximately 30 (29 subjects analysed) subjects based on a previous genetic analysis they had completed which had given 80% power. After an initial analysis of the data a power analysis showed that a further 11 subjects were required to detect a significant difference in the pSTAT5 assay with 90% power. Therefore the pSTAT5 analysis was based on a population of 41 rather than 29 subjects from the GSK volunteer panel. The ELISA for serum IL-7 was also completed on 29 subjects (R+D systems Cat No: HS750).

2.3.2 PREPARATION OF GSK2618960 FOR ANTIBODY TITRATION

As GSK2618960 was added to blood in a 1:1 ratio, a 200µg/ml concentration would have a working concentration of 100 µg/ml. Serial dilutions of GSK2618960 (in µg/ml) was conducted on a logarithmic scale - 100 (positive control), 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 and 0 (negative control).

2.4 RECEPTOR OCCUPANCY ASSAY

The main purpose of the receptor occupancy assay was to demonstrate the binding of the GSK antibody to IL-7Rα receptors on human T lymphocytes. Although this assay was originally developed in-house by GSK it has been used elsewhere for measuring IL-7Rα occupancy (Kern et al., 2016).

A PE labeled GSK2618960 IL-7Rα antagonist identified unbound IL-7Rα receptors following incubation of subjects' blood with unlabeled GSK2618960 IL-7Rα antagonist. As unlabeled GSK antibody levels increased, there were lower median fluorescence intensities of PE (due to less binding of PE labeled GSK antibody), when assessed by flow cytometry.

12 ml of blood was collected in two 6 ml sodium heparin blood tubes from volunteers from the GSK volunteer panel according to genotype (please refer to section 2.3.1). Volunteers from each genotype group were selected for blood draw in a randomised manner (in order to make sure genotypes were analysed throughout the assay period with no bias towards a particular genotype throughout the period the receptor occupancy assay was conducted) and blinded to the person doing the assay.

50µl of unlabeled GSK antibody (please refer to section 2.3.2 for details of the unlabeled GSK antibody titration which was performed prior to the receptor occupancy assay) was added to 50µl of the subject's blood in FACS tubes and incubated for 30 minutes at room temperature.

The subjects' T lymphocytes were identified by antibody staining of CD3+ cells (1µl CD3 V450). The IL-7 receptor (2µl CD127 AF647) was also stained (this antibody binds to a different epitope on IL-7Rα from unlabeled GSK2618960, enabling a calculation of total IL-7Rα). 7µl of PE-labeled GSK antibody was added to the 'antibody mix' (10µl of 'antibody mix' made up of the different antibodies used in this assay was added to the 100µl in the FACS tube) to identify unbound IL-7 receptors. Antibody concentrations were applied, as described above, at concentrations determined by titration experiments. Once the 'antibody mix' was added to the FACS tube, samples were incubated at room temperature for 30 minutes.

1ml of fix/lyse buffer was added to the samples and incubated for 10 minutes at 37 degrees Celsius. The fix/lyse buffer has been shown to preserve the light scattering

properties of cells and whole blood lysis has been shown to be as effective as density gradient centrifugation in the preparation of PBMCs for lymphocyte subset analysis (Renzi and Ginns, 1987).

Following this incubation step, the samples were centrifuged for 6 minutes at 500 g. The samples were then washed in 2ml phosphate buffered saline and centrifuged again for 6 minutes at 500 g.

The supernatant was poured off and the cells were re-suspended in their residual volume with 300µl of phosphate buffered saline. The samples were run immediately on a BD FACS CANTO flow cytometer. Data was analysed using FlowJo software v10.0.4 (Treestar) to calculate the Median Fluorescence Intensity (MFI) of PE, which was used to determine the percentage of unbound IL-7 receptors.

The MFIs from the different doses were calculated to represent a percentage of maximal signal to derive a dose response curve for each subject:

(MFI – MFI negative control) / (MFI positive control – MFI negative control) x 100

The MFI of the negative control was used to account for background signal. The inverse of this would then account for the numbers of receptors occupied by GSK2618960 rather than the numbers of unbound receptors. The receptor occupancy results have been graphically represented in this manner.

Please refer to figure 4.1 for an overall scheme of the receptor occupancy assay.

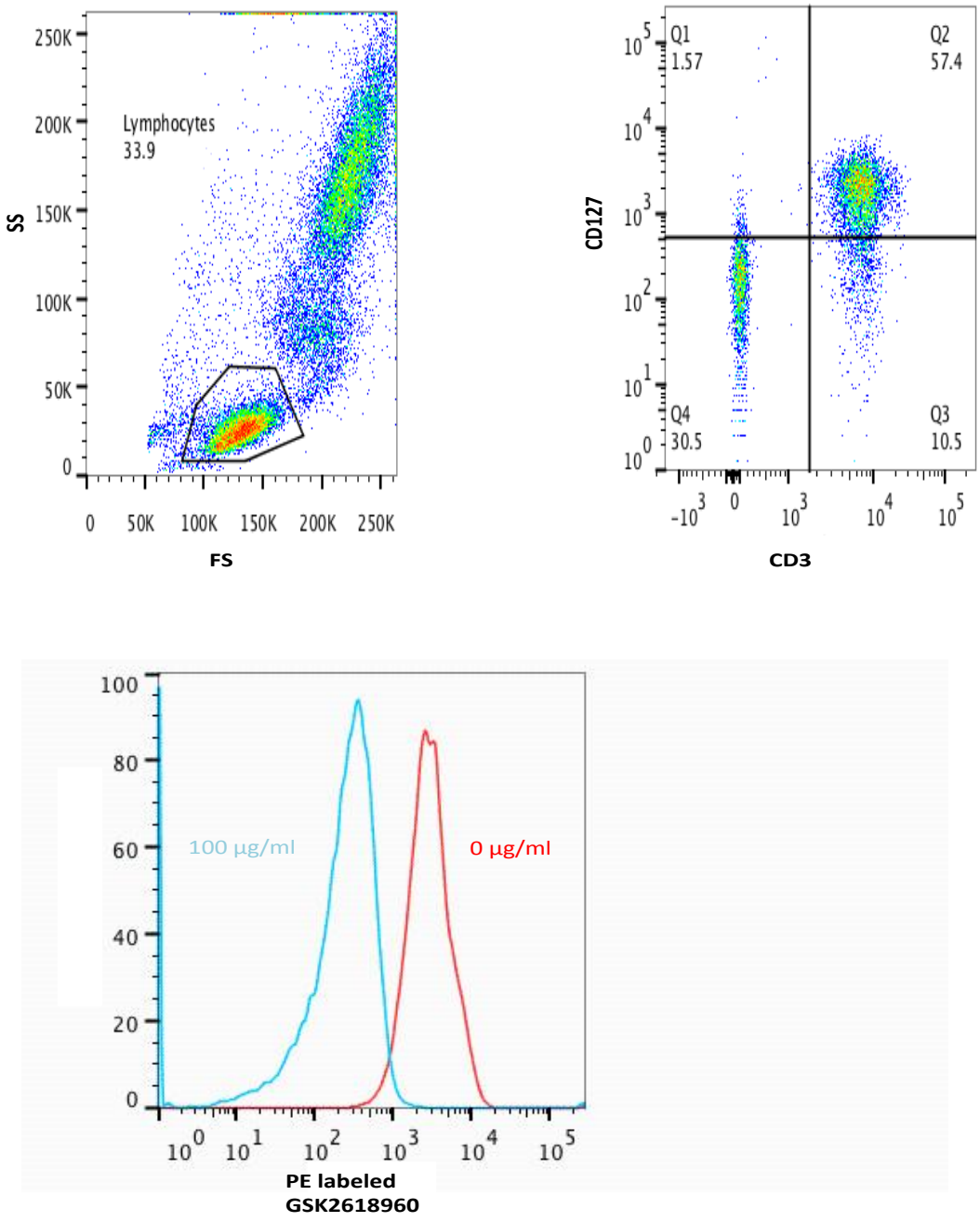

Figure 2. 1: Receptor Occupancy

Lymphocytes derived from whole blood were gated based on forward scatter (FS) and side scatter (SS). CD3+ T cells expressing IL-7Rα (CD127) were selected (upper right quadrant). The histogram represents PE labeled GSK2618960 which depicts the difference between the complete occupation of IL-7 receptors with 100 µg/ml of GSK2618960 (blue) and the number of unbound IL-7 receptors with 0 µg/ml GSK261896 (red).

2.5 PHOSPHORYLATED STAT5 ASSAY

The STAT5 phosphorylation (pSTAT5) assay measured the levels of pSTAT5 in CD3+ cells, taken from whole blood after incubation with a range of concentrations of IL-7Rα antagonist and IL-7.

12 ml of blood was collected in two 6 ml sodium heparin blood tubes from volunteers from the GSK volunteer panel according to genotype (please refer to sections 2.3.1 and 2.4).

50µl of unlabeled GSK antibody (please refer to section 2.3.2 for details of the unlabeled GSK antibody titration which was performed prior to the receptor occupancy assay) was added to 50µl of the subject's blood in FACS tubes and incubated for 30 minutes at room temperature. Negative and positive controls (comprised of 50µl phosphate buffered saline) were also added to 50µl of the subject's blood in a FACS tube for 30 minutes at room temperature.

Following this the cells were concurrently stimulated with 5ng/ml rhIL-7 (PBS used for negative control) for 20 minutes, whilst being stained with 1µl CD3 V450 and 2µl CD4 APC surface antibodies (antibody concentrations determined by titration experiments) for 20 minutes at room temperature.

Red blood cells were lysed by 1 ml of 1X fix/lyse buffer, which was incubated at 37 degrees Celsius for 10 minutes (as described in section 2.4). Following this step, the samples were washed twice in PBS, and centrifuged for 6 minutes at 500 g.

For the intracellular detection of the transcription factor pSTAT5, reagents were used to permeabilise the cells to allow intracellular staining. 1 ml of Perm Buffer was added to the FACS tubes containing the cells and incubated on ice for 30 minutes.

Following this step, the samples were washed twice in FACS buffer, centrifuged for 6 minutes at 500 g. Cells were then stained with 5µl of pSTAT5 for 30 minutes on ice, in the dark.

The supernatant was removed and the cells were re-suspended in their residual volume. 300µl of phosphate buffered saline was added and the samples were run immediately on a BD FACS CANTO flow cytometer. Data was analysed using

FlowJo software v10.0.4 (Treestar) to calculate the Median Fluorescence Intensity (MFI) of pSTAT5.

The MFIs from the different doses were calculated to represent a percentage of maximal signal, as described in section 2.4, deriving a dose response curve for each subject.

Please refer to figure 4.2 for an overall scheme of the pSTAT5 assay.

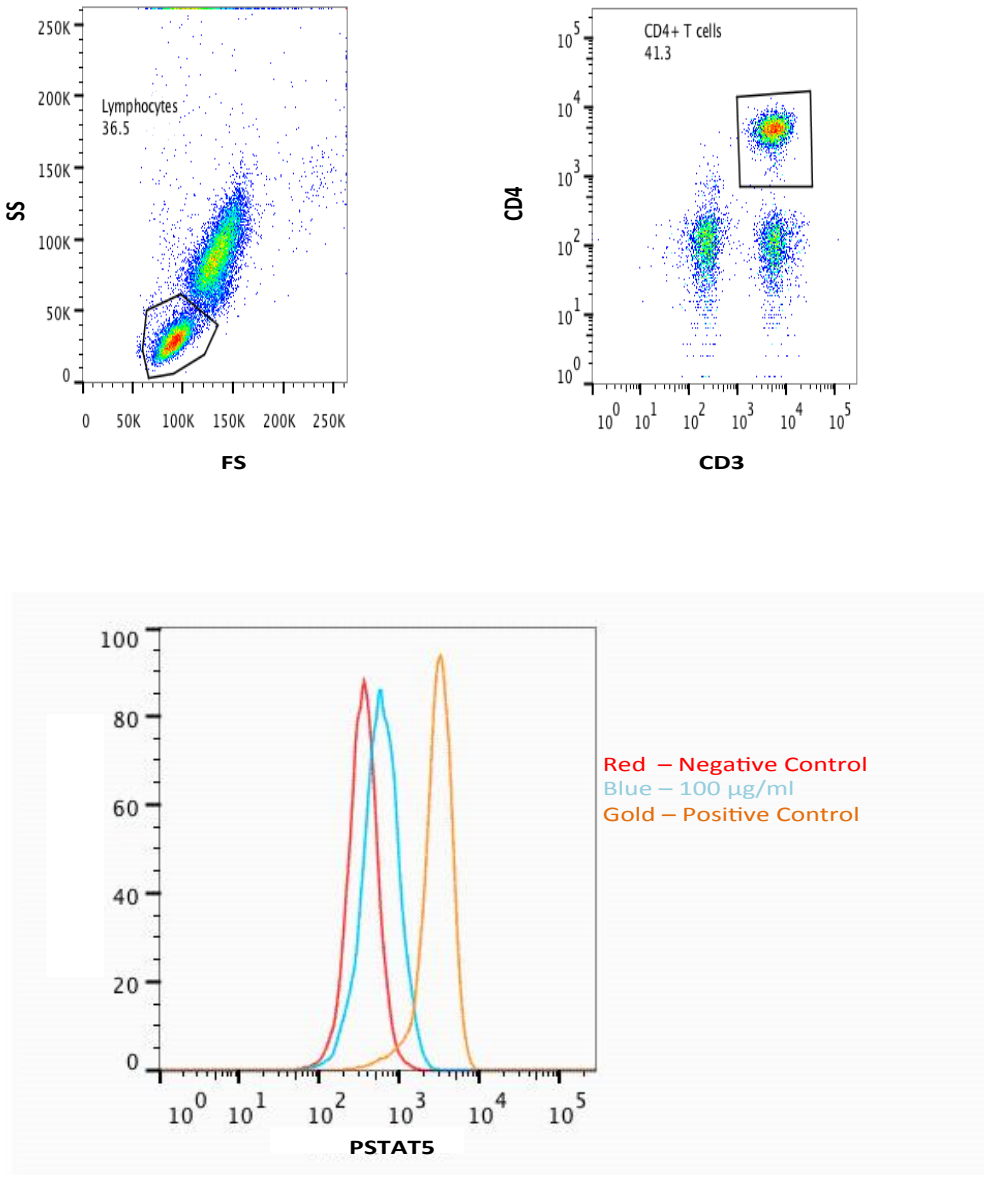

Figure 2. 2: Phosphorylated STAT5

Lymphocytes derived from whole blood were gated based on forward scatter (FS) and side scatter (SS). CD3+ CD4+ T cells were selected. The histogram represents downstream activation through the IL-7 receptor as depicted by phosphorylated STAT5 (pSTAT5). The histogram shows the partial agonist effect of GSK2618960 with abrogation of the pSTAT5 signal when compared to the positive (5 ng/ml rhIL-7) and negative (PBS) controls.

2.6 IN VIVO STIMULATION OF IL-7Rα

An overall scheme of the methods is depicted in the diagram below.

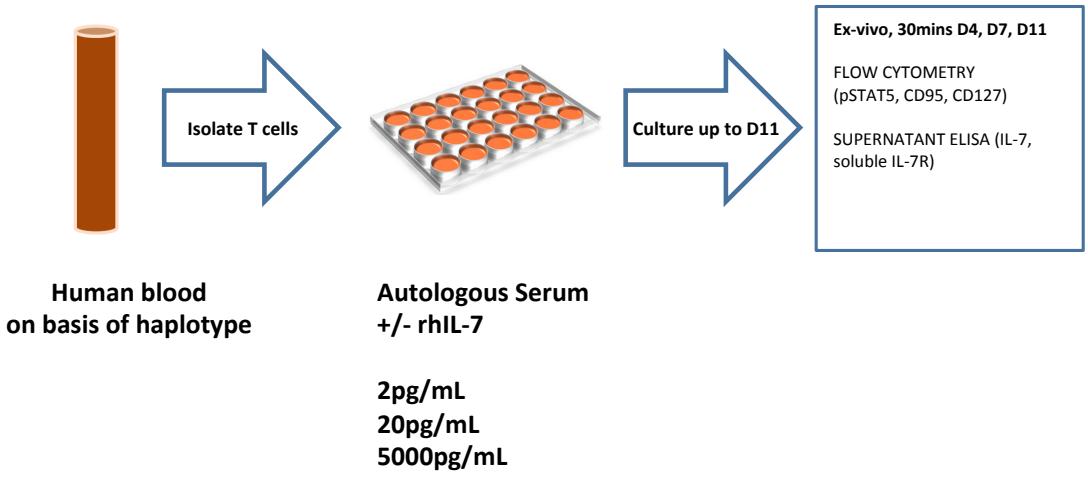

Figure 2. 3: In vitro stimulation of IL-7Rα

T lymphocytes were isolated from whole blood by ficoll gradient. T lymphocytes were then cultured with an IL-7 titration up to 11 days. Flow cytometry and ELISAs on the cell culture supernatant were then measured.

2.6.1 DEMOGRAPHICS

22 Volunteers were drawn from the GSK volunteer panel. Subjects were selected based on their genotype for the rs6897932 polymorphism in IL-7Rα. 14 subjects were included in the at risk group and 8 subjects in the protective group. The ELISAs on serum soluble IL-7Rα, supernatant serum soluble IL-7Rα and supernatant IL-7 were also completed on 22 subjects.

2.6.2 PREPARATION OF T CELLS FROM WHOLE BLOOD

Blood was collected from subjects in heparinized tubes. 2 ml of T cell cocktail from Rosette Sep was added to 40ml of blood. Rosette Sep was used as it is a highly efficient method of sorting T lymphocytes from whole blood without the need for magnetic separation kits (Stem Cell Technologies, 2018). The blood and T cell cocktail was mixed using a vortex and incubated for 20 minutes at room temperature.

20 ml of Phosphate buffered saline (PBS) and 2% foetal calf serum (FCS) was gently mixed with the blood after incubation with the Rosette Sep. This mixture was separated using Ficoll density gradient centrifugation (Ficoll Paque Plus; GE Healthcare Life Sciences, cat. no. 17-1440-03).

T cells were counted using a Beckman-Coulter cell counter.

2.6.3 PREPARATION OF COMPLETE MEDIUM

Subjects' blood contained in sodium-heparin tubes were incubated at room temperature for 30 minutes. These tubes were centrifuged for 10 minutes at 1300 g. The serum was taken for preparation of complete medium.

To make up 25 ml of the complete medium, 19.5 ml of RPMI 1640 was mixed with 5 ml of serum, 0.25 ml of Glutamax-I and 0.25 ml of Penicillin/Streptomycin.

Following Ficoll density gradient centrifugation, the cells were re-suspended in as minimal an amount of residual fluid as possible. The appropriate amount of complete medium was added to the falcon tube in order to have 1 x 10^6 cells per ml (e.g. If you

have 3.76 x 10^6 cells in the falcon tube then 3.76 ml of complete medium should be added to the falcon tube). In order to do this assay a minimum of 32 million T cells were needed per subject with 1 ml of cells in complete medium added to each well in the culture plate.

2.6.4 IL-7 TITRATION

The IL-7 titrations were based on the molar ratios (soluble IL-7Rα:IL-7) from the Lundstrom paper of 5000:1, 500:1 and 50:1 (Lundstrom et al., 2013). In the Lundstrom paper IL-7 activity was augmented over time only at 'middle molar ratios' of approximately 500:1, which equated to the ratio found in vivo.

Therefore this assay attempted to recreate the different molar ratios used in the Lundstrom paper using the rhIL-7 mixed with the soluble IL-7Rα present in the autologous serum in the culture. Previous studies had measured average soluble IL-7Rα concentration in serum at approximately 100 ng/ml soluble IL-7Rα (Lundstrom et al., 2013). Therefore I used this calculation to predict the average amount of soluble IL-7Rα present within the cell culture.

In this assay 1ml of complete medium was used in which 20% of the complete medium was serum. Therefore an approximate soluble IL-7Rα concentration of 20 ng/ml was present within the cell culture. This gave a molar concentration of 0.4nM. Therefore 2pg/ml (5000:1), 20pg/ml (500:1) and 5000pg/ml (2:1 but used as the positive IL-7 control) rhIL-7 gave similar molar ratios as the Lundstrom paper.

There were 4 titrations - an IL-7 negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml IL-7 wells. There were duplicates of each of the IL-7 titrations at each time point.

For the 2 pg/ml IL-7 titration, 2 µL of 1 ng/ml rhIL-7 was added to 1 ml cell culture.

For the 20 pg/ml IL-7 titration, 20 µL of 1 ng/ml rhIL-7 was added to 1 ml cell culture.

For the 5000pg/ml IL-7 titration positive control, 50 µL of 100 ng/ml rhIL-7 was added to 1 ml cell culture.

50 µL of PBS was added to the IL-7 negative control wells.

Once IL-7 was added, the plates were incubated at 37 degrees Celsius in a cell culture incubator.

2.6.5 POST CELL CULTURE

The time points used in this assay were days 0, 4, 7 and 11.

Once a particular time point was reached the plates were taken out of the cell culture incubator and centrifuged at 500g for 6 minutes.

950 µL of cell culture supernatant was removed and stored at -80 degrees Celsius for later ELISA analysis.

1ml of PBS was then added to each well and the cells were centrifuged again at 500g for 6 minutes.

The supernatant was then removed and the cells were re-suspended in 600 µL of PBS. Each well therefore had two samples, one for cell surface staining (please refer to table 2.3) and one for intracellular staining (please refer to table 2.4).

Samples underwent cell surface staining and intracellular staining with the staining panels depicted below.

Compensation controls were prepared using BD biosciences compensation beads. 500 µL of PBS was added to a FACS tube and then 4 drops of both positive and negative beads were added. This tube was mixed by a vortex and 80 µL was added to 6 FACS tubes (one FACS tube for each antibody used in the assay). 2 µL of each antibody was added to its appropriately labeled tube.

Antibody	Company	Catalogue Number	Amount (µL)
CD3 V450	BD	560365	1
CD8 V500	BD	561617	2
CD4 PerCP5.5	BD	560650	2
CD127 AF647	Biolegend	317605	2
CD95 PE	Biolegend	305608	5

Table 2. 3: Cell surface antibody staining panel

Antibody	Company	Catalogue Number	Amount (µL)
CD3 V450	BD	560365	1
CD8 V500	BD	561617	2
CD4 APC	BD	555349	2
pSTAT5 PE	BD	612567	5

Table 2. 4: Intracellular antibody staining panel

All samples stained were incubated at room temperature for 30 minutes. The samples were washed twice in PBS, and centrifuged for 6 minutes at 500g.

The supernatant was removed and the cells were re-suspended in their residual volume with 300µl of phosphate buffered saline. The samples were run immediately on a BD FACS CANTO flow cytometer. Data was analysed using FlowJo software v10.0.4 (Treestar).

For the intracellular detection of the transcription factor pSTAT5, reagents were used to permeabilise the cells to allow intracellular staining. Samples for intracellular staining had 1 ml of Perm Buffer III added followed by incubation for 30 minutes on ice.

The samples were washed twice in FACS buffer, and centrifuged for 6 minutes at 500 g. Cells were then stained with 5µl of pSTAT5 for 30 minutes on ice, in the dark.

The supernatant was removed and the cells were re-suspended in their residual volume. 300µl of phosphate buffered saline was added and the samples were run immediately on a BD FACS CANTO flow cytometer. Data was analysed using FlowJo software v10.0.4 (Treestar) to calculate the Median Fluorescence Intensity (MFI) of pSTAT5.

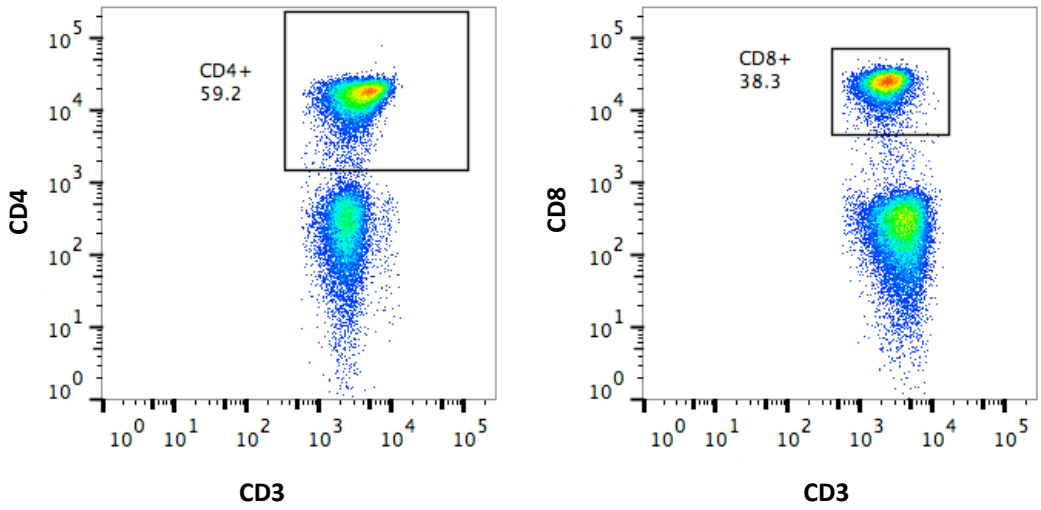

Figure 2. 4: Gating strategy for IL-7 Stimulation assay

After gating for lymphocytes, gating for CD4+ and CD8+ T cells was performed as above. From this MFIs for CD95 (Fas Ligand), IL-7Rα and pSTAT5 were recorded.

2.7 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISAs)

The purpose of the serum ELISAs was to measure the cytokines that were relevant to the IL-7 signaling pathway within the cohort used. This would allow for analysis to determine if the haplotypes used mirror what has been described previously in the literature.

Serum was taken from subjects on day 0. This was stored in a -80 degrees Celsius freezer until ready for ELISA analysis. Duplicate serum samples were used for analysis.

Analysis of the supernatant ELISAs allowed examination of the effect of stimulation of IL-7Rα on the production and bioavailability of soluble IL-7Rα and IL-7, and the difference, if any, between the genotype groups.

The supernatants were harvested from T lymphocytes stimulated with differing amounts of IL-7 after 30 minutes, 4,7 and 11 days. Samples were stored in a -80 degrees Celsius freezer until ready for ELISA analysis. Duplicate supernatant samples were used for analysis.

IL-7 was measured using the commercially available IL-7 high sensitivity ELISA kit by R&D Systems. This is described in more detail in section 2.7.2.

The soluble IL-7 receptor ELISA, described in section 2.7.1, measured soluble IL-7 receptor with a highly sensitive non-isotopic time-resolved fluoroimmunoassay using dissociative fluorescence enhancement (DELFIA; PerkinElmer). This assay was used in collaboration with Ricardo Ferreira who developed the assay, based on his previous experience using DELFIA assays (Ferreira et al., 2013).

2.7.1 SOLUBLE IL-7Rα ELISA

2.7.1.1 TABLE OF REAGENTS

Product Description	Catalogue/Item Number	Supplier
ELISA Coating Buffer (5x)	421701	Biolegend
Foetal Bovine Serum	Lab Stock	In-house
Phosphate Buffered Saline	10010-056	Gibco
Tween	Lab Stock	In-house
Monoclonal anti-IL7R Antibody (Capture Antibody)	MAB 306	R&D Systems
Delfia Eu-N1 Streptavidin	1244-360	PerkinElmer
Co-star 3590 flat bottom 96 well plates without lid	07-200-36	Fisher Scientific
Anti-Human IL7R-Biotin (Detection Antibody)	13-1278	eBioscience
Recombinant Human IL7Rα-Fc chimera	306-IR	R&D Systems
Delfia Assay Buffer	CR85-100	PerkinElmer
Delfia Enhancement Solution	1244-104	PerkinElmer

Table 2. 5: Reagents used for the soluble IL-7Rα ELISA

Circulating soluble IL-7 receptor concentrations were measured using a highly sensitive non-isotopic time-resolved fluorescence ELISA assay based on the dissociation-enhanced lanthanide fluorescent immunoassay technology (DELFIA; PerkinElmer).

The assay diluent contained 1x phosphate buffered saline and 10% foetal bovine serum. The wash Buffer contained 1x Phosphate Buffered Saline and 0.05% Tween. This ELISA was completed over the course of three days.

On Day 1, in order to enable binding of soluble IL-7 receptor in the serum, the 96 well plate was coated with 100 µL Capture antibody, which had been diluted in Coating buffer to a final concentration of 1 µg/ml. The plates were sealed overnight and incubated at 4 degrees Celsius.

On Day 2, the plates were 'washed' by aspirating the liquid in the wells and adding 250 µL wash buffer. This was completed manually using a multi-pipette tool (or alternatively a plate washing machine could be used). This process was completed three times.

After washing, the ELISA plates were 'blocked', reducing non-specific binding of other proteins in the serum or cell culture supernatant, with 200 µL of assay diluent added to each well. The plates were incubated for 90 minutes at room temperature.

The ELISA Standards were derived from recombinant human IL7Rα/Fc chimera diluted in assay diluent to a final concentration of 10 ng/ml. Six further 1:2 dilutions were made to make up a standard curve.

Serum samples were diluted 1:20 with assay diluent before being added to the ELISA plate. Cell culture supernatant samples were diluted 1:2 with assay diluent before being added to the ELISA plate.

The plates were washed three times as described above. 100 µL of standards and samples were added according to the desired plate layout. The ELISA plates were incubated at room temperature for two hours before being incubated overnight at 4 degrees Celsius.

On Day 3, the plates were washed five times as described above. In order to detect the soluble IL-7 receptor bound by the capture antibody, a biotinylated mouse anti-CD127 monoclonal antibody was diluted in assay diluent to a final concentration of 50 ng/ml, before 100 µL was added to each well. The plates were incubated for one hour at room temperature.

The plates were washed three times as described above. Europium-Streptavidin was then diluted in DELFIA buffer to a concentration of 0.1 µg/ml. 100 µL was added to each well. The ELISA plates were incubated for one hour at room temperature.

The plates were washed three times as described above. 100 µL of DELFIA enhancement solution was added to each well. The ELISA plates were incubated at room temperature for ten minutes (when colour change occurs) before being read on the Victor plate reader using the factory-set DELFIA Europium protocol (excitation at 340 nM and emission at 615 nM). Quantification of test samples was obtained by fitting the readings to a human recombinant IL-7Rα serial dilution standard curve plated in duplicate on each plate.

2.7.2 INTERLEUKIN 7 HIGH SENSITIVITY QUANTIKINE ELISA

The following ELISA was performed as per the manufacturer's instructions using a commercially available kit from R+D systems (Cat No: HS750). This ELISA was used on both the serum and cell culture supernatant samples. The methods for this ELISA are briefly described below.

The assay employed the quantitative sandwich enzyme immunoassay technique. In order to bind IL-7, a monoclonal antibody specific for human IL-7 had been pre-coated onto the 96 well plates. The IL-7 Standard (lyophilized recombinant human IL-7 in a buffered protein base) was reconstituted with 'calibrator diluent' (a protein buffered base). Serial two-fold dilutions were performed in order to generate a seven point standard curve. 200 µL of standards and samples were pipetted into the wells and the immobilised antibody bound any IL-7 present. The 96 well plates were kept in a moist environment to minimize the edge effect that may occur in immunoassays and it was incubated at room temperature overnight (for at least 14 hours).

In order to wash away unbound substances a series of wash steps were performed. Wash buffer was prepared by diluting the 100 ml of stock 'wash buffer concentrate' with 900 ml of distilled water. Liquid was aspirated from each of the wells in the plate. 400 µL of wash buffer was added to each of the wells. This process was completed six times. After the final wash the wells were aspirated to dryness.

200 µL of 'IL-7 conjugate' (an enzyme-linked polyclonal antibody specific for human IL-7, conjugated to alkaline phosphatase) was added to each well. The 96 well plates were incubated at room temperature for two hours at this point.

In order to remove any unbound antibody-enzyme reagent, the plates were washed six times as described above. 50 µL of 'substrate solution' was added to the wells and incubated for 45 minutes at room temperature. Without further washing, 50 µL of 'amplifier solution' was added to the wells, and left to incubate for 45 minutes at room temperature. It is at this step that colour developed in proportion to the amount of IL-7.

The colour development was stopped using a stop solution (composed of 2N sulphuric acid) and the intensity of the colour was measured.

Absorbance was read on the MultiSkan Ascent Reader within 30 minutes at 490/690 Nm. Quantification of samples was obtained by fitting the readings to the standard curve.

2.8 STATISTICAL ANALYSIS FOR ANTAGONISM AND STIMULATION OF IL-7Rα

For analysis from the receptor occupancy and phosphorylated STAT5 curves from antagonism of the IL-7 receptor this was completed with the help of GSK statistician Philip Overend.

Curves from the raw data were generated from the duplicate results for each subject.

Each curve was split into 4 parts:

A – curve start (e.g. minimum receptor occupancy/maximum pSTAT5 response)
B – slope of the curve
C – log10 EC50 (the halfway maximal response)
D – curve end (e.g. maximal receptor occupancy/minimal pSTAT5 response)

The mean and standard error was generated from the data for each part of the curve for each subject. Subsequently the means and standard errors were pooled within each genetic group. 'Weighted' analysis of the results was used for the curve parameters based on the standard error from the duplicate results for each subject. Subjects with the smaller standard error were given more importance in the 'weighted' analysis of variance.

Analysis of variance (ANOVA) was used, as this was the statistical test that could compare multiple means across a normalized distribution avoiding inflating the risk of the type 1 error rate by making too many comparisons within the data.

I completed the statistical analysis of the ELISAs, the effect of the drug up to and beyond the EC50 and the flow cytometry results from stimulation of IL-7Rα.

Generation of the Fas Ligand receptor (CD95), IL-7Rα and pSTAT5 results:

The median fluorescence intensity of membrane bound IL-7Rα, Fas Ligand (CD95) and pSTAT5 was taken from each sample at day 0, 4, 7 and 11. Duplicate samples were taken for each titration point. The mean of these duplicate data points were then entered into the analysis.

To analyse both the flow cytometry results and the ELISAs, multiple t tests were used to compare the means of the different genetic groups at the various time points at different concentrations of IL-7. This was corrected using the Bonferroni method to arrive at p values indicating if there was a significant difference between the genetic groups.

2.9 GSK TRIAL OF AN IL-7 RECEPTOR ANTAGONIST

2.9.1 PATIENTS AND PROCEDURES

This was a Phase I randomised, double-blind, placebo-controlled study performed in a single centre in Cambridge. The study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki 2013, and local regulations. The protocol was approved by the local ethics committee (14/LO/1670, National Research Ethics Service Committee, London, UK) and all study subjects provided written informed consent. The study was registered on Clinicaltrials.gov (identifier: NCT01808482).

Healthy male participants were included in the trial if they were between 18 and 55 years of age at the time of signing the informed consent with a body weight 50-100 kg and BMI within the range 19.0–29.9 kg/m^2, with history of current vaccination status for tetanus, diphtheria, pertussis, measles, mumps and rubella (or consent to vaccination at screening); with history of current vaccination status for influenza or who consent to receive influenza vaccine at screening; with no suicide risk; no live vaccination within one month of screening; no history of anaphylaxis or severe allergic reaction and consented to use contraception from the time of the first dose of study medication until the final follow-up visit.

Exclusion criteria for healthy male participants included history of smoking within the previous 6 months; history or evidence of alcoholism; inability to refrain from the use of prescription medications and if the participant had received another investigational product within 30 days or 5 half-lives of the product (whichever was longer).

Multiple sclerosis patients were included in the trial if they had relapsing-remitting MS according to the 2010 revisions of the McDonald criteria; had at least 2 relapses; have demonstrated active disease activity within the previous 12 months; expanded disability status score (EDSS) of ≤5.0 at the screening; male or female between 18 and 55 years of age at the time of signing informed consent; with history of current vaccination status for tetanus, diphtheria, pertussis, measles, mumps and rubella (or consent to vaccination at screening); with history of current vaccination status for influenza (or who consent to receive influenza vaccine at screening) and consented to use of contraception from the time of the first dose of study medication until the final follow-up visit.

Exclusion criteria for multiple sclerosis patients within the trial included intolerance to undergo MRI scanning; treatment with steroids for a relapse or otherwise within 30 days of dosing; within the previous 6 weeks treatment with first line disease modifying therapies such as glatiramer acetate or beta-interferons; within the previous 12 months treatment with alemtuzumab, natalizumab, mitoxantrone, cladribine, fingolimod, methotrexate, azathioprine, or any other immunosuppressant or cytotoxic therapy; a history of malignancy, or a history of clinically significant autoimmunity other than multiple sclerosis.

Subjects were screened up to 28 days (42 days for those subjects consenting to vaccination) before admission to the clinical unit on Day -1 (pre-dose). All subjects remained in the unit for at least 24 hours following dosing and were monitored at least weekly during the period of full receptor occupancy and then every 4 weeks thereafter until week 24 (in case of latent lymphopenia).

The dose level of GSK2618960 that was predicted to provide not more than 30 days maximal receptor occupancy (RO) was 12.0 mg/kg. The no observed adverse effect (NOAEL) dose level in the 4-weekly repeat IV bolus cynomolgus monkey toxicity study was 300 mg/kg (Leung et al., 2012).

2.9.2 TRIAL OUTLINE

2.9.2.1 Part A

Based on preclinical repeat dose toxicology studies in cynomolgus monkeys (I was not involved with this pre-clinical work), dose levels were set at 0.001, 0.006, 0.03, 0.15, 0.6, 2, 6 and 12 mg/kg. Predicted human pharmacokinetics was calculated based on the preclinical monkey data and a safety margin was built into the planning for the study. 24 healthy subjects were due to be dosed in Part A.

2.9.2.2 Part B

Healthy volunteers in this part of the study would undergo repeat doses of the drug (i.e. second dose approximately 4-5 weeks after the first dose). The aim was to have full receptor occupancy for 8-10 weeks. The size of dose and the frequency of doses used in Part B would be decided by the dose escalation committee after the finish of Part A.

For this part of the study an initial 12 subjects were due to be recruited, but another 12 subjects would undergo multiple doses if receptor occupancy from a single dose turned out to be less than 4 weeks (after a dose escalation committee decision that this would be safe).

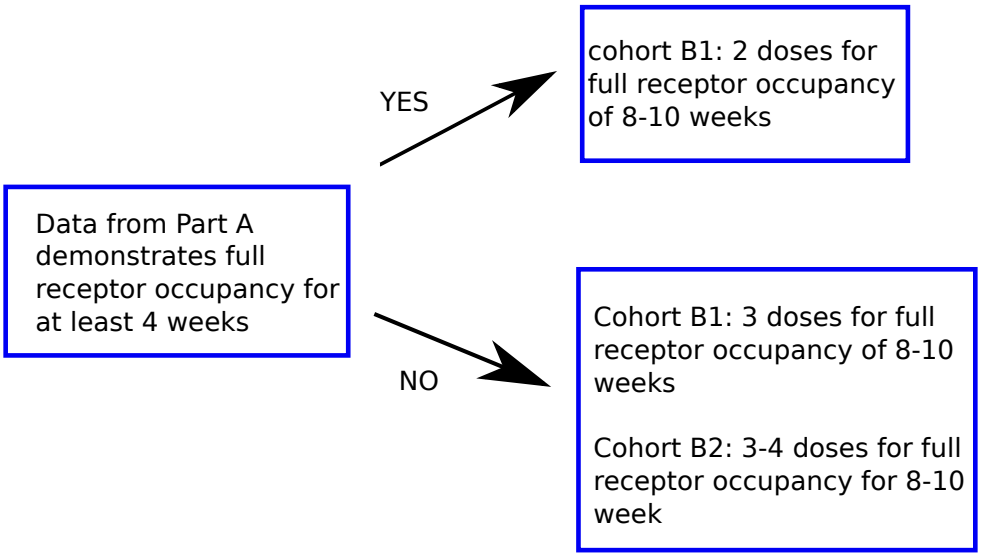

Figure 2. 5: Outline to Part B

Conduct of cohort B2 was optional and based on the decision of the dose escalation committee.

2.9.2.3 Part C

Part C was due to involve 20 multiple sclerosis patients. Safety, tolerability, pharmacokinetics and pharmacodynamics were due to be investigated. Several pre and post-dose MRI scans to investigate subclinical disease activity were also planned, exploring any potential for rebound disease activity (whilst also being able to assess any potential benefit from the drug).

Laboratory experiments would also be carried out looking at lymphocyte subsets including regulatory T cells and downstream signaling from the IL-7 receptor to see what effect antagonising IL-7Rα would have on the T cell population.

Dose selection for part C would be dependent on what happened in Parts A and B and the final decision on this would be taken by the dose escalation committee.

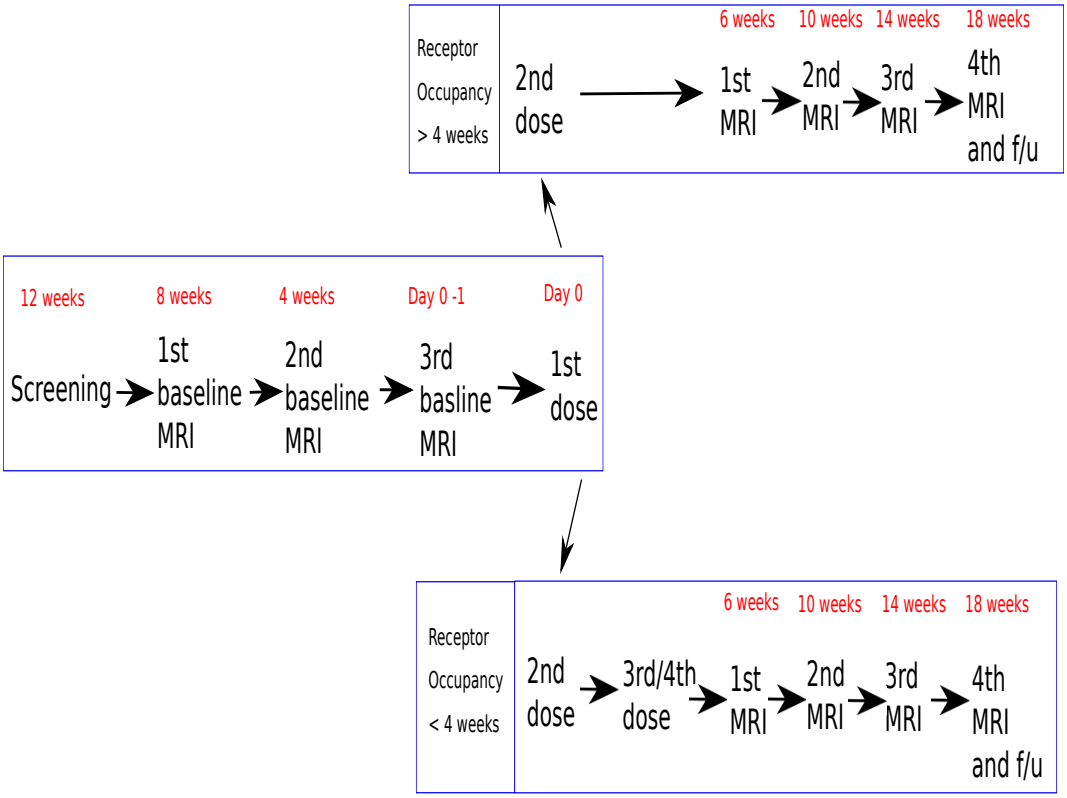

Figure 2. 6: Outline to Part C

The upper arm to this figure denotes the course of part C with 2 doses of the drug with the lower arm outlining the course of Part C if the drug had less than 4 weeks full IL-7 receptor occupancy.

2.9.3 LABORATORY ANALYSIS OF SAMPLES

During the trial as part of my role assessing clinical safety I had access to and regularly reviewed blood results including full blood counts (without lymphocyte subsets), liver function tests and ECGs. During the dose escalation meetings I was able to see results involving the pharmacokinetics of the drug. There was also some pharmacodynamic data reviewed such as the percentage of receptors the drug was occupying.

I was not involved in the laboratory analysis of blood samples from the trial as I was involved in the clinical work in the trial and could possibly have been unblinded to the trial participants. Specific laboratory results referred to in the results section but not undertaken by myself are outlined below. These analyses were undertaken internally within GSK.

- Flow cytometry investigating the receptor occupancy on CD3+ T cells by GSK2618960. This used PE labeled GSK2618960 IL-7Rα antagonist to identify the percentage of IL-7Rα receptors that were unbound after ex-vivo incubation of the subjects' blood with unlabeled GSK2618960 IL-7Rα antagonist.
- Flow cytometry investigating intracellular STAT5 phosphorylation (a measure of downstream IL-7Rα signaling) in CD4+ T cells after ex-vivo incubation of whole blood with IL-7.
- Flow cytometry of ex-vivo blood investigating lymphocyte subsets including B cells, CD3+ T cells, NK cells, regulatory T cells, recent thymic emigrants, CD4+ T cells, CD8+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, and central memory CD4+ T cells.
- IL-7 and soluble IL-7Rα ELISAs from blood plasma.
- Presence and titre of antibodies to GSK2618960.
- Blood sampling for pharmacokinetics of GSK2618960.

No statistical analyses were carried out due to the small number of subjects in the trial. A description of what happened is outlined in the results chapter.

2.10 LYMPHOCYTE RECOVERY AFTER ALEMTUZUMAB DOES NOT PREDICT MULTIPLE SCLEROSIS DISEASE ACTIVITY

2.10.1 PATIENTS AND PROCEDURES

All patients had relapsing-remitting multiple sclerosis (RRMS) and had participated in CAMMS223 (a Phase 2 randomised control trial) and CAMMS 224 or SM3 (both investigator-led, open label studies). CAMMS223 key eligibility criteria were disease onset within 3 years, at least two clinical relapses during the previous 2 years and a score of 3 or less on the Expanded Disability Status Scale (EDSS). Patients were included in CAMMS 224 and SM3 if they had at least 1 relapse in the previous year, an EDSS score of 6.0 or less, with disease duration of less than ten years. Subsequently all patients entered either CAMSAFE (an investigator led long-term observational study), or the extension phase of the CAMMS223 trial. The first patient from this cohort was treated on 22 November 1999 with the date for final collection of data on 1 January 2013.

All studies were approved by a regional ethics board and institutional research committee. All patients gave written informed consent.

2.10.2 CLINICAL TREATMENT AND FOLLOW-UP PROTOCOL

All patients received at least 2 elective cycles of alemtuzumab given annually, with the potential for further cycles if there was clinical or radiological evidence for on-going disease activity. Patients were reviewed at 1 and 3 months and then quarterly for the first two years after each treatment cycle. For the following two years, they were seen biannually and then at least annually thereafter. Patients were also seen whenever a relapse was suspected.

2.10.3 OUTCOME ASSESSMENTS

For participants in the CAMMS223 study, EDSS scores were determined quarterly in a blinded fashion by a neurologist who also adjudicated possible relapses. The same assessor measured the EDSS of patients in the CAMMS224 and SM3 studies, albeit less frequently. Sustained accumulation of disability was defined as an increase of 1.5 EDSS points from a baseline of 0, or an increase of ≥1.0 if the baseline was ≥1.0

confirmed over 6 months. A relapse was defined as new neurological symptoms attributable to multiple sclerosis, lasting >48 hours with an objective change in neurological examination.

Peripheral blood mononuclear cell phenotyping was performed at baseline and then quarterly for the first 36 months and then at least annually (including - total lymphocyte count, CD4+, CD8+, CD19+, CD56-NK, and monocyte counts).

Brain MRI scans were performed in most patients with a suspicion of active disease prior to re-treatment with alemtuzumab. Monthly MRI scans were performed in a subset of patients from the SM3 study. A number of clinically inactive patients had interval MRI scans to look for subclinical activity, and to provide a means for comparison in case of future disease activity.

2.10.4 STATISTICAL ANALYSIS

Statistical analysis was done in consultation with Mr Richard Parker, a statistician at the University of Cambridge.

Median time for recovery to the lower limit of normal (LLN) was calculated for each cell subset. All data was categorised depending on the cycle of alemtuzumab treatment. Patients were placed into 'active' or 'non-active' groups independent of when an event took place within a particular treatment cycle. Therefore, within each cycle, patients were defined as being 'relapse-free' or 'relapsing', 'disability-free' or having 'accumulated disability', or having reached a 'positive composite endpoint' (defined as: having relapsed, and/or accumulated disability, and/or having had an 'active' MRI scan); or with a 'negative composite endpoint' based on all three negative outcomes. A subgroup of patients (n=91), scanned after treatment, were classified as MRI 'active' or 'non-active'.

To assess differential lymphocyte reconstitution between groups, a linear mixed effects regression method was undertaken with CD4+/CD8+/CD19+/CD56+/ monocytes or total lymphocyte count as the outcome variable, and 'relapse/ disability/ active MRI/ composite score' and time point as explanatory variables.

This method was used as it was the optimum way to explore the relationship between the outcome variable (e.g. CD4+ count) and the different explanatory variables (e.g. time point and relapse), which needed to be accounted for.
A quadratic term (time point squared) was also included due to the observed relationship between time point and outcome. A separate linear mixed effects model was fitted within each cycle. A continuous autoregressive (order 1) correlation structure was assumed for all models. Model coefficients were presented with 95% confidence intervals and p-values.

A Fisher exact test was used to assess whether a CD4+ count of 388.5 x 10^6/mL or greater at 12 months predicts disease activity - either clinically or radiologically. A Fisher exact test was used as it was the optimum way to compare categorical data and to see if there is a relationship between those values.

The standard 5% significance level was used throughout, and no adjustment made for multiple testing in order to avoid inflating the Type II error rate. The linear mixed effects regression method was implemented in R software using the 'nlme' package (Pinheiro et al., 2013). R software was also used to compute the Fisher's exact tests. All other analyses were performed in GraphPad PRISM (version 5.00 for Windows; www.graphpad.com).

CHAPTER 3 - FIRST TIME IN HUMAN TRIAL OF IL-7 RECEPTOR ANTAGONIST

3.1 INTRODUCTION

GSK2618960 was developed by GSK for a first time in human study in healthy subjects but also in MS patients.

3.1.1 THE RATIONALE FOR THE DEVELOPMENT OF AN IL-7 RECEPTOR ANTAGONIST

As previously described in section 1.10 of the main introduction there is a need within the MS treatment armamentarium for the development of therapies with fewer side effects and less rigorous monitoring requirements. There has been some interest in the IL-7 pathway and MS for a number of years. By 2008 increased IL-7 levels were found within MS lesions (Kremlev et al., 2008) (Jana et al., 2014). Yδ cells are one of the first cells involved in the MS plaque (Wucherpfennig et al., 1992) and IL-7 is involved in the maturation of T lymphocytes in the thymus, which leads to the production of Yδ cells (Mackall et al., 2011).

Subsequently the discovery of IL-7Rα as one of the first SNPs in MS outside of HLA (Teutsch et al., 2003) further strengthened the case for IL-7Rα within the pathophysiology of MS. Furthermore in 2007 increased levels of soluble IL-7Rα were discovered in individuals with the at risk SNP for IL-7Rα (Gregory et al., 2007) (Lundmark et al., 2007).

In EAE there is increased levels of IL-7 at the onset and peak of the disease (Arbelaez et al., 2015). It has also been demonstrated that partial or complete deficiency of IL-7Rα reduces the severity of EAE or prevents the disease entirely (Walline et al., 2011) (Ashbaugh et al., 2013). IL-7 has been purported to increase the levels of GM-CSF, which has been shown to increase the pathogenicity of T lymphocytes in EAE (Sheng et al., 2014). The use of IL-7Rα antagonists has also been shown to ameliorate EAE (Lee et al., 2011, Ashbaugh et al., 2013) (Liu et al., 2010).

Following the success by GSK scientists of the IL-7 antagonist in EAE (Liu et al., 2010), GSK decided to develop this drug for a wide range of autoimmune indications including multiple sclerosis. Pre-clinical work showed that the drug was well tolerated

by cynamolgous monkeys (Leung et al., 2012). However there was some evidence in this pre-clinical work that the monkeys developed anti-drug antibodies (ADA). Therefore the trial design included repeat dosing of subjects in an attempt to detect ADAs at an early stage.

3.1.2 EARLY INCLUSION OF MS PATIENTS IN DRUG DEVELOPMENT

It was planned for MS patients to be included at the early stages of drug development (i.e. within this first time in human trial), as there had been some conjecture about if there would be a difference seen between MS patients and healthy human subjects. MS patients can also benefit in being part of the drug development process at an early stage, as they will be able to receive drug treatments in clinical trials many years prior to the drug becoming available if it passes through phase 3 trials and regulatory approval.

The 'exon skipping hypothesis', developed by Gregory and colleagues in Cambridge, was based on the increased amounts of soluble IL-7Rα in those carrying the at risk SNP for multiple sclerosis. This was the result of an amino acid change from threonine to isoleucine in the transmembrane section of the protein, leading to skipping of exon 6 and formulation of greater levels of soluble IL-7Rα (Gregory et al., 2007). T lymphocytes would have lower levels of available IL-7 as increased levels of soluble IL-7Rα compete with cell-associated IL-7Rα for IL-7. Following this hypothesis to its logical conclusion would suggest that administering IL-7 would ameliorate multiple sclerosis, while antagonism of this pathway would lead to disease worsening (Gregory et al., 2007) (Mazzucchelli et al., 2012).

This goes against the evidence seen with EAE. When IL-7 was administered in EAE this led to a worsening of disease rather than an improvement as suggested by Gregory and colleagues (Bebo et al., 2000). In the main introduction in section 1.13.2 I have described how antagonism of IL-7Rα led to an improvement in EAE clinical scores (Ashbaugh et al., 2013) (Liu et al., 2010) (Lee et al., 2011).

The exon skipping hypothesis as proposed by Gregory et al also assumed the function of soluble IL-7Rα was to compete with cell-associated IL-7Rα for IL-7, thereby limiting the amount of IL-7 available to T lymphocytes in patients with multiple sclerosis. However as discussed in the main introduction in section 1.12.5

Lundstrom and colleagues (Lundstrom et al., 2013) have demonstrated increased IL-7 bioavailability over time with increased levels of soluble IL-7Rα. This would be entirely consistent with the studies in EAE of amelioration with the use of IL-7Rα antagonists.

However a note of caution should always be used when extrapolating from studies in EAE mice, which have led to increased disease severity in individuals with MS, as has been demonstrated with other treatments, most notably anti-TNF drugs (The Lenercept Multiple Sclerosis Study Group, 1999). Therefore to truly understand how the drug might affect MS patients is to test the drug in the MS population early in the drug development process.

3.1.3 AIMS

To assess the safety of the drug GSK2618960 in humans in a first-in-human trial and explore secondary endpoints of pharmacokinetics and pharmacodynamics of GSK2618960.

To assess the safety of the drug in multiple sclerosis patients but also to look at secondary MRI endpoints which may give an indication of efficacy and safety of GSK2618960.

3.1.4 HYPOTHESIS

The drug GSK2618960 will be safe in humans.

The drug GSK2618960 will show efficacy in treating multiple sclerosis, by reducing the formation of new MRI MS lesions.

3.2 TRIAL METHODS

Outlined below is my personal involvement within the trial, which was initially meant to be involvement in Parts A and B of the trial (please refer to figure 3.1), learning about Phase 1 clinical trials as part of my training as a translational doctor. Finally I was due to lead and oversee part C of the trial, an open label study of GSK2618960 in MS patients.

3.2.1 PERSONAL INVOLVEMENT IN THE GSK TRIAL

I was involved in the design of the trial, as well as writing parts of the protocol focusing on the multiple sclerosis patients. I also attended planning meetings on the practical aspects of the trial and helped to prepare the clinical trials unit team within GSK at Cambridge by speaking to them about the trial and helping to prepare useful documents, which could be used by members of the team, when referring to the trial, as needed. Finally I was heavily involved with the application for ethical approval of the trial through IRAS (Integrated Research Application System) by writing the ethics application and by writing the participant information sheets for the trial. This also involved attending the ethics committee hearing for the trial. The participant information sheet for part C can be viewed in the appendix (please refer to section 9.1).

During meetings focused on the trial protocol, I was also involved in discussions regarding the development of the assays to be used on the trial. However, I did not participate in these assays during the trial to prevent unblinding.

I was heavily involved in the clinical work on the trial, including screening and consenting participants for the trial and then supervising each participant as they went through the trial. This involved dosing and monitoring of the participants whilst helping to review results from clinical investigations and responding to adverse events. I drafted the clinical safety report prior to each of the dose escalation committee meetings which would help to inform those present whether to proceed onto the next dose level. I gathered this information using the PIMS (Phase 1 Management System) computer system at GSK, which has all the information on dosing, adverse events and the investigations carried out on subjects during the trial. This information would include my opinion on if a particular adverse event was

related to the drug. I would present at the dose escalation committee meetings the main clinical findings. I would also participate in the discussions, however the final decision on whether to proceed to the next dose would be made by the principal investigator and the medical monitor for the trial.

I was not involved in the preclinical toxicology studies on cynomolgus monkeys that led to some of the decisions made on minimum anticipated biological effect level (MABEL) for the initial doses used in Part A. I was also not involved in the laboratory assays (to prevent unblinding of subjects during the trial), which led to some of the results given, and also which helped to inform the discussions at the dose escalation meetings during the trial.

The original intention was that I would lead the trial through to completion but unfortunately the study was terminated early, due to concerns within GSK about the data on which the study rationale was based. This meant that I was not able to complete all the aims from this part of my research.

3.2.2 TRIAL OUTLINE

I have given here a broad outline of the trial design. For a more detailed description of each part of the trial please refer to chapter 2.9.2.

The study was due to be conducted in three parts. Part A in 24 healthy individuals with single ascending doses of the drug being given. This part was designed in such a way that one individual could receive two different doses of the drug after a period where the drug was deemed to have been cleared and was not having any on-going effects in the body (predicted to be 12 weeks for this study although in this adaptive trial design this could change). Part B would have been in 12 or 24 healthy volunteers with repeat doses given to individuals. Part C was due to be in 20 multiple sclerosis patients where repeat doses of drug would have been given.

Study subjects for Parts A and B were recruited via the GSK recruitment team. They were healthy men screened to make sure they had no underlying illness or intercurrent infection. Screening and consent for the trial ensured they fit the inclusion criteria for the trial as outlined in the trial protocol. Multiple Sclerosis patients for Part C were due to be recruited nationally from referral from neurologists

to Cambridge for consideration of the trial. Demographics for the subjects used in the trial are shown in the results section.

Throughout the study clinical data would be collected by interview, with the use of investigations such as ECGs; with laboratory data looking at the full blood count (particularly lymphocyte counts), liver and renal function. Blood samples were drawn for laboratory analysis of pharmacokinetics (PK), receptor occupancy (RO), phosphorylated STAT5 (pSTAT5) and lymphocyte subsets (determined by flow cytometry).

Parts A and B were due to be a double-blind, placebo-controlled, randomised trial. A dose escalation committee comprising of the Principal Investigator, medical monitor and key trial staff (including myself), would review dose escalations. Part C would be an open-label study.

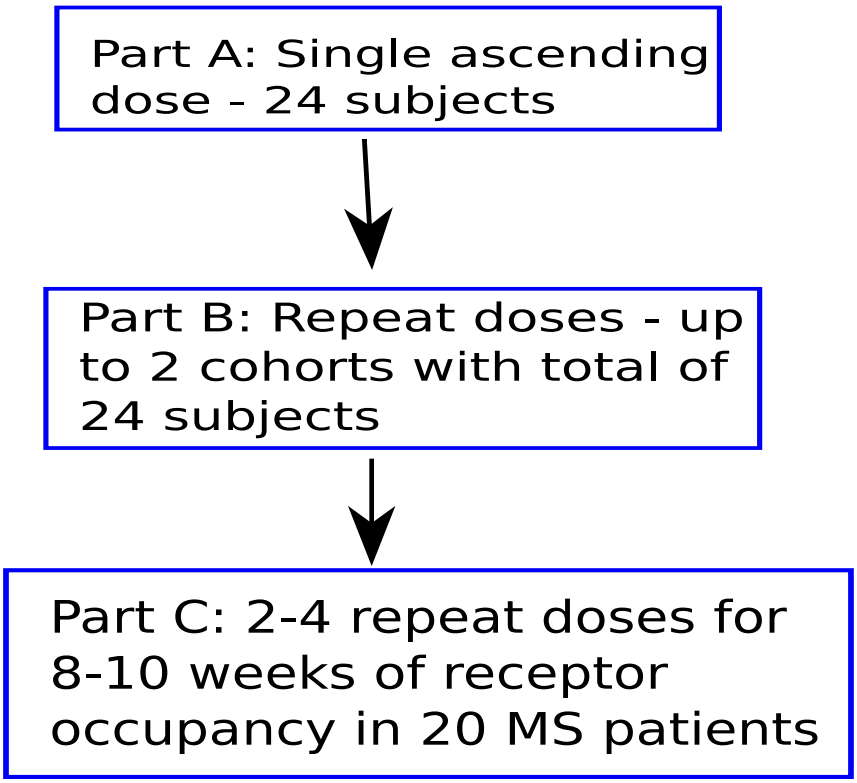

Figure 3. 1: Outline of Trial with Parts A, B and C

'Receptor occupancy' refers to binding of GSK2618960 at the membrane-bound IL-7 receptor.

3.3 GSK TRIAL RESULTS

Although the overall intended methods of the trial have been presented, in fact the study was terminated after the fourth dose level (0.15mg/kg) in Part A due to data misrepresentation in an important preclinical study (Liu et al., 2013).

3.3.1 DEMOGRAPHICS

Participants in the trial were recruited by GSK. The demographics of the study population can be viewed in table 3.1 below.

Age in Years, Mean (SD)	38.9, (5.86)
Sex, n	
Female:	0
Male:	16
BMI (kg/m^2), Mean (SD)	25.53, (2.580)
Height (cm), Mean (SD)	179.00, (5.831)
Weight (kg), Mean (SD)	82.06, (11.492)
Race, n	
Asian – South East Asian Heritage	1
White – White/Caucasian/European Heritage	14
Mixed	1

Table 3. 1: the demographics of the population in the first time in human trial

3.3.2 CLINICAL SAFETY RESULTS

16 patients entered the trial before trial termination. There were 4 dose levels undertaken during the trial – subjects received 0.001 mg/kg (or placebo) as an intravenous bolus dose (subjects 1001, 1002, 1003, 1004), 0.006 mg/kg (or placebo) as an intravenous infusion for 5 min (subjects 2001, 2902, 2003, 2004), 0.03mg/kg (or placebo) infusion for one hour (subjects 3001, 3002, 3003, 3004) and 0.15mg/kg (or placebo) infusion for one hour (subjects 4001, 4002, 4003, 4004).

There were 19 Adverse Events in total. There were no Serious Adverse Events. Three adverse events were as a result of the study drug. For detailed adverse event statistics please refer to table 3.2.

Subject 3003 had an irregular broad complex tachycardia lasting for 7 beats at 200 beats per minute at approximately 11 hours post dosing. This subject was asymptomatic with no evidence of QT prolongation or any other morphological change on 12-lead ECG monitoring. The Cardiology department reviewed this subject and no further action was deemed necessary. This adverse effect was unrelated to the study drug.

Two subjects had a lymphopenia (subjects 2001 and 3002). Lymphopenia was a theoretical side effect that had been predicted prior to the trial commencing, as an IL-7Rα antagonist would prevent proliferation of T lymphocytes. However, both of these episodes resolved without any specific action taken. Subject 2001 (0.006 mg/kg GSK2618960) had a baseline lymphocyte count of 1.49 $x10^6$ cells/μL (normal range 1.2-3.65 $x10^6$ cells/ μL) and this reduced to 1.02 $x10^6$ cells/μL on day 8 and resolved by day 30. This adverse effect was due to the study drug although the reduction in lymphocyte counts was so small it is difficult to be certain of this conclusion.

Subject 3002 (placebo) had a baseline lymphocyte count of 1.84 $x10^6$ cells/μL that reduced to 0.66 $x10^6$ cells/μL on day 8 and resolved by day 41. This was related to a concurrent viral illness and not due to the study drug.

There were no adverse events, which led to subject withdrawal. All adverse events were resolved prior to study completion, with the exception of one episode of seasonal allergy, which was resolving at the time of follow up.

Adverse Event	Placebo (n=6)	GSK2618960 0.001mg/kg (n=1)	GSK2618960 0.006mg/kg (n=1)	GSK2618960 0.03mg/kg (n=1)	GSK2618960 0.15mg/kg (n=1)	Total (n=19)
Lymphopenia	1	0	1*	0	0	2
Tachycardia	0	0	0	1	0	1
Vomiting	1	0	0	0	0	1
Catheter site	0	0	1	0	0	1
Seasonal allergy	2	0	0	0	0	2
Nasopharyngitis	1	0	1	0	1	3
Hand fracture	0	0	1	0	0	1
Laceration	0	0	1	0	0	1
Neck pain	0	0	1	0	0	1
Dysgeusia	0	0	0	1*	0	1
Headache	0	1*	0	1	0	2
Paraesthesia	0	0	0	1	0	1
Oropharyngeal	0	0	0	1	0	1
Rhinorrhea	1	0	0	0	0	1

Table 3. 2: Adverse Events during the Trial

The table is presented as the total number of subjects reporting the event for each dosing session. Asterisked numbers indicate drug related adverse effects.

3.3.3 PHARMACOKINETIC RESULTS

These pharmacokinetic (PK) results were measured from plasma samples. I reviewed the PK results as part of the dose escalation committee of the study. I did not partake in the PK assay. PK results enabled investigators to examine how long the drug was present within the body and if this was coherent with the predictions made from the pre-clinical studies. This would give investigators information on the length of time the drug was active within humans.

Due to the limited plasma concentrations of the study drug GSK2618960 at low doses, pharmacokinetic parameters were only available for the two higher dose levels (0.03 mg/kg and 0.15 mg/kg). Subjects within the first dose level of 0.001mg/kg did not reach the lower limit of quantification (LLQ) for pharmacokinetic results to be derived. One subject reached the LLQ on the second dose level of 0.006mg/kg and therefore only one result was available at one-hour post dose.

Unexpectedly only 2 subjects (rather than the expected 3 subjects with one subject treated with placebo) were given the study medication for the third dose level of 0.03mg/kg (an internal investigation took place to investigate why this happened but no clear explanation was found for why two subjects in this group were dosed with placebo). The maximal concentration of the drug (Cmax) was about half of what was predicted from the previously calculated mathematical modeling of the preclinical monkey data.

For dose level 0.15mg/kg, maximal concentrations of the study medication were close to what was predicted (Cmax 1820 ng/ml to last for 60 hours). However the drug was cleared more quickly than expected, so the terminal phase predictions were incorrect. The study medication was cleared between 48 and 72 hours (please refer to figure 3.2).

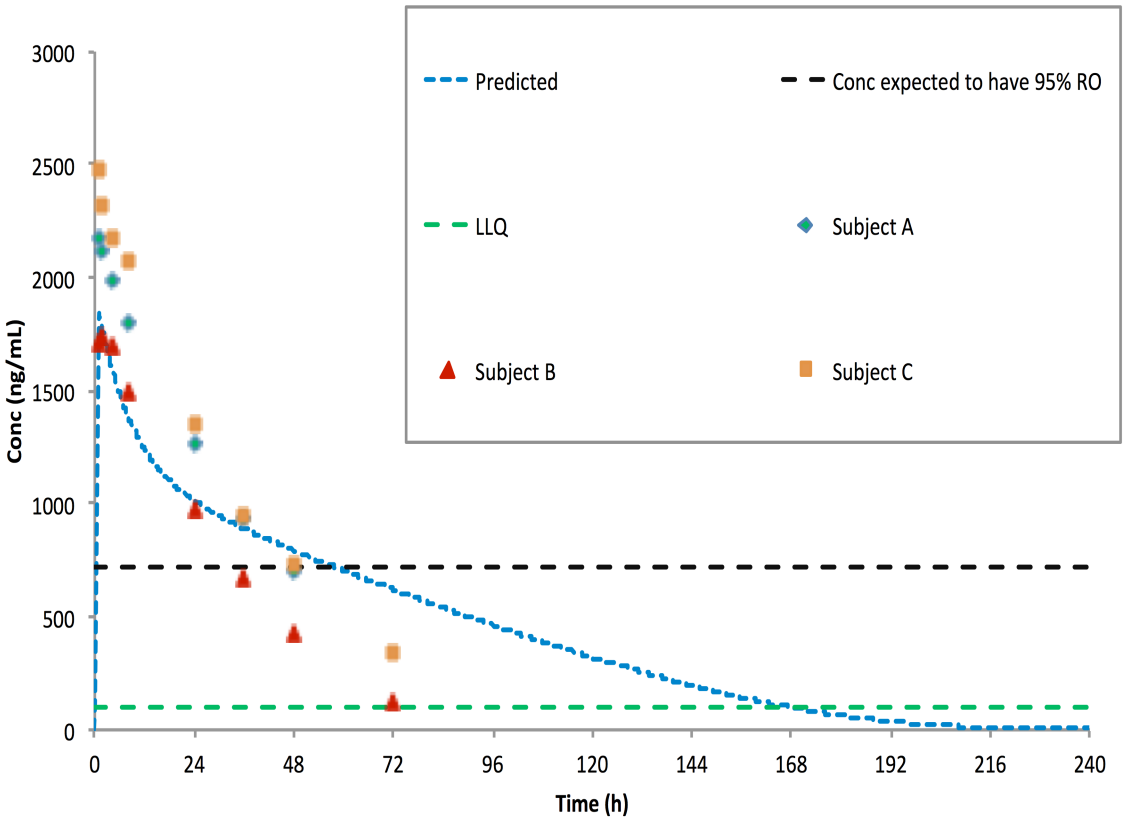

Figure 3. 2: Predicted and Observed Pharmacokinetics at 0.15mg/kg

Maximal concentrations of the drug were close to what was predicted from pre-clinical studies, the drug was cleared more quickly than expected between 48-72 hours. 3 subjects (A, B, and C) received the study drug. Y-axis represents the concentration of the drug GSK2618960 (ng/ml) with the x-axis representing time post dosing in hours. The green dash line represents the lower limit of quantification (LLQ). The black dash line represents the concentration of the drug expected from 95% receptor occupancy (RO) of IL-7Rα by GSK2618960. The blue dash line represents the predicted concentration of GSK2618960 (ng/ml) from pre-clinical studies.

3.3.4 PHARMACODYNAMIC RESULTS

3.3.4.1 RECEPTOR OCCUPANCY OF IL-7Rα WITH GSK2618960

Results from Receptor Occupancy (RO) of GSK2618960 at the IL-7 receptor were reviewed during the dose escalation committee meetings. The RO showed if the drug was binding to T cells and how long this lasted. This enabled the investigators to see if the drug bound to its target and how long it was having an effect on T cells.

For the 3 subjects dosed at the first dose level of 0.001mg/kg receptor occupancy was measured at 10.6%, 5.4% and 0.3% at 1 hour post dose. It is difficult to comment on receptor occupancy results with such a small dose of GSK2618960.

For the 3 subjects dosed at the second dose level of 0.006mg/kg receptor occupancy values 1 hour post dose was between 66.1 and 82.1%, with a mean RO of 75.0%. At 4 hours post dose, the mean RO was 53.0%. At 24 hours post dose, the mean RO was less than 10%.

For the 2 subjects dosed with 0.03mg/kg, greater than 90% RO was found at the 1 and 4 hours post-dosing, decreasing to 50.0% at the 24 hours post-dosing.

For those subjects who received 0.15mg/kg, full receptor occupancy (>90%) was seen for 24-48 hours. At 72 hours, RO values for the 3 subjects were 90.6, 60.4, and 91.5% (Please refer to figure 3.3).

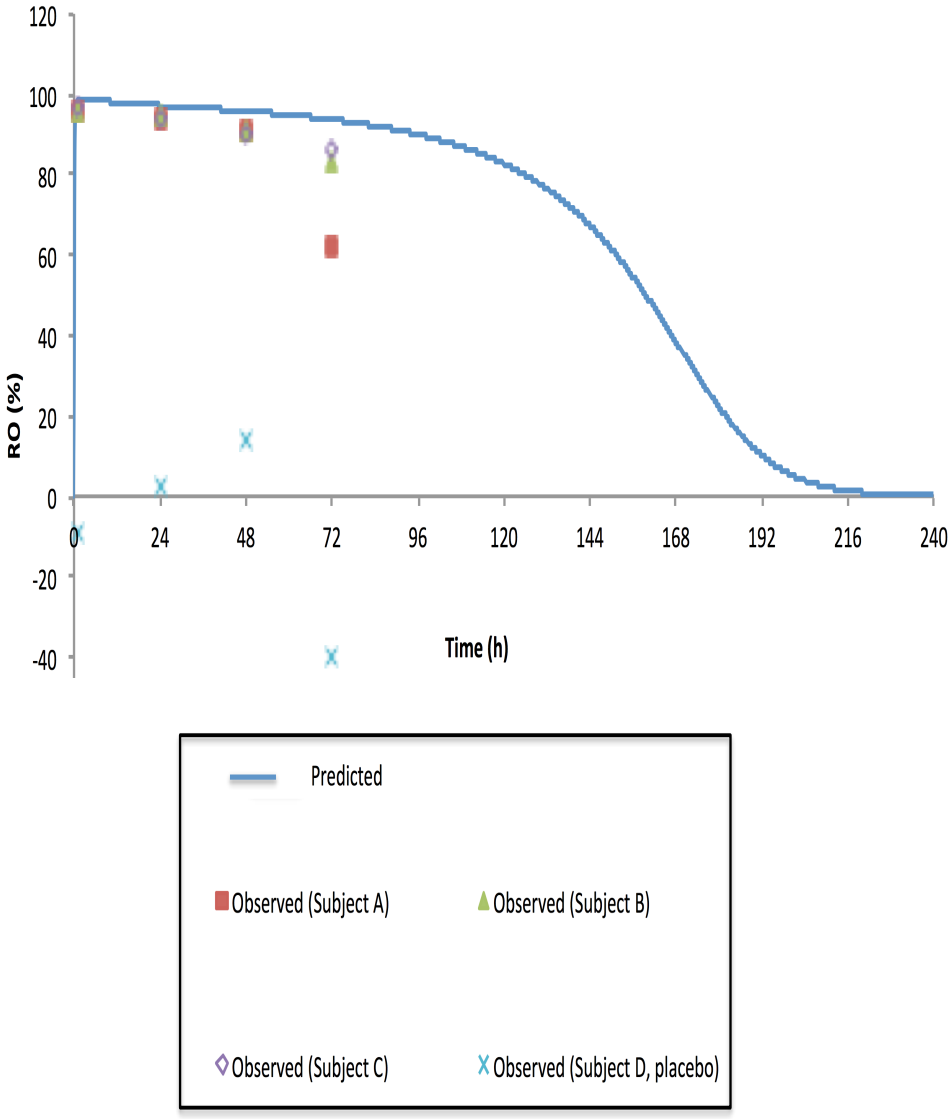

Figure 3. 3: Predicted and Observed Receptor Occupancy (RO) at 0.15mg/kg

Subjects A, B and C received the study drug. Subject D received placebo. The Y-axis represents the percentage of IL-7 receptors occupied by GSK2618960. The X-axis represents the time post dosing in hours. The blue line indicates the predicted RO percentage of GSK2618960 from pre-clinical studies. Subjects dosed with GSK2618960 demonstrated RO >90% for 48 hours.

3.3.4.2 PHOSPHORYLATED STAT5

Other pharmacodynamic markers measured by flow cytometry such as phosphorylated STAT5 (pSTAT5) and lymphocyte subsets were not yet available at the time of the dose escalation meetings but were available from the final clinical study report. I was not involved in the assays or the analysis of this data.

For pSTAT5 (a classical marker for downstream signaling from IL-7 receptor activation by IL-7) data from subjects dosed with 0.15mg/kg showed that mean pSTAT5 inhibition was greater than 90% up to and including 72 hours post-dosing.

As a consequence of early termination of the trial, the limited number of data points for lymphocyte subsets, plasma soluble IL-7Rα and plasma IL-7 precludes meaningful interpretation.

3.3.4.3 IMMUNOGENICITY

I was not involved in measuring anti-drug antibodies, however anti-drug antibodies were not detectable at day 1, day 15 or week 8 in any of the subjects.

Interestingly in a follow-up study (Ellis et al., 2019) anti-drug antibodies were detected in 5/6 subjects administered 0.6 mg/kg and in 6/6 subjects administered 2.0 mg/kg GSK2618960.

3.4 THE PREMATURE TERMINATION OF THE TRIAL

I first learnt that there was a problem with the continuation of the trial on the 7th June 2013. This was from telephone calls from both principal investigators for the trial Joseph Cheriyan and Alasdair Coles.

They alerted me to the accusations that had been made on internet forums of fraudulent data concerning some of the preclinical data in the lead up to the trial from China (FierceBiotech, 2013) (McBride, 2013a). The rest of the clinical team on the trial were informed after the final dose escalation committee meeting on the 13th June 2013. For this meeting the medical monitor of the trial Frank Gray was present along with the Scientific Lead for the trial Paul Thompson. Marina Zvartau-Hind represented Senior GSK management.

GSK were aware of the allegations on 31st March 2013 after an internal allegation was made (before the first subject was dosed). At this stage they had an internal investigation of this allegation but comments made by senior GSK management to the GSK Clinical Trials Unit in Cambridge suggested that from 'time to time they received allegations' and after an initial inquiry into the matter, GSK's leadership did not see that it was necessary to launch any further investigations. GSK's leadership certainly did not see the need to stop the trial before a subject had been dosed. This information was revealed during a meeting with the Cambridge GSK Clinical Trials Unit on 13th June 2013.

It is difficult to know if the allegations made over the internet were more substantial than the allegations made at the end of March, however GSK's leadership were now of the opinion that the trial needed to be stopped immediately before any further subjects were dosed (McBride, 2013b) and the author of the Nature Medicine paper in question (Liu et al., 2010) (Liu et al., 2013) was suspended pending further investigation (GlaxoSmithKline, 2013). Those aware of the allegations about the veracity of some of the data, which according to GSK was fundamental to the rationale of this trial, did not inform the Principal Investigators until many months later. In the meantime healthy volunteers were exposed to the drug during this time period.

At this stage GSK launched a more thorough inquiry into the data misrepresentation. They revealed in the meeting on 13th June 2013 with the other members of the

Cambridge GSK Clinical Trials Unit that it was believed the misrepresented data was not of the experimental allergic encephalomyelitis mouse data but of the in vitro multiple sclerosis patient data which was actually healthy volunteer data (Liu et al., 2013). If this was the case then the scientific rationale for the trial was still very strong and would not in itself have meant that the trial needed to be terminated.

GSK approved all other work to continue on the IL-7/IL-7Rα pathway on the 16th July 2013 after an initial termination of all work on the pathway within the company. Work has already started on seeking approvals for a new study, again looking at healthy volunteers in Parts A and B, with the new potential indication of psoriasis as a potential therapeutic target instead of multiple sclerosis.

From the multiple sclerosis point of view it is very disappointing that GSK have, for now, dropped their interest in this disease. It is very difficult to know why this is the case based on scientific reasoning, particularly as the EAE mouse data is thought to be good data which has also been reported by another group (Lee et al., 2011). This however would not take into account the political pressure that was on GlaxoSmithKline due to the nature of this fraudulent material entering the public domain. It is true to say GSK were always planning to use the drug for other indications such as Psoriasis and Inflammatory Bowel Disease but this does not explain the lack of interest now seen in multiple sclerosis. Again in the meeting held with the Cambridge Clinical Trials Unit team on 13th June 2013, no clear explanation was given for the change in indication for the drug, although the perception in the wider public may be any further work in multiple sclerosis by GSK may always be clouded by the perception of scientific fraud.

3.5 DISCUSSION

More information is required to know if this drug will proceed further in the drug discovery process. Another trial in healthy volunteers was completed (Ellis et al., 2019), as there was insufficient data to reach firm conclusions due to the premature conclusion to the trial due to fraud in some of the pre-clinical trial data (Liu et al., 2013).

The conclusion we can draw from this first-in-human trial of 16 patients dosed in this curtailed study is that so far the drug is safe and tolerable. There were no deaths or serious adverse events. There were no adverse events that led to the withdrawal from the trial of a study subject, and no infusion reactions or acute immune system stimulation was observed. The mild lymphopenia seen in one subject was short-lived. It remains to be determined if at higher doses this would have become more of a problematic side effect. Having a low lymphocyte count would mean great care would have to be taken of the subject's long-term risk of potential opportunistic infections. No opportunistic infections were acquired as a result of the short-term lymphopenia in one subject as a result of the study drug in this cohort.

Some pharmacokinetic data was derived from the study with a suggestion that the drug was cleared more quickly than had been predicted from preclinical monkey studies. Although no evidence of anti-drug antibodies could be found perhaps the immune system had a role in removing the drug from the body as anti-drug antibodies were found in pre-clinical testing in cynomolgus monkeys (Leung et al., 2012). With the relatively short follow-up period of eight weeks when anti-drug antibodies were measured, and a failure of the study to reach the repeat dosing stage it is not possible to say if GSK2618960 at the dose level used in this study, is capable of producing anti-drug antibodies in humans. It is interesting to note the appearance of anti-drug neutralising antibodies at slightly higher doses of the study drug (Ellis et al., 2019). In this follow-up study they were unable to comment whether the anti-drug antibodies contributed to the faster than predicted clearance of GSK2618960, as anti-drug antibodies were only detected after 21 days, which coincides with the expected clearance for IgG monoclonal antibodies such as GSK2618960.

Although few firm conclusions can be made about this drug due to the premature termination of the drug trial, this early work with the drug does not reduce its potential

to be a drug which could benefit multiple sclerosis and other autoimmune diseases in the future if further drug trials are conducted of this antibody or similar antibodies targeted to the IL-7 receptor. However the appearance of anti-drug antibodies in this subsequent study would severely limit the potential of GSK2618960 as a potential therapeutic option for MS patients.

CHAPTER 4 - ANTAGONISM AND STIMULATION OF THE IL-7 RECEPTOR

4.1 INTRODUCTION AND AIMS

This chapter describes the laboratory work I have done during my PhD, which consists of two main projects (i) understanding whether IL-7Rα genotype affects T cell responses to GSK2618960, a novel IL-7Rα antagonist being developed by GSK as a potential new treatment for autoimmunity and (ii) whether IL-7Rα genotype affects IL-7 bioavailability and signaling *in vitro*. Each project will be introduced below.

Prior to the IL-7Rα trial with GSK2618960 there were concerns about worsening of disease activity with antagonism of the IL-7 receptor, despite the evidence with use of the drug in EAE mice demonstrating disease improvement with IL-7Rα antagonism. This stems back to the 2007 paper (Gregory et al., 2007), which depicted greater soluble IL-7Rα in the at risk group with potentially less cell-associated IL-7Rα and potentially a lower level of IL-7 baseline activity in this group. This pharmacogenomic work could also identify genetic groups in which the drug was more dangerous or potentially more effective.

The aim of the *in vitro* work was to explore the effects the SNP rs6897932 had on the IL-7Rα pathway. This would explore the increase in soluble IL-7Rα and investigate if this reduced cell signaling through the IL-7 pathway. In the Lundstrom paper it points towards the opposite effect with increased bioactivity of IL-7 over time with the increased soluble IL-7Rα levels with the at risk SNP for rs6897932 (Lundstrom et al., 2013).

Increased soluble IL-7Rα may not be the sole method by which IL-7 signaling is altered. The at risk SNP causes an amino acid change from threonine to isoleucine at codon 244 (T244 to I244). Threonine is a polar amino acid with isoleucine being hydrophobic. This could lead to a change in signaling strength (Mazzucchelli et al., 2012). The at risk group could exhibit increased signaling through IL-7Rα which could be an alternative method of augmenting IL-7 bioactivity. Gain of function mutations have been shown to act as oncogenes in T-ALL and B-ALL (Shochat et al., 2011). These mutations are generally insertion of bases encoding a cysteine and a proline into exon 6 of IL-7Rα in the extracellular domain at the border with the

transmembrane region close to the T244I residue. The cysteines form divalent bonds causing homodimerisation of IL-7Rα causing ligand independent signaling through the IL-7Rα pathway. This first example of a gain of function mutation causing increased signaling points towards another mechanism for potentiating the IL-7Rα signal in the at risk group for rs6897932.

Lundstrom et al also demonstrated differences in negative feedback pathways of IL-7Rα with increased levels of soluble IL-7Rα (Lundstrom et al., 2013). The two major examples of these pathways are downregulation of IL-7Rα and upregulation of the Fas Ligand receptor (CD95), which causes programmed cell death. Lundstrom demonstrated reduced upregulation of CD95 with reduced Fas-mediated cell death in vitro with increased soluble IL-7Rα levels. Therefore soluble IL-7Rα changed the normal upregulation in CD95 that occurs with increased levels of IL-7. Downregulation of IL-7Rα was associated with increased levels of soluble IL-7Rα, mimicking augmented IL-7 bioactivity. In the second part of this chapter these pathways are explored with *in vitro* IL-7 stimulation in genetically stratified populations according to the SNP rs6897932.

This chapter will investigate not only if there are differences between the genetic groups with antagonism of IL-7Rα, but in the second part of this chapter with *in vitro* rhIL-7 stimulation are there differences in IL-7 physiology between the genetic groups, particularly in downstream signaling and negative feedback mechanisms of the IL-7 receptor.

4.2 PROJECT 1: THE EFFECT OF IL-7Rα GENOTYPE T CELL RESPONSES TO GSK2618960

As discussed in the main introduction, IL-7 has been implicated as a cofactor in a number of autoimmune diseases – including lupus (Gonzalez-Quintial et al., 2011), EAE (Lee et al., 2011) and autoimmune diabetes (Penaranda et al., 2012).The mechanism by which IL-7 leads to autoimmunity is not fully understood, but is believed to include promoting T cell survival, and enhancing the proliferative responses of T-cells to weak self-antigens (Fry and Mackall, 2005). Given this, IL-7 has been considered as a potential therapeutic target.

GSK2618960 is humanized IgG1 monoclonal antibody, developed by GSK, which binds to the extracellular domain of human IL-7Rα, which as part of a heterodimer with the common gamma receptor forms the IL-7 receptor. It functions as an antagonist, competitively inhibiting IL-7 binding and therefore downstream phosphorylation of STAT5 (Leung et al., 2012). Although a significant amount of pre-clinical work had been done by GSK prior to the start of my PhD, no one had asked whether or not the effect of GSK2618960 was influenced by IL-7Rα genotype.

This *in vitro* project was performed in parallel with a first-time-in-human clinical trial using GSK2618960 in which I was the chief sub-investigator. The trial is discussed in Chapter 3.

4.3 METHODS

This section provides an overview of the experimental methods used for each part of this chapter. For more detailed information about the methods used please refer to the methods section in chapter 2.

4.3.1 PARTICIPANTS

Healthy subjects from the GSK volunteer panel donated blood for the project according to IL-7Rα genetic stratification as outlined in the methods section 2.1. However I will briefly revisit why the panel was stratified by IL-7Rα in the manner used during this study.

The 'at risk' allele for soluble IL-7Rα is the C allele for rs6897932. This is in high linkage disequilibrium with all identified SNPs in IL-7Rα for multiple sclerosis and all other autoimmune diseases. In the Lundstrom paper in 2013, they demonstrated that there was an allele dose effect on soluble IL-7Rα levels measured in the serum, when comparing the differing genotypes CC (homozygous at risk), CT and TT (homozygous protective) at rs6897932. The Lundstrom paper had previously demonstrated the highest levels of soluble IL-7Rα with homozygous CC at rs6897832 and the lowest levels of soluble IL-7Rα with homozygous TT at rs6897932. The heterozygous CT showed intermediate levels of soluble IL-7Rα and therefore inclusion of this would reduce the power of the effect that was being investigated.

As the effects of soluble IL-7Rα on IL-7-IL-7Rα physiology were being investigated and how this was driven by the different genotypes of rs6897932, I decided to compare the 'at-risk' group (homozygous CC at rs6897932) with the 'protective' group (homozygous TT at rs6897932) in order to enhance the power of the effect being investigated.

There were two flow cytometry assays, the receptor occupancy assay and the phosphorylated STAT5 assay. There were two enzyme-linked immunosorbent assays (ELISAs) for soluble IL-7Rα and IL-7.

4.3.2 DETERMINING THE RECEPTOR OCCUPANCY OF IL-7Rα BY GSK2618960

For the receptor occupancy assay and the ELISAs 30 subjects in total, 10 from each genotype group, were investigated. For an overview of the receptor occupancy assay please refer to figure 4.1. For more detailed information on the receptor occupancy assay and the ELISAs please refer to the methods section 2.4 and 2.7.

4.3.3 MEASURING PSTAT5 FOLLOWING BINDING OF GSK2618960

For the pSTAT5 assay the investigation was initially on 30 subjects but following a preliminary analysis a power calculation to 90% power indicated to reach statistical significance further subjects would need to be added. Therefore the pSTAT5 assay was completed on 41 subjects in total. For an overview of the pSTAT5 assay please refer to figure 4.2.

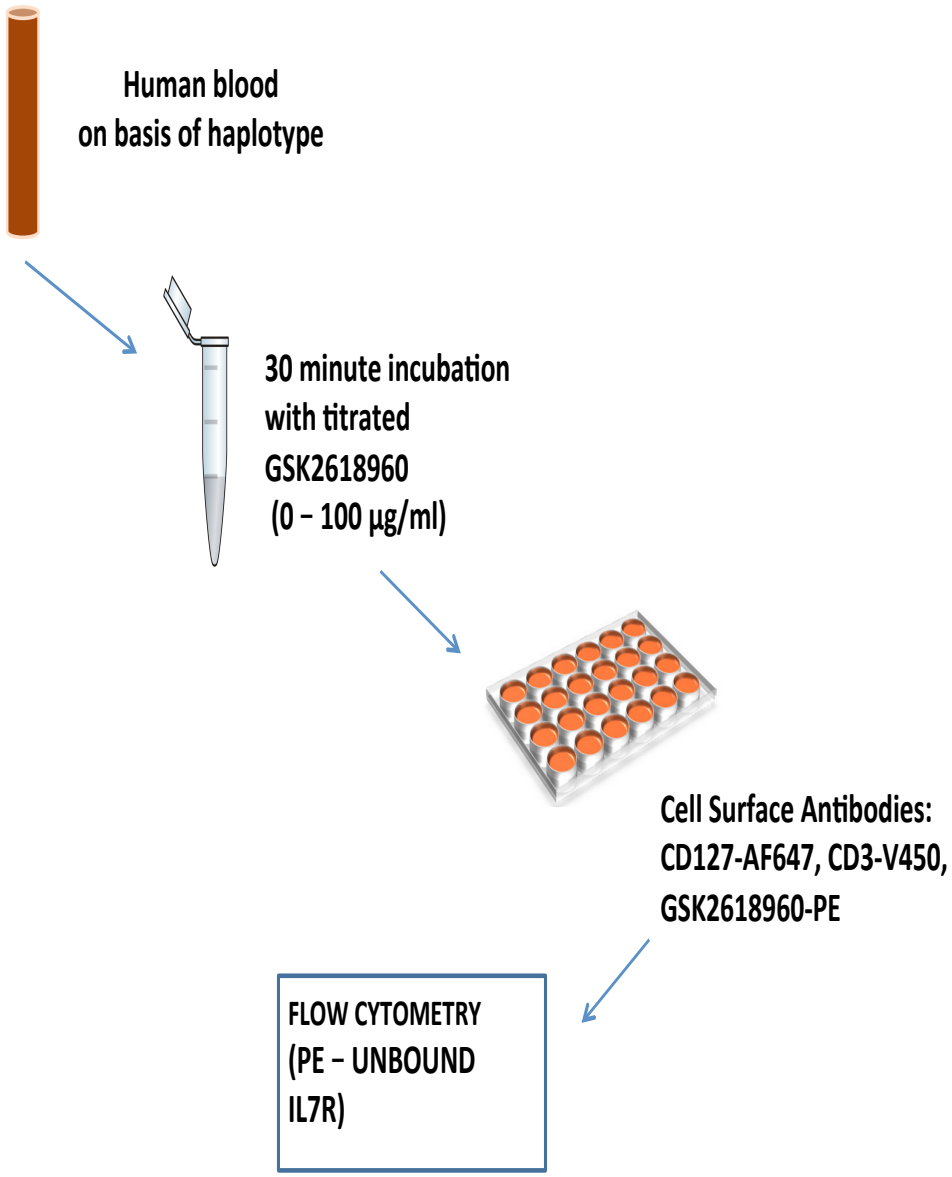

Figure 4. 1: Receptor Occupancy Assay

A PE labeled GSK2618960 IL-7Rα antagonist was used to identify the percentage of unbound IL-7Rα receptors following incubation of the subjects' blood with unlabeled GSK2618960 IL-7Rα antagonist.

T lymphocytes were identified by antibody staining of CD3+ cells (CD3 V450). The IL-7 receptor (CD127 AF647) was also stained (this antibody binds to a different epitope on IL-7Rα from GSK2618960). PE labeled GSK antibody was added to identify unbound IL-7 receptors.

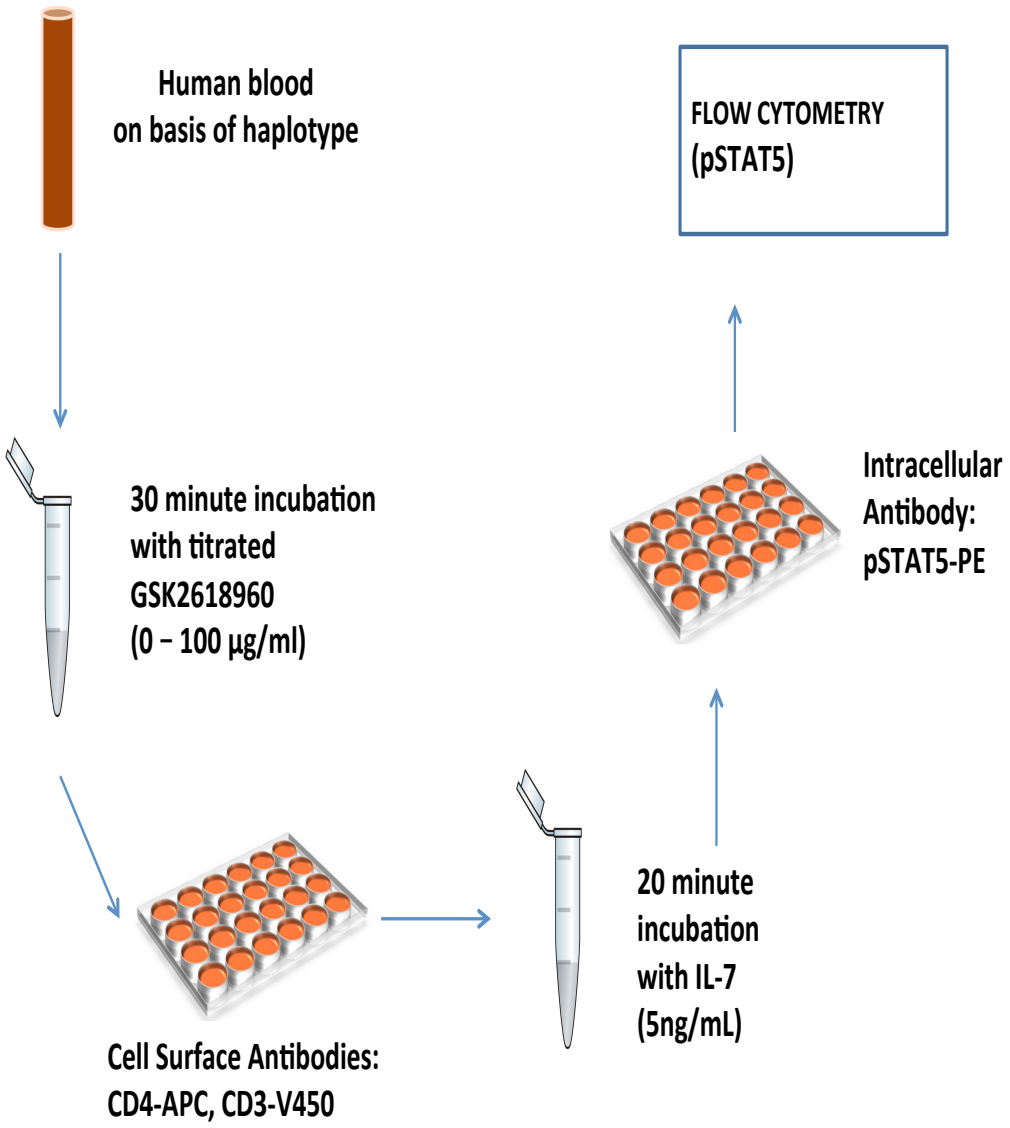

Figure 4. 2: pSTAT5 assay

Whole blood was incubated with GSK2618960 for 30 minutes. Cell surface antibody staining (CD3 V450 and CD4 APC) was followed by a 20-minute incubation with IL-7 prior to intracellular staining with pSTAT5.

4.4 RESULTS

4.4.1 SERUM SOLUBLE IL-7Rα

First I attempted to replicate the reported effects of IL-7Rα genotype on serum soluble IL-7Rα levels in my patient cohort (n=22). Using ELISA, serum soluble IL-7Rα was found to be significantly higher in subjects in the at risk groups compared to the protective group (Figure 4.3 mean 78.6 ng/mL vs 29.7 ng/mL; $p < 0.0001$).

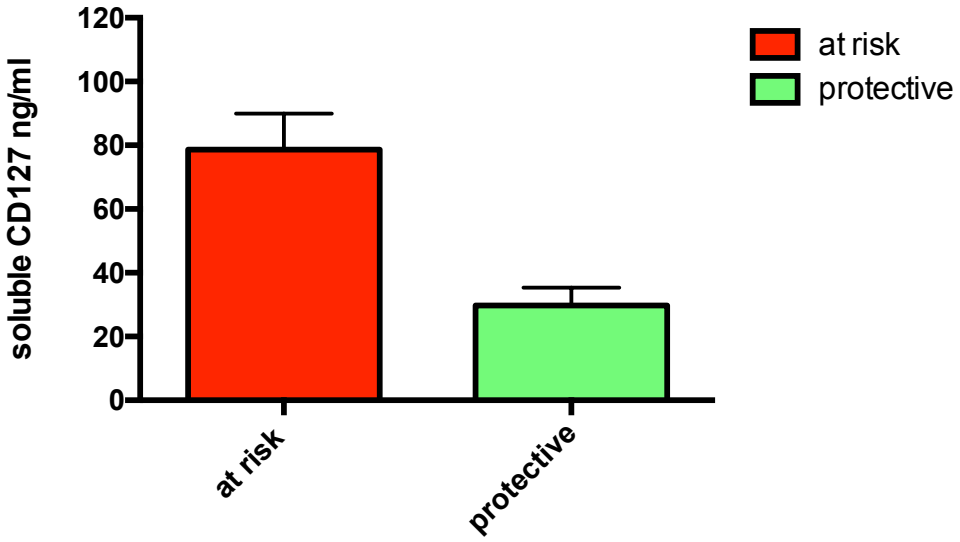

Figure 4. 3: ELISA of serum soluble IL-7Rα

This confirms the genetic differences in soluble IL-7Rα levels based on IL-7Rα genetic stratification by genotype with higher levels in the at risk group compared to the protective group (mean 78.6 ng/mL vs 29.7 ng/mL; $p < 0.0001$). Each genetic group is represented on the x-axis (at risk group 14 subjects, protective group 8 subjects, total number = 22) and soluble IL-7Rα (CD127) on the y-axis. The error bars indicate the 95% confidence interval.

4.4.2 SERUM INTERLEUKIN 7

Next I went on to determine if serum IL-7 varied by IL-7Rα genotype as had previously been reported (Lundstrom et al., 2013). IL-7 was measured by ELISA. There was no significant difference in this cohort (n=29) between those in the at risk group compared to subjects in the protective group, although there was a trend for increased IL-7 levels in the at risk group, similar to what was discovered in the Lundstrom paper (Figure 4.4, mean 8.22 pg/mL vs 6.02 pg/mL; p = 0.0643).

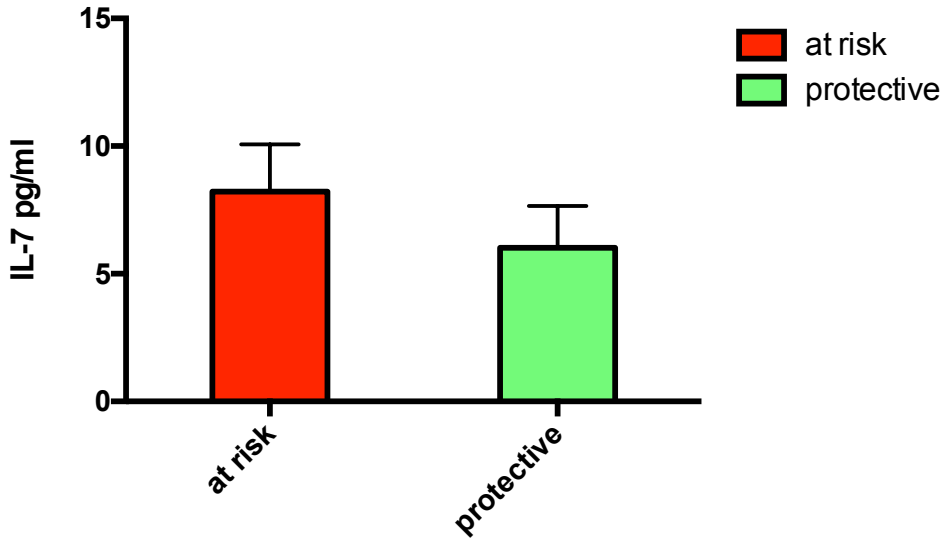

Figure 4. 4: ELISA of serum IL-7 levels based on IL-7Rα genotype

There was no significant difference between the genetic groups, although there was a trend for higher IL-7 levels in the at risk group (mean 8.22 pg/mL vs 6.02 pg/mL; p = 0.0643). Each genetic group is represented on the x-axis (at risk group 19 subjects, protective group 10 subjects, total number = 29) and IL-7 on the y-axis. The error bars indicate the 95% confidence interval.

4.4.3 EXPLORING THE EFFECT OF GSK2618960 ON IL-7Rα EXPRESSION AND DETERMINING IF THIS IS AFFECTED BY IL-7Rα GENOTYPE

The data presented below was obtained by performing receptor occupancy assays, and was analysed with the help of Mr Philip Overend, a statistician from GSK.

In brief the receptor occupancy assays involved incubation of whole blood with unlabeled titrated GSK2618960 followed by staining for CD3+ T cells and IL-7Rα to measure overall expression of total surface IL-7Rα on T cells. PE labeled GSK2618960 was then added to determine the overall expression of unbound IL-7Rα. The inverse of this expression determined the overall receptor occupancy of IL-7Rα with unlabeled GSK2618960. Please refer to figure 4.1 for an overview of this assay.

From this assay I was able to determine (i) IL-7Rα surface expression of untreated cells, and how this varied by genotype (ii) how IL-7Rα expression was affected by GSK2618960 and (iii) receptor occupancy and whether it was influenced by genotype.

4.4.4 NO SIGNIFICANT DIFFERENCE IN EXPRESSION OF SURFACE IL-7Rα BY GENOTYPE

In order to compare the genotypic effect on IL-7Rα expression the groups were compared with no drug present. There was no statistically significant difference between the genetic groups. However, there was a trend for the protective group to have greater expression of IL-7Rα than the at risk groups (please refer to figure 4.5) but this did not reach statistical significance (mean MFI 1696.80 vs 1524.08; p=0.2266).

The trend for the protective group to express higher levels of IL-7Rα has been reported elsewhere (Hoe et al., 2010).

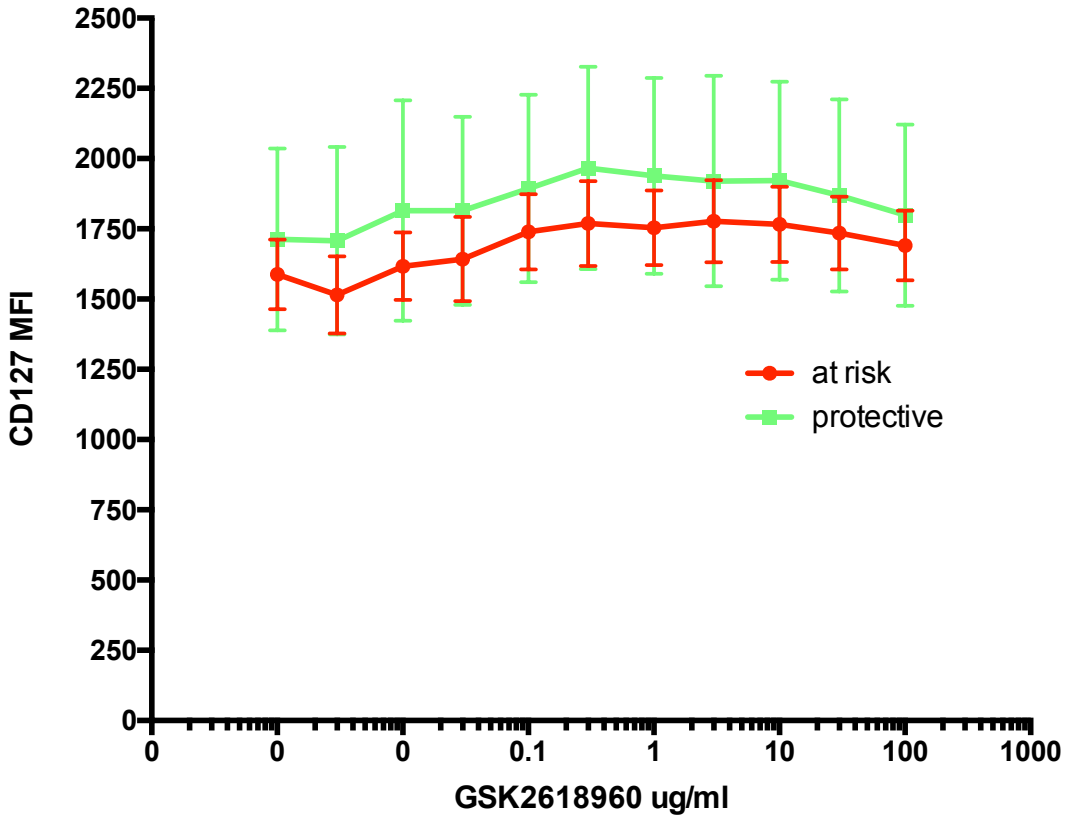

Figure 4. 5: CD127 MFI according to genetic group

This figure demonstrates a non-significant trend for greater values of cell-associated IL-7Rα (CD127) median fluorescence intensity (MFI) in the protective group (mean MFI 1696.80 vs 1524.08; p=0.2266). The MFI values for IL-7Rα are on the y-axis, with the Log10 GSK2618960 concentration on the x-axis. IL-7Rα genetics was stratified by genotype (at risk group 20 subjects, protective group 10 subjects, total number = 30).

4.4.5 GSK2618960 LEADS TO INCREASED SURFACE IL-7Rα EXPRESSION

After reviewing the data from figure 4.5, I hypothesized that increasing doses of GSK2618960 up to 0.1 μg/ml caused an initial upregulation of IL-7Rα due to a negative feedback loop from antagonism of IL-7 signaling, as a result of the blockade of signaling via IL-7Rα. From 0.1 μg/ml to maximum dosing of GSK2618960 there was a subsequent plateau of this effect.

I tested this hypothesis by analysing the effect of the drug on IL-7Rα expression by comparing the mean of IL-7Rα MFI from zero concentration of GSK2618960 to the concentration of drug that gives half-maximal response (EC50), as measured by pSTAT5, at 0.1 μg/ml. The mean of IL-7Rα MFI covering the plateau phase (from the EC50 at 0.1 μg/ml to 100 μg/ml GSK2618960) was also compared.

4.4.6 GSK2618960 INCREASES IL-7Rα MFI UP TO EC50

A paired t test was used to compare the mean of IL-7Rα MFI at zero concentration of GSK2618960 with the concentration of drug that gives half-maximal response (EC50) as measured by pSTAT5, which was 0.1 μg/ml.

This showed a significant difference between the IL-7Rα MFI at zero concentration of drug compared to the EC50 (mean MFI 1582 vs 1791; $p = 0.0215$). The difference between the groups is represented in figure 4.6.

4.4.7 GSK2618960 IS NOT RESPONSIBLE FOR THE PLATEAU IN IL-7Rα MFI BETWEEN THE EC50 AND 100 μG/ML

A paired t test was used to compare the mean of IL-7Rα MFI at 0.1 μg/ml, the EC50 of GSK2618960, with 100 μg/ml, the concentration of drug at maximum receptor occupancy.

This did not show a difference in IL-7Rα MFI between 0.1 μg/ml and 100 μg/ml (mean MFI 1791 vs 1727; $p = 0.4738$). This data is represented in figure 4.7.

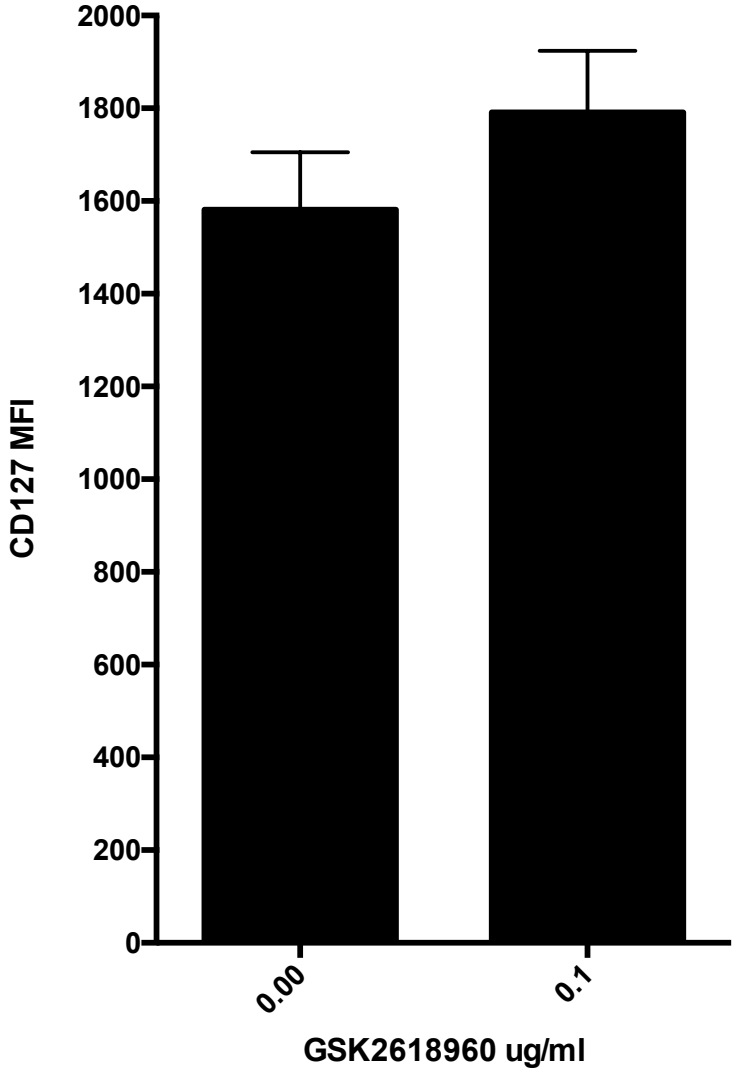

Figure 4. 6: IL-7Rα surface expression from baseline up to EC50

The mean IL-7Rα (CD127) MFI of the cohort at zero concentration and the EC50 of GSK2618960 was compared, with a statistically significant increase in CD127 MFI at 0.1 µg/ml, indicating that GSK2618960 is responsible for the increase in CD127 MFI (mean MFI 1582 vs 1791; p = 0.0215; n=30). The columns represent the mean with the 95% confidence interval.

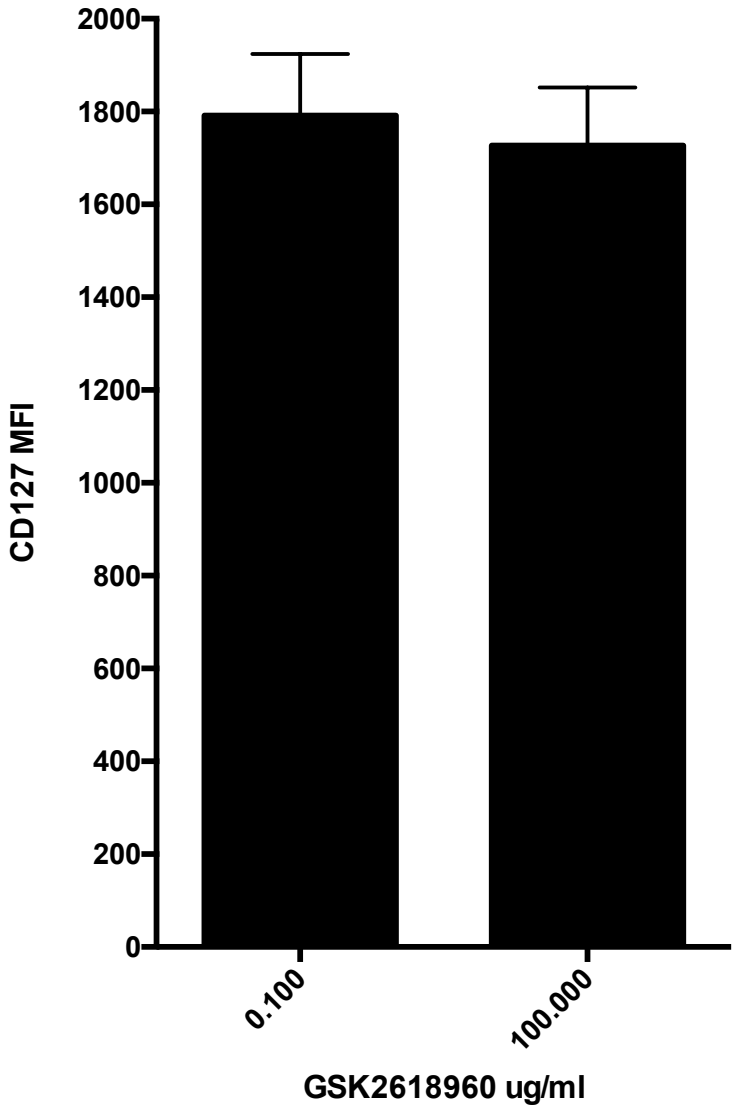

Figure 4. 7: IL-7Rα surface expression from EC50 to maximum receptor occupancy

The mean IL-7Rα (CD127) MFI at the EC50 of GSK2618960 and at maximum receptor occupancy (100 µg/ml GSK2618960) was compared, with no difference found between the groups (mean MFI 1791 vs 1727; p = 0.4738; n=30). The columns represent the mean with the 95% confidence interval.

4.4.8 RECEPTOR OCCUPANCY

Next I investigated the effect of genotype on receptor occupancy of GSK2618960. This would investigate if differences in IL-7Rα genotype would lead to a difference in binding capacity of the drug to the IL-7 receptor.

As described in the methods section, curve fitting was completed for each subject and split into four parts:

Part A - minimum receptor occupancy
Part B - the slope of the curve
Part C - the log10 EC50
Part D – maximal receptor occupancy

The data (please refer to figure 4.8) shows that as the concentration of GSK2618960 increases, as expected, receptor occupancy increases.

4.4.9 COMPARISON OF RECEPTOR OCCUPANCY BETWEEN THE GENETIC GROUPS

There were no statistical differences between the genetic groups, including for Part D of the curve at maximal receptor occupancy with GSK2618960.

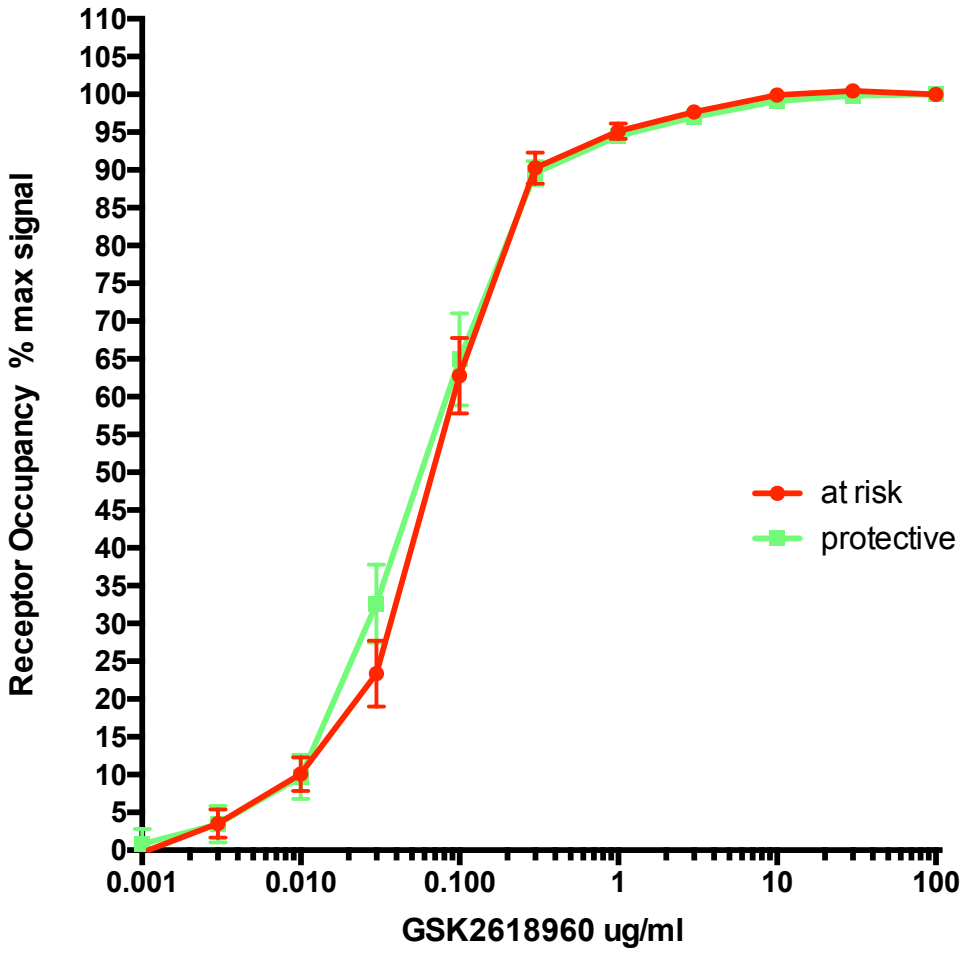

Figure 4. 8: Percentage IL-7 receptor occupancy with GSK2618960

As the concentration of GSK2618960 increased the percentage IL-7 receptor occupancy increased. This effect did not show a difference (e.g. Part D of curve at maximal receptor occupancy - mean at risk 99.33 vs mean protective 98.67; p = 0.1140) between the genetic groups (at risk group 20 subjects, protective group 10 subjects, total number =30). Calculation was from the inverse of the signal from unbound IL-7Rα MFI on the y-axis, with the Log10 GSK2618960 concentration on the x-axis. IL-7Rα genetics was stratified by genotype. Results are representative of two biological duplicates.

4.4.10 PHOSPHORYLATED STAT5 MEDIAN FLUORESCENCE INTENSITY

Next I measured pSTAT5 as a measure of downstream signalling from the IL-7 receptor. I investigated if there was a difference between the genetic groups in abrogation of signaling from the IL-7 receptor with increasing doses of GSK2618960.

As described in the methods section, curve fitting was completed for each subject and split into four parts:

Part A - maximum response of pSTAT5
Part B - the slope of the curve
Part C - the log10 EC50
Part D – minimum response of pSTAT5

The data (please refer to figure 4.9) shows that as expected, as the concentration of GSK2618960 increases phosphorylation of STAT5 decreases, due to antagonism through IL-7Rα.

4.4.11 ANTAGONISM BY GSK2618960 IS CAUSED BY PARTIAL AGONISM

At maximal receptor occupancy of GSK2618960 there continued to be stimulation through IL-7Rα, indicating that the drug works by partial agonism as evidenced by the continued signaling in Part D of the curve for the at risk group (mean 3.75) and the protective group (mean 7.03).

This was noted in pre-clinical testing before the commencement of the first-time-in-human trial. GSK2618960 was used between 0.3 and 100 µg/ml with human whole blood T cells, which demonstrated a low but significant phosphorylation of STAT5 (Leung et al., 2012).

4.4.12 AT MAXIMAL RECEPTOR OCCUPANCY THERE IS GREATER ONGOING STIMULATION IN THE PROTECTIVE GROUP

For Part D of the curve, at maximal doses of GSK2618960, there was significantly greater stimulation through IL-7Rα, as evidenced by pSTAT5, in the protective group compared to the at risk group (mean 7.03 vs 3.75; p = 0.0360).
For the other parts of the curve there were no statistical differences between the genetic groups.

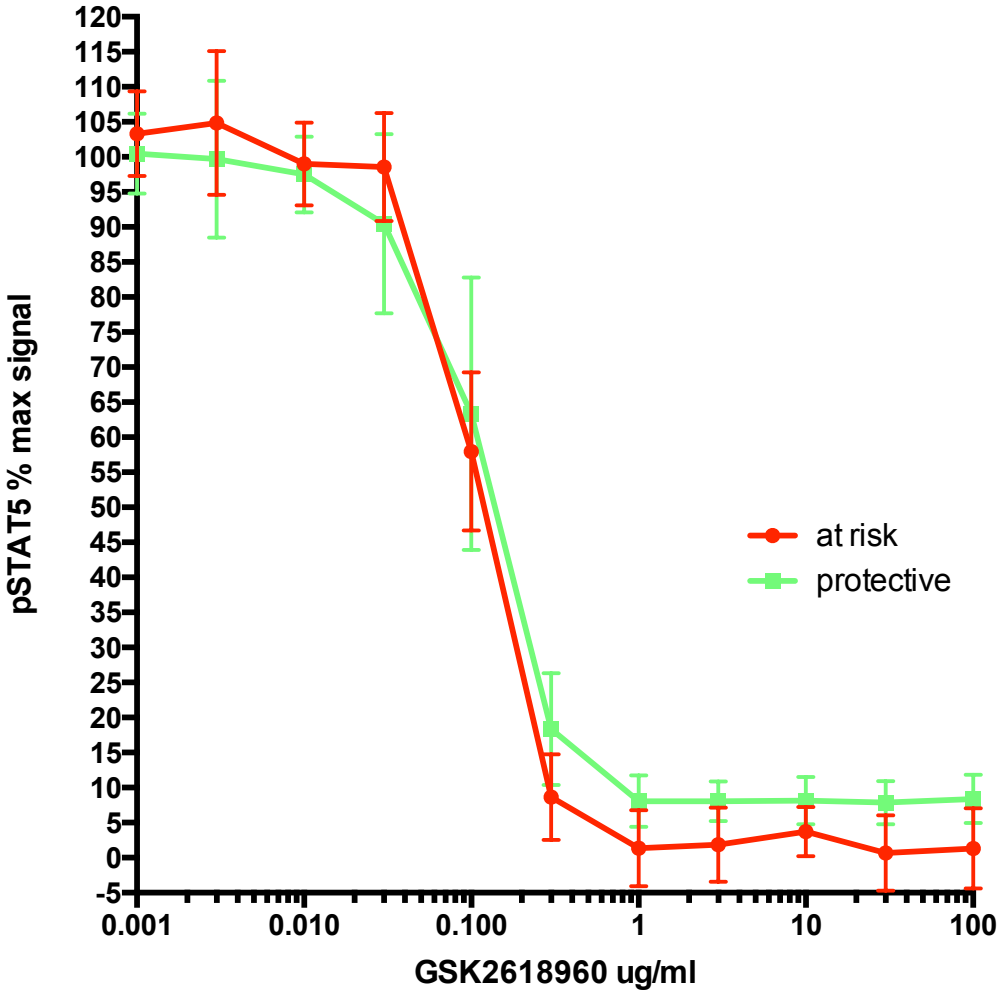

Figure 4. 9: pSTAT5 signaling with GSK2618960

At maximal doses of GSK2618960, there was greater signaling through the IL-7 receptor in the protective group (e.g. Part D of curve at minimum response of pSTAT5 - mean 7.03 vs 3.75; p = 0.0360). Percentage maximum signal of pSTAT5 MFI is on the y-axis and the Log10 GSK2618960 concentration on the x-axis. IL-7Rα genetics was stratified by genotype (at risk group 20 subjects, protective group 9 subjects, total number =29). Results are representative of two biological duplicates.

4.5 PROJECT 2: THE EFFECT OF IL-7Rα GENOTYPE ON IL-7 BIOAVAILABILITY AND SIGNALING

As discussed in the introduction, in 2013 Lundstrom et al (Lundstrom et al., 2013) reported that soluble IL-7Rα (sIL-7Rα) competes with cell-associated IL-7Rα complex for binding to IL-7, leading to reduced IL-7 consumption and overall increased IL-7 bioavailability and bioactivity. For example, the addition of sIL-7Rα to human T cells cultured in the presence of rhIL-7 reduced initial signaling (as evidenced by diminished STAT5 phosphorylation); however at later time points sIL-7Rα augmented IL-7 induced effects – such as IL-7Rα down regulation and CXCR4 up-regulation. Furthermore IL-7 levels were higher at the end of cultures when sIL-7Rα was present, confirming its reduced consumption.

In this section I asked, does genotype rs6897932, which is known to effect soluble IL-7Rα levels, alter early and/or late IL-7 signaling *in vitro*? My hypothesis was that those carrying the risk variant (homozygous for CC at rs6897932) would lead to reduced IL-7 signaling at early time-points, but increased IL-7 signaling overall with more prolonged culture. As per the Lundstrom paper I assessed IL-7 signaling by measuring IL-7Rα down regulation, and pSTAT5. I also measured CD95, which was shown in the Lundstrom paper to have decreased up-regulation in the presence of soluble IL-7Rα compared to IL-7 alone. In the Lundstrom paper this was shown to have significant effects with reduced Fas ligand mediated cell death.

For this experiment, rather than adding recombinant soluble IL-7Rα to the cell culture media I chose to culture the cells in 20% autologous serum – this was done in order to determine if the magnitude of difference seen between genotypes was sufficient to have a biologically meaningful effect. So as to mimic the molar ratios of sIL-7Rα: rhIL-7 used in the Lundstrom paper 2, 20 and 5000 pg/ml of rhIL-7 was added to the media. My early and late time points were 30 minutes, 4, 7 and 11 days.

4.5.1 CELL CULTURE MEDIA SOLUBLE IL-7Rα DIFFERENCES BETWEEN GENOTYPE

Significant differences were found between the at risk group and the protective group in soluble IL-7Rα levels in the cell culture supernatants (please refer to figure 4.10).

This confirms that the genetic differences in serum soluble IL-7Rα was present in our cohort (demonstrated in figure 4.3), and as reported in the literature (Gregory et al., 2007) (Lundstrom et al., 2013) maintained in the cell culture media.

4.5.2 NO DIFFERENCE IN IL-7 CONSUMPTION BETWEEN THE GROUPS

Multiple t tests with bonferroni correction were used to compare IL-7 levels in the cell culture supernatants between the genetic groups. There was no significant difference found in IL-7 levels between the genetic groups (please refer to figure 4.11).

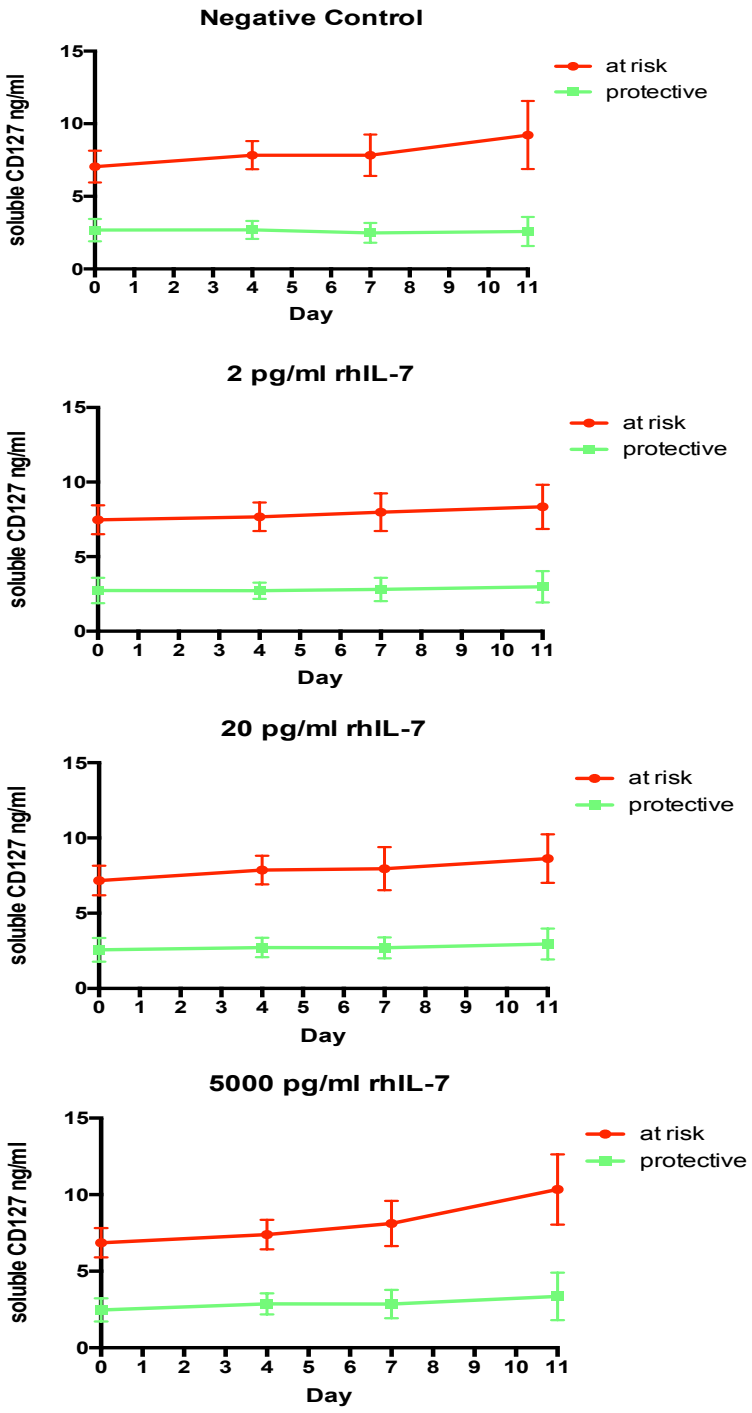

Figure 4. 10: ELISA of cell culture supernatant soluble IL-7Rα levels

The genetic differences between the groups was confirmed by the significant differences in soluble IL-7Rα with higher levels in the at risk group compared to the protective group. Multiple t tests with bonferroni correction generated multiple p values, each one below the significance threshold of p=<0.05. Each graph showed a particular rh-IL7 titration from the negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml. IL-7Rα genetics was stratified by genotype (at risk group 14 subjects, protective

group 8 subjects, total number =22). The time point is on the x-axis, with the mean of the duplicate median fluorescence intensity (MFI) values of soluble IL-7Rα on the y-axis. The 95% confidence intervals represent the error bars. Results are representative of two biological duplicates.

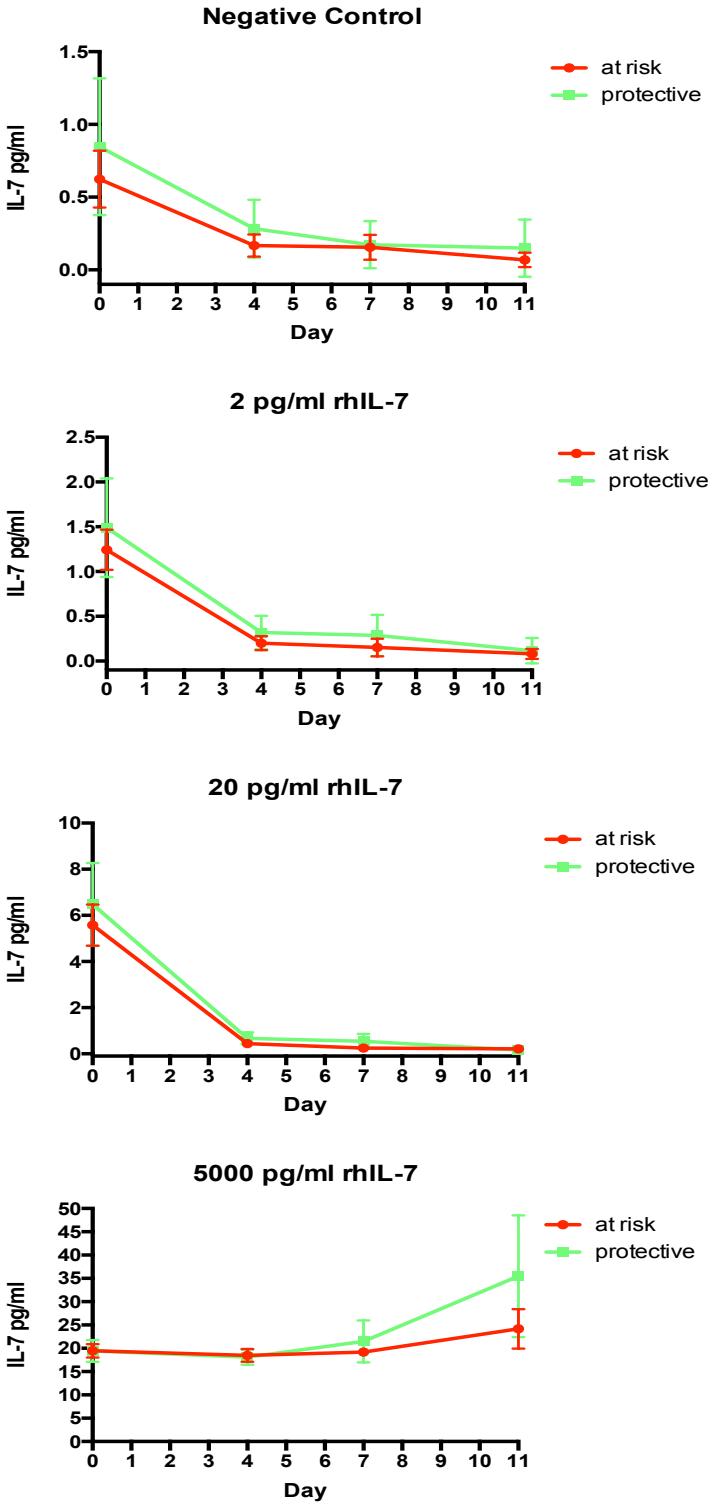

Figure 4. 11: ELISA of cell culture supernatant IL-7 levels

There was no difference in IL-7 consumption between the genetic groups. Multiple t tests with bonferroni correction generated multiple p values, each one above the significance threshold of p=<0.05. Each graph showed a particular rh-IL7 titration from the negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml. IL-7Rα genetics was stratified by genotype (at risk group 14 subjects, protective group 8 subjects, total

number =22). The time point is on the x-axis and the mean of the duplicate median fluorescence intensity (MFI) values of IL-7 on the y-axis. The 95% confidence intervals represent the error bars. Results are representative of two biological duplicates.

4.5.3 DOWNREGULATION OF IL-7Rα

Independent of genetic group, using t tests to compare IL-7Rα MFI at each dose titration of IL-7 at Days 4, 7 and 11, with IL-7Rα MFI at Day 0, there was a significant decrease in IL-7Rα MFI at each timepoint. For example at 5000 pg/ml there were significant decreases compared to Day 0 at Day 4 (mean MFI difference -908.62, p= < 0.0001), Day 7 (mean MFI difference -494.2, p= < 0.0001) and Day 11 (mean MFI difference -187.5, p= 0.0320).

Multiple t tests were used to compare the downregulation of IL-7Rα between the genetic groups. There was no significant difference found in downregulation of IL-7Rα between the genetic groups, in particular there was no trend for a decrease in IL-7Rα downregulation from the protective group compared to the at risk group (the potential mechanism for the results seen with pSTAT5 with project 1).

Figure 4.12 depicts the downregulation of IL-7Rα in CD4+ and CD8+ cells following stimulation with rhIL-7, comparing the at risk group with the protective group.

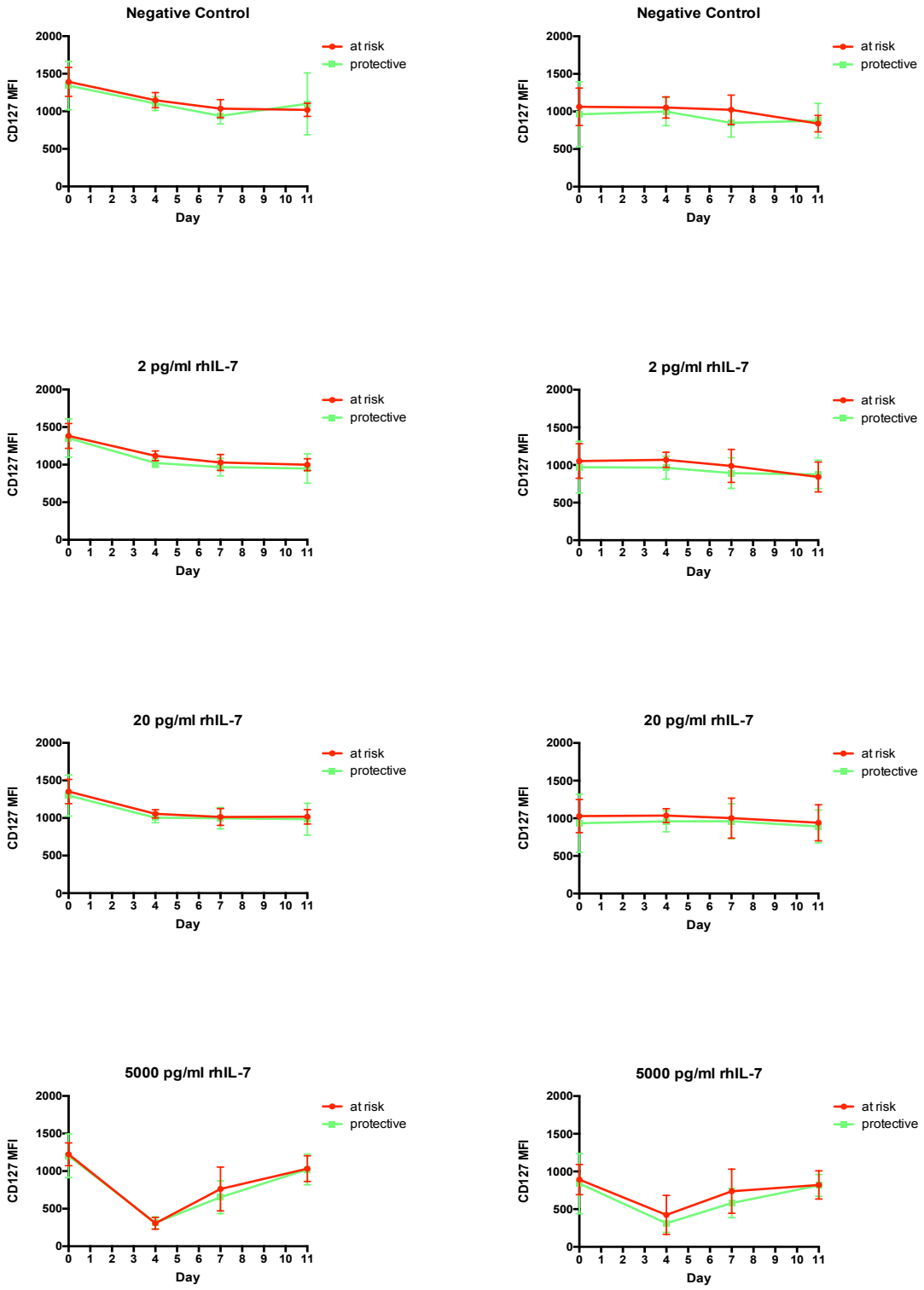

Figure 4. 12: IL-7Rα (CD127) downregulation following rhIL-7 stimulation

No difference was seen for CD4+ (left panel) and CD8+ (right panel) T cells between the genetic groups in downregulation of IL-7Rα. Multiple t tests with bonferroni correction generated multiple p values, each one above the significance threshold of p=<0.05. Each graph showed a particular rh-IL7 titration from the negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml. IL-7Rα genetics was stratified by genotype (at risk

group 14 subjects, protective group 8 subjects, total number =22). The time point is on the x-axis and the mean of the duplicate median fluorescence intensity (MFI) values of CD127 on the y-axis. The 95% confidence intervals represent the error bars. Results are representative of two biological duplicates.

4.5.4 UPREGULATION OF CD95

Independent of genetic group, using t tests to compare the Fas Ligand receptor (CD95) MFI at each dose titration of rhIL-7 at Days 4, 7 and 11, with CD95 MFI at Day 0, there was a significant upregulation of CD95 at 5000 pg/ml of rhIL-7 for days 4 (mean MFI difference 5113, p= 0.0003) and 7 (mean MFI difference 4820, p= 0.0007). For Day 11 there was a trend towards upregulation without reaching significance (mean MFI difference 1088, p= 0.2489). Smaller doses of rhIL-7 added to the culture did not upregulate of CD95 expression.

Multiple t tests were used to compare the upregulation of CD95 between the genetic groups. There was no significant difference found in upregulation of CD95 in CD4+ and CD8+ cells between the genetic groups (please refer to figure 4.13).

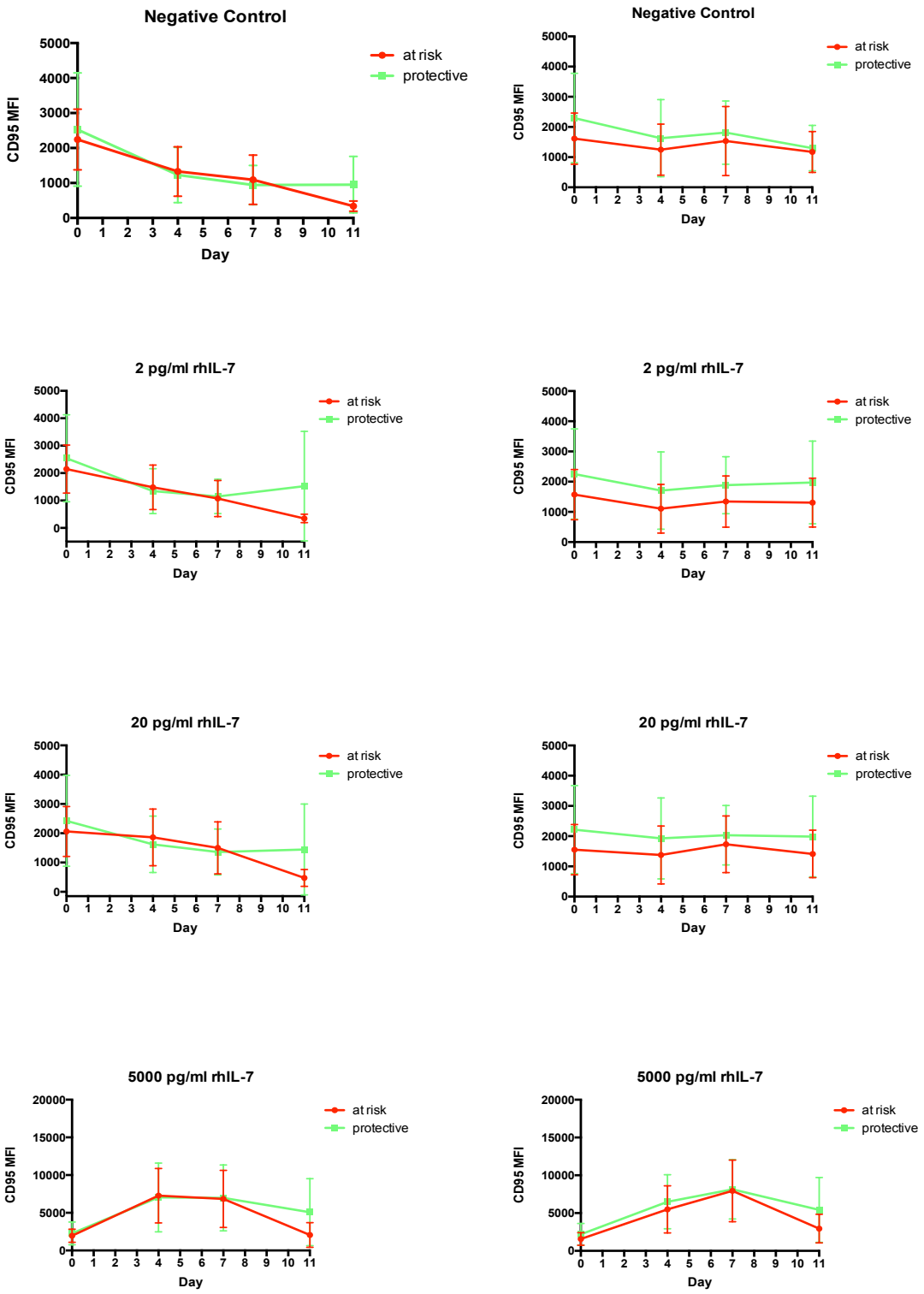

Figure 4. 13: CD95 upregulation following rhIL-7 stimulation

No difference was seen for CD4+ (left panel) and CD8+ (right panel) T cells between the genetic groups in upregulation of CD95 (Fas Ligand Receptor). Multiple t tests with bonferroni correction generated multiple p values, each one above the significance threshold of p=<0.05. Each graph showed a particular rh-IL7 titration from the negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml. IL-7Rα genetics was

stratified by genotype (at risk group 14 subjects, protective group 8 subjects, total number =22). The time point is on the x-axis and the mean of the duplicate median fluorescence intensity (MFI) values of CD95 on the y-axis. The 95% confidence intervals represent the error bars. Results are representative of two biological duplicates.

4.5.5 NO DIFFERENCE IN DOWNSTREAM SIGNALING VIA PSTAT5 BETWEEN THE GROUPS

Independent of genetic group, using t tests to compare pSTAT5 MFI at each dose titration of IL-7 at Days 4, 7 and 11, with pSTAT5 MFI at Day 0, there was no significant increase in pSTAT5 MFI with IL-7 stimulation over the 11 days of the culture. Over a short time period there was a significant increase in pSTAT5 MFI on Day 0 (30 minutes incubation with IL-7) between the negative control and 5000 pg/ml (mean MFI difference 1076, $p = < 0.0001$).

Multiple t tests were used to compare downstream signaling from IL-7Rα, measured by the MFI of pSTAT5, between the genetic groups. There was no significant difference found in pSTAT5 in CD4+ and CD8+ cells between the genetic groups (please refer to figure 4.14).

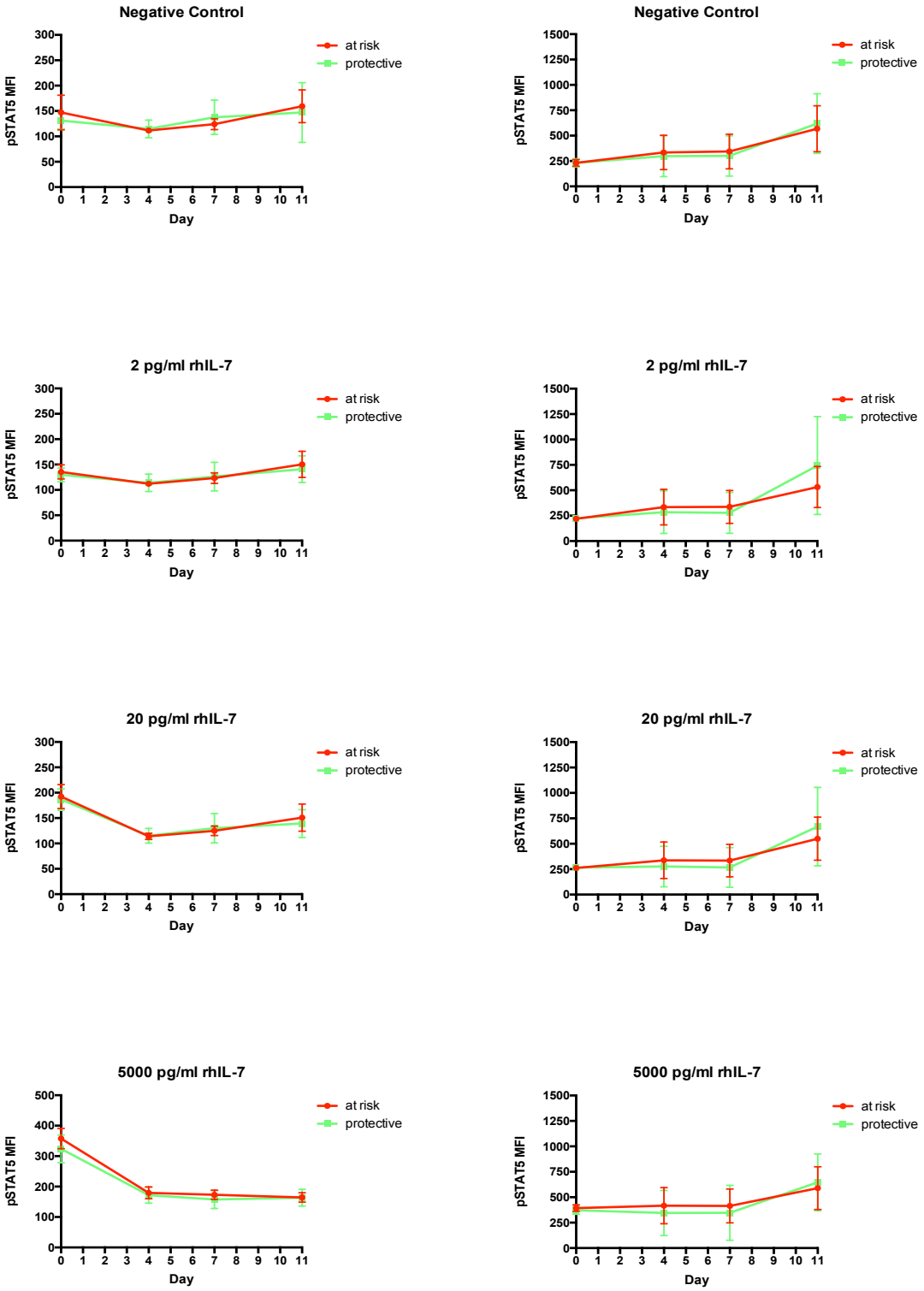

Figure 4. 14: pSTAT5 following rhIL-7 stimulation

There was no difference in phosphorylation of STAT5 for CD4+ (left panel) and CD8+ (right panel) T cells between the genetic groups. Multiple t tests with bonferroni correction generated multiple p values, each one above the significance threshold of p=<0.05. Each graph showed a particular rh-IL7 titration from the negative control, 2 pg/ml, 20 pg/ml and 5000 pg/ml. IL-7Rα genetics was stratified by genotype (at risk

group 14 subjects, protective group 8 subjects, total number =22). The time point is on the x-axis and the mean of the duplicate median fluorescence intensity (MFI) values of pSTAT5 on the y-axis. The 95% confidence intervals represent the error bars. Results are representative of two biological duplicates.

4.6 DISCUSSION

4.6.1 MANIPULATION OF THE IL-7Rα PATHWAY BY GSK2618960

GSK2618960 antagonises IL-7Rα by partial agonism. This is independent of genetic group, however its partial agonistic effects are most easily seen in the protective group. The literature does not describe the mechanisms of other IL-7Rα antagonists used so it is difficult to comment if the partial agonist mechanism is specific to GSK2618960.

GSK2618960 increased the expression of IL-7Rα caused by the reduced stimulation through IL-7Rα with antagonism of the drug. This resulted in a steady increase in IL-7Rα expression up to the half maximal concentration of the drug (EC50). However although the subsequent plateau in the curve was not found to be statistically significant, increased downregulation of IL-7Rα with partial agonism by increasing concentrations of GSK2618960 is biologically plausible. This is the first time an IL-7Rα antagonist has had this effect described, although elsewhere in the literature a dose dependent reduction in pSTAT5 following IL-7Rα antagonism in mice is described and it would be reasonable to assume similar effects on IL-7Rα was also observed, however this report focuses on the use of IL-7Rα antagonists in mice rather than humans (Lee et al., 2011).

4.6.2 INHERENT DIFFERENCES BETWEEN THE GENETIC GROUPS

There were increased levels of soluble IL-7Rα in the serum of the at risk group. This was the first *in vitro* study investigating the genetic differences in IL-7 activity over several days, based solely on the expression of serum soluble IL-7Rα as determined by IL-7Rα genotype. Autologous serum was used which successfully transferred the genetic differences seen with levels of soluble IL-7Rα to the cell culture assay demonstrated by the ELISA of the cell culture supernatant. Other studies have found greater soluble IL-7Rα in the at risk groups, with this difference first found with mRNA from PCR (Gregory et al., 2007) and then subsequently using ELISA (Hoe et al., 2010) (Lundstrom et al., 2013).

There was a trend for greater expression of IL-7Rα in the protective group. Although this trend was not found to be statistically significant, the study was not powered to assess this observation. However this is not the first study to have seen this trend as

it was also observed in a paper from 2010 (Hoe et al., 2010). This difference could account for the effect on pSTAT5 signaling seen in the protective group, although it would not account for why this effect is only seen at maximal concentrations of IL-7Rα antagonist.

This led to the hypothesis that differences in negative feedback of the IL-7 pathway was the mechanism responsible for the difference seen between the genetic groups at maximal concentrations of IL-7Rα antagonism. Therefore in the second part of the chapter I explored the negative feedback mechanisms of IL-7Rα downregulation and CD95, which had previously been investigated by Lundstrom *in vitro* with excess soluble IL-7Rα. This had previously not been investigated in genetic groups stratified by IL-7Rα genotype.

4.6.3 STIMULATION OF IL-7Rα BY IL-7 CAUSES DOWNREGULATION OF IL-7Rα MFI

The data following stimulation of IL-7Rα with IL-7 showed significant increases in downregulation of IL-7Rα independent of genetic group. This has been demonstrated previously in other studies (Park et al., 2004). IL-7Rα is the only γc cytokine receptor that downregulates in response to activation (Park et al., 2004). This enables the most efficient use of IL-7, a limited resource in vivo, normally produced by stromal cells and monocytes (Mackall et al., 2011) to maintain the diversity of the peripheral T cell pool (Park et al., 2004).

4.6.4 STIMULATION OF IL-7Rα BY HIGH DOSE IN VITRO IL-7 CAUSES UPREGULATION OF CD95

Lundstrom et al demonstrated that increased levels of soluble IL-7Rα reduced the normal IL-7 induced upregulation of CD95. This resulted in reduced Fas mediated cell death (Lundstrom et al., 2013). This study sought to investigate this relationship between *in vivo* dose levels of IL-7 (2-20 pg/ml) and upregulation of CD95. This study did not demonstrate upregulation of CD95 at *in vivo* doses of IL-7 but did show at higher doses of IL-7 (5000 pg/ml) an upregulation of CD95. There was no difference between the genetic groups at higher doses of IL-7. This data highlights the uncertainty around the role of the IL-7 pathway *in vivo* in the extrinsic apoptosis pathway as mediated by the Fas ligand receptor. This data suggests that at in vivo

levels of IL-7, reduction in CD95 upregulation, as suggested by Lundstrom et al, by increased soluble IL-7Rα is not possible. However within lymph nodes and areas of lymphopoiesis IL-7 is secreted by stromal cells and vascular endothelium and IL-7 levels will be higher than the normal range of IL-7 in the plasma (2-8 pg/ml) (Sprent and Surh, 2012). Also raised IL-7 levels within a lymphopenic environment could invoke this mechanism and warrants further investigation.

4.6.5 STIMULATION OF IL-7Rα BY IL-7 CAUSES EARLY RATHER THAN LATE INCREASES IN SIGNALING OF PSTAT5

The data in this study demonstrated significant early increases (approximately 30 minutes incubation on Day 0) in pSTAT5 signaling with administration of IL-7. This is concordant with the data from other studies (Lundstrom et al., 2013). The data does not support ongoing later activation of pSTAT5 from IL-7Rα activation. The duration and intensity of IL-7Rα signaling that supports survival and homeostatic proliferation of T cells is unknown (Carrette and Surh, 2012). It is unlikely that T cells are supported by a continuous stimulation with high concentrations of IL-7, particularly considering that IL-7 is present in low concentrations in vivo and on IL-7 binding to IL-7Rα this promotes IL-7Rα downregulation as evidenced from the data in this chapter.

4.6.6 DIFFERENCES BETWEEN THE GENETIC GROUPS FOLLOWING MANIPULATION OF THE IL-7Rα PATHWAY

There was greater pSTAT5 signaling in the protective group at maximal concentrations of GSK2618960. The partial agonist effect of this IL-7Rα antagonist enabled this observation of this difference between the protective genotype group and the at-risk groups. A mean difference of -3.28 ($p=0.0360$) when IL-7Rα receptors were fully bound with the IL-7Rα antagonist pointed towards differential activation through the IL-7Rα pathway between genetic groups, with greater activation through IL-7Rα in the protective group.

Up until this point, there has been no demonstrable genotype effect on IL-7 signaling via pSTAT5 in cells. This novel observation on cell signaling is particularly important as the only previously significant genetic difference between individuals based on IL-

7Rα SNPs was the amount of soluble IL-7Rα, first with soluble IL-7Rα mRNA (Gregory et al., 2007) in PBMCs and cell lines and later with CD4+ T cells and dendritic cells (Lundstrom et al., 2013) (Hoe et al., 2010), but also at the level of protein expression of soluble IL-7Rα (Hoe et al., 2010). In the lymphopenic setting in individuals with HIV, patients with 'haplotype 2' (protective for rs6897932 with lower levels of soluble IL-7Rα) showed faster reconstitution of T cells following treatment with HAART (Rajasuriar et al., 2010). However these studies did not demonstrate these genetic differences by pSTAT5 and this study is the first to demonstrate differential pSTAT5 signaling based on IL-7Rα genotype.

A potential mechanism to explain the phenomenon of greater signaling in the protective group at maximal concentrations of IL-7Rα antagonist could be a difference in the relative strength of negative feedback signaling between the genetic groups. Based on our findings, the protective group would have less negative feedback regulation than the at risk group. The major mechanism of negative feedback in IL-7Rα physiology is downregulation of IL-7Rα receptors upon exposure to IL-7. As discussed in the introduction to this thesis, there are many mechanisms which cells use to downregulate IL-7Rα, including increased JAK3 activity on IL-7Rα leading to greater lysosomal degradation of IL-7Rα (Henriques et al., 2010). Following IL-7 stimulation, there is increased expression of Gfi-1, a transcriptional repressor which downregulates new protein synthesis of IL-7Rα (Park et al., 2004).

The second part of the chapter focused on stimulation of IL-7Rα, which tested the hypothesis of differential relative negative feedback of IL-7Rα between the genetic groups being responsible for the increased pSTAT5 signal at maximal antagonism of IL-7Rα by GSK2618960.

There were no differences found between the genetic groups for the negative feedback mechanisms (downregulation of IL-7Rα and upregulation of CD95) investigated in this study. This is contrary to the evidence that was presented by Lundstrom et al that increased soluble IL-7Rα, the effective difference between the genetic groups, would lead to differences in downregulation of IL-7Rα.

There was also no difference between the genetic groups in IL-7 consumption, as demonstrated by IL-7 ELISA of the cell culture supernatants, which was the major mechanism suggested by Lundstrom et al for the differences seen between the genetic groups.

There were also no significant differences seen in pSTAT5 signaling between the genetic groups with stimulation of IL-7Rα. Although this was not demonstrated in previous publications according to IL-7Rα genotype, Lundstrom et al demonstrated that independent of genotype, an increasing ratio of soluble IL-7Rα to IL-7 led to a reduction of the pSTAT5 signal at early timepoints. This effect was not reproduced in this study.

One limitation of the study is that it could be argued that the study did not have the power to detect differences between the genetic groups. Perhaps the actual levels of IL-7 and soluble IL-7Rα used were too small to detect a difference. For example we detected with the soluble IL-7Rα ELISA between 6-10 ng/ml in the at risk group and 2-3 ng/ml in the protective group in the supernatants used in the cell culture. This value for soluble IL-7Rα was lower than what we originally predicted and did not equate to the middle molar ratios seen in the Lundstrom paper when a significant effect from soluble IL-7Rα was seen. This middle molar ratio was also postulated to be the ratio seen *in vivo* between soluble IL-7Rα and IL-7 as soluble IL-7Rα circulates in relative molar excess to IL-7. Based on previous studies we initially predicted approximately 100ng/ml as the average serum soluble IL-7Rα concentration. In our study cohort there was 80 ng/ml soluble IL-7Rα for the at risk group and 30 ng/ml approximately for protective group. This would mean lower levels of IL-7 were needed to reach the 500:1 middle molar ratio as postulated by Lundstrom et al.

However, the 500:1 molar ratio has not been reproduced in subsequent studies (Cote et al., 2015) and even in the Lundstrom paper it is not clear if the 500:1 molar ratio was significant when working with human T cells. In one study (Cote et al., 2015) using recombinant soluble IL-7Rα a minimum of 200 µg/ml and a maximum of 1000 µg/ml was used which is several thousand fold more than what was found in the cultures used in our study. In this study they found a dose response relationship between the amount of soluble IL-7Rα and increased IL-7 activity. In the Lundstrom paper they used smaller amounts of soluble IL-7Rα but nevertheless started at a minimum of 1 µg/ml and went up to 10 µg/ml, which was several hundred fold more than the cultures used in our study. Perhaps in the effort to recreate *in vivo* conditions within our cultures, too low a level of soluble IL-7Rα and IL-7 was used to see differential effects between the genetic groups.

In summary this was the first study to find differences between the genetic groups for IL-7Rα downstream signaling with pSTAT5. Although this pointed towards a difference in negative feedback between the genetic groups I was unable to demonstrate this with *in vitro* rhIL-7 stimulation, using rhIL-7 levels analogous to that found *in vivo*.

CHAPTER 5 – THE CAMTHY TRIAL

5.1 INTRODUCTION TO THE CAMTHY TRIAL

CAMTHY is a study aimed at increasing thymopoiesis of T lymphocytes in order to re-balance and diversify the population of lymphocytes post alemtuzumab, which was hypothesized to reduce the autoimmune side effects, associated with alemtuzumab.

In this section of the thesis I describe the outcome of the dose tolerability sub-study part of the CAMTHY trial, as I was heavily involved at this stage of the trial. Please refer to section 5.2.4 for more details on my role within the CAMTHY trial.

Alemtuzumab is a monoclonal antibody, which is directed against CD52, a protein of unknown function on lymphocytes. Alemtuzumab causes a lymphopenia, following which homeostatic reconstitution leads to prolonged alteration of the immune repertoire.

Lymphocytes recover at different speeds following treatment, with B lymphocytes recovering the fastest, reaching the lower limit of normal (LLN) at 3 months. CD8+ T lymphocytes recover to the LLN at 19.5 months post treatment. CD4+ T lymphocytes are slowest to recover at 32 months (Kousin-Ezewu et al., 2014).

Despite the high clinical efficacy of alemtuzumab in reducing the disability in MS patients (Cohen et al., 2012) (Coles et al., 2012b) (Coles et al., 2012a), its use does come with side effects, specifically the development of secondary autoimmunity in 30-40% of patients following administration (Tuohy et al., 2015) (Kousin-Ezewu and Coles, 2013). The efficacy and secondary autoimmunity experienced with alemtuzumab use is explained in more detail in the main Introduction in sections 1.10 and 1.10.2.2.

The lymphopenia following alemtuzumab and the subsequent reconstitution of lymphocytes occurs when the clones of lymphocytes that remain following depletion are activated by cytokines such as IL-7, IL-15 and IL-21 (Jameson, 2002). IL-7 is a particularly important cytokine, which stimulates increased thymic production of T lymphocytes, activation of anti-apoptotic signaling via the IL-7 receptor on

lymphocytes and the expansion and survival of peripheral T lymphocytes (Fry and Mackall, 2005).

There is also continuous engagement of the T cell receptors with self-peptide/MHC complexes, particularly in the lymphopenic environment that prevails after alemtuzumab (Takeda et al., 1996) (Tanchot et al., 1997). Together with the release of homeostatic cytokines this leads to the process of controlled peripheral (i.e. outside of the thymus) repopulation of lymphocytes referred to as 'homeostatic proliferation'.

Joanne Jones et al investigated the reconstitution of lymphocytes post alemtuzumab and compared this to the occurrence of secondary autoimmunity (Jones et al., 2013). It had previously been established that there was no difference in clinical efficacy of alemtuzumab for MS between patients affected by secondary autoimmunity and those not affected (Habek et al., 2012). Jones et al established that there was no difference in the speed of lymphocyte reconstitution between the autoimmune and non-autoimmune groups post alemtuzumab (Jones et al., 2013).

The paper eloquently described measuring thymic output by analysing naïve T cells. T cell receptor excision circles, small sections of DNA that are produced following T cell maturation in the thymus that persist in mature T cells (Douek et al., 1998), were also measured. It has been previously demonstrated that MS patients had reduced thymic thymopoiesis (Hug et al., 2003). The paper by Jones et al showed that this was exacerbated by alemtuzumab (Jones et al., 2013).

Alemtuzumab also increased peripheral expansion of the remaining lymphocyte clones, as measured by CDR3 and TCR spectratyping and sequencing, leading to a less diverse lymphocyte pool. The clones of cells remaining after alemtuzumab have previously been shown to be enriched for autoreactive cells (Jones et al., 2009). In the early reconstitution phase post alemtuzumab, the peripheral expansion of lymphocytes was greater in CD8+ cells than CD4+ cells. CD28-CD57+CD8+TEMRAS, previously associated with Grave's disease (Sun et al., 2008), particularly dominated the CD8+ population post alemtuzumab treatment.

It is interesting that early peripheral reconstitution of T cells affects CD8+ cells when the cell count is lowest, as this is when IL-7 levels will be high. It has already been established that IL-7 is an important cytokine in homeostatic proliferation (Fry and

Mackall, 2005) and it has been demonstrated that CD8+ lymphocytes are more sensitive to IL-7 than CD4+ lymphocytes (Guimond et al., 2009). This results in greater homeostatic proliferation in CD8+ lymphocytes during early reconstitution following alemtuzumab (Jones et al., 2013).

The observation that those with secondary autoimmunity post alemtuzumab were more likely to have reduced thymic function and greater homeostatic proliferation (Jones et al., 2013), lead to the hypothesis that altering this process by increasing thymic output with keratinocyte growth factor (palifermin), would reduce secondary autoimmunity following alemtuzumab. This is the hypothesis that led to the CAMTHY trial.

5.2 METHODS

5.2.1 STUDY DESIGN AND PARTICIPANTS

The final 3 patients from the sub-study would enter the main 'CAMTHY' trial, a single centre, randomised, double-blind, placebo-controlled trial, which was powered to investigate 80 patients with relapsing-remitting MS to assess if preserving thymic function with the use of Palifermin would help to reduce the autoimmune side effects associated with alemtuzumab. All patients gave written informed consent in accordance with Good Clinical Practice (REC 12/LO/0393).

The inclusion criteria for the trial were patients who were between 18 and 50 years of age with relapsing-remitting multiple sclerosis; disease duration of 10 years or less; at least two relapses in the previous 2 years with at least one in the previous 12 months (untreated or on beta interferon or glatiramer acetate) and an expanded disability status scale (EDSS) score of 5.0 or less.

Exclusion criteria for the trial included progressive forms of multiple sclerosis; previous thymectomy; previous treatment with alemtuzumab, natalizumab, mitoxantrone, cyclophosphamide, cladribine, rituximab or any other immunosuppressant or cytotoxic therapy; a history of malignancy, or a history of clinically significant autoimmunity other than multiple sclerosis.

5.2.2 DRUG TREATMENTS

Please refer to figure 5.1 for an overview of the trial. All patients received 12mg/day alemtuzumab for 5 days at baseline and 3 days at month 12. Following a dose-tolerability sub-study, patients were assigned (1:1) to receive placebo or palifermin, given for 3 days immediately prior to and after each cycle of alemtuzumab, with repeat doses at month 1 and month 3.

The sub-study was an open-label dose escalation tolerability study, which investigated if the doses of palifermin to be used in the study would be tolerable. The sub-study was made up of 9 patients in total with 3 different dose levels of palifermin – 90 mcg/kg/day, 120 mcg/kg/day and 180 mcg/kg/day. 3 patients were used at each dose level. Although the top dose of 180 mcg/kg/day had been used

previously in humans (Vadhan-Raj et al., 2010), it had never been used with a repeated dosing strategy at such frequent intervals in human studies.

Palifermin was given as an intravenous bolus injection on days -5, -4 and -3 prior to each cycle of alemtuzumab and on days 8, 9 and 10. Three further doses were given at month 1 and month 3 after each cycle of alemtuzumab.

Each dose level was separated by a minimum of 10 days (from the day 10 dose) and escalation between doses only occurred if no more than mild (grade 1) or moderate adverse events (grade 2) occurred, according to the Common Terminology Criteria for Adverse Events version 3.0 (Trotti et al., 2003).

Subjects were seen at 3-month intervals for standard monitoring of alemtuzumab and at the same time were monitored for adverse effects from palifermin up to month 30.

Following completion of the sub-study, the trial steering committee would be consulted with the clinical results. If 180 mcg/kg/day of palifermin was deemed tolerable (no adverse events greater than grade 2), the main CAMTHY study would be able to proceed.

The major primary endpoint of the main CAMTHY trial was measurement of thymic reconstitution with naïve CD4+ (CD45RA+CCR7+) cell count at month 6. The results from the main CAMTHY trial can be viewed in the paper that has been published in the appendix (Coles et al., 2019).

5.2.3 STATISTICAL ANALYSIS

The decision to proceed to the main CAMTHY trial from the sub-study was made by the trial steering committee following review of the sub-study adverse events; therefore no formal statistical analysis was needed. In the main study 28 patients were needed for 80% power to calculate a 50% increase in the percentage of naïve CD4+ cells at month 6. For further information on the statistical analysis please refer to the manuscript of the CAMTHY trial in the appendix.

5.2.4 MY PERSONAL ROLE WITHIN THE CAMTHY TRIAL

At the beginning of my research period, prior to the commencement of the IL-7Rα trial, I was heavily involved with some of the administrative work before the CAMTHY trial began. This involved liaising with local general practitioners about the taking and monitoring of blood results for patients on the trial with the help of the Cambridge Local Research Network (CLRN). I also liaised with the staff on the Clinical research Facility (CRF), where the patients would be dosed with palifermin and alemtuzumab. I administered the first dose of palifermin to the first patient on the sub-study.

I helped to consent patients for the trial and explained to them the rationale for the CAMTHY trial and what they could expect from participating in the trial.

Following this I was the main doctor on the sub-study assessing patients during their treatment, monitoring for side effects from palifermin and helping to document the adverse events that occurred. This was during the treatment that patients received but also in the follow-up clinic visits. I helped to field and reply to queries from patients during the trial period outside of these scheduled visits.

As part of this work I took blood from patients for immune phenotyping. Within the laboratory I helped to prepare the peripheral blood mononuclear cell (PBMC) layer for use in subsequent assays. However I was not involved in further laboratory or statistical analysis of the samples taken.

The information and knowledge gathered during the completion of the sub-study and the beginning of the main CAMTHY trial helped me to prepare for the IL-7Rα trial, which aided my development as a translational clinician.

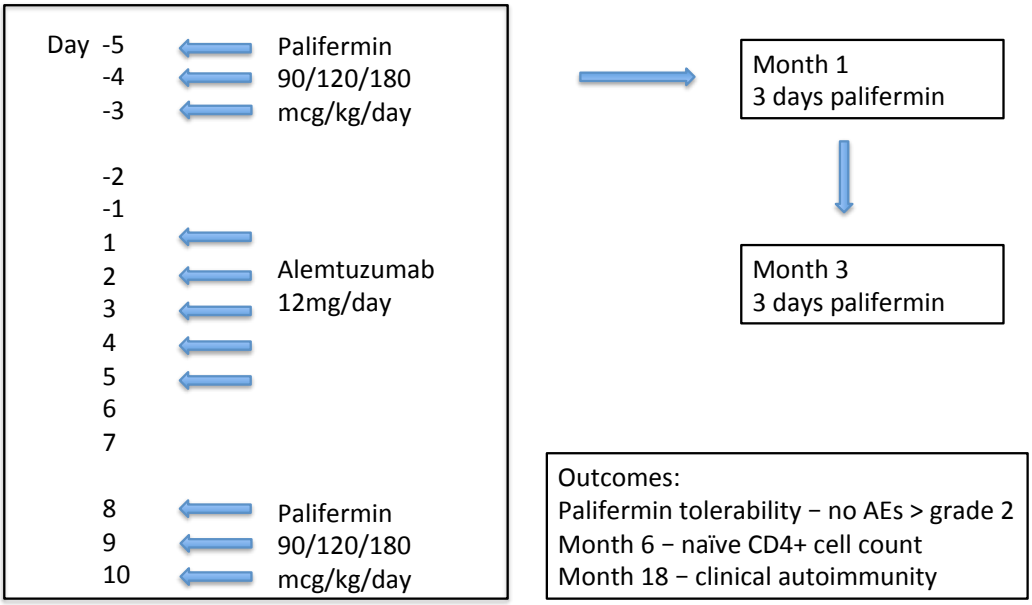

Figure 5. 1: Overview of the CAMTHY trial and tolerability sub-study.

Patients for the open label dose escalation tolerability sub-study were given either 90, 120 or 180 mcg/kg/day of palifermin. The treatment cycle outlined above was repeated at month 12. If doses were tolerated with no serious adverse events (AEs), the 3 patients dosed with 180 mcg/kg/day of palifermin would proceed to the main CAMTHY trial of 80 patients, which was a placebo-controlled trial with outcomes measuring thymic function (e.g. naïve CD4+ cell count) and clinical autoimmunity.

5.3 RESULTS

5.3.1 CLINICAL RESULTS OF THE DOSE TOLERABILITY SUB-STUDY

Palifermin was deemed tolerable by the trial steering committee as no patient had an adverse event that was deemed serious or life threatening (all adverse events grade 2 and below). Therefore the main CAMTHY trial of 28 patients was allowed to proceed following the sub-study in 9 patients.

Please refer to table 5.1 for a full outline of the adverse events that occurred during this sub-study. Here I will detail some of the more common side effects that were encountered by patients during the study.

Palifermin caused an infusion syndrome in which patients developed an erythematous rash that would later lead to some experiencing peeling of the skin, hair thinning and in a few cases hair loss.

Patients also experienced swelling of the hands and face. Some patients noticed discolouration of the tongue with altered taste for a short period after the transfusion.

Palifermin administration prior to alemtuzumab did not alter the adverse effects experienced during alemtuzumab which has been well described elsewhere (Coles et al., 1999).

		90mcg/kg/day	120mcg/kg/day	180mcg/kg/day
Infusion-associated symptoms at baseline	Erythematous skin rash	3	3	3
	Oral symptoms	3	3	3
	Oedema (facial/hands)	1	3	3
	Urticarial skin rash	1	0	2
	Discoloured tongue	3	3	3
	Pyrexia	3	2	1
	Headache	2	0	2
	Skin sensitivity	0	0	2
	Fatigue	0	0	1
	Chest tightness	2	0	1
	GI upset	1	1	2
Infusion-associated symptoms at months 1 and 3	Erythematous skin rash	2	3	2
	Oral symptoms	3	3	3
	Oedema (facial/hands)	3	3	3
	Discoloured tongue	3	3	3
	Skin sensitivity	0	0	1
	Peeling skin	0	1	0
	Pyrexia	1	1	0
	GI upset	1	0	1
Adverse Events unrelated to infusions	Hair loss	1	1	2
	Dry skin	2	1	2
	Nail changes (ridging)	1	0	1
	Upper respiratory tract infection	2	0	1
	Fatigue	1	0	0
	Low mood	1	1	0

Table 5. 1: Adverse event data for sub-study patients by dose of Palifermin

This displays the incidence of all adverse events during the sub-study. The severity of the adverse events were categorised as either grade 1 or 2. Adjusted with permission from Joanne Jones, principal investigator of the CAMTHY trial.

5.3.2 SUMMARY OF THE MAIN CAMTHY TRIAL RESULTS

The main trial was stopped following a pre-planned interim analysis after 28 patients had reached month 6 post-treatment.

The primary outcome of the CAMTHY trial assessed thymic production of T lymphocytes by measuring the CD4+ naïve T cell count (CD45RA+ CCR7+) at month 6. Patients treated with palifermin displayed less naïve CD4+ cells than patients treated with placebo at month 6 ($2.229x10^7$/L vs. $7.733x10^7$/L; p=0.007).

Thymopoiesis within the dose tolerability sub-study was assessed following this result. A dose dependent effect was seen on the reduction of thymic reconstitution by palifermin. Naïve CD4+ T-cells at 6 months were lower in the 90mcg/Kg arm of the sub-study compared to placebo. Naïve CD4+ counts continued to reduce in the three patients on 120mcg/Kg and 180mcg/Kg of palifermin (table 5.2).

There was no difference in rates of autoimmunity between the two treatment groups after 30 months of follow-up with 4/14 palifermin versus 5/13 placebo developing clinical autoimmunity (one patient was lost to follow-up; Fisher's exact test, two-sided, p=0.69).

For further details of the results from the main CAMTHY trial please refer to the appendix of the paper from the trial, which has been published (Coles et al., 2019).

Variable	Group	Statistic	Baseline	M1	M3	M6
Sample Numbers	Low dose (90mcg/Kg)	n	2	3	2	2
	Median dose (120mcg/Kg)		3	3	3	3
	High doses (180mcg/Kg)		3	2	3	3
Total CD4 Count ($x10^9$/mL)	90mcg/Kg	Mean (SD) Median Min, Max	0.870 (0.270) 0.740 0.69, 1.18	0.065 (0.099) 0.010 0.006, 0.18	0.130 (0.130) 0.060 0.05, 0.28	0.127 (0.050) 0.120 0.08, 0.18
	120mcg/Kg	Mean (SD) Median Min, Max	1.043 (0.463) 1.140 0.54, 1.45	0.006 (0.00) 0.006 0.006, 0.006	0.077 (0.046) 0.050 0.05, 0.13	0.087 (0.064) 0.060 0.04, 0.16
	180 mcg/Kg	Mean (SD) Median Min, Max	0.88 (0.352) 0.920 0.51, 1.210	0.024 (0.031) 0.006 0.006, 0.060	0.039 (0.029) 0.050 0.006, 0.060	0.057 (0.015) 0.060 0.04, 0.070
% of naïve CD4 (CCR7+CD45RA+)	90mcg/Kg	Mean (SD) Median Min, Max	44.65 (0.495) 44.650 44.3, 45.00	3.55 (2.686) 5.020 0.45, 5.180	1.62 (0.877) 1.620 1.00, 2.240	23.45 (11.950) 23.450 15.0, 31.90
	120mcg/Kg	Mean (SD) Median Min, Max	37.8 (16.441) 45.700 18.9, 48.80	2.617 (2.724) 1.820 0.38, 5.65	4.19 (2.745) 2.820 2.4, 2.35	7.587 (8.325) 2.810 2.75, 17.20
	180mcg/Kg	Mean (SD) Median Min, Max	30.567 (6.527) 27.000 26.6, 38.10	1.575 (0.134) 1.575 1.48, 1.670	7.83 (3.189) 8.230 4.46, 10.80	10.963 (9.595) 7.540 3.55, 21.80
Naïve CD4 (CCR7+CD45RA+) count ($x10^7$/mL)	90mcg/Kg	Mean (SD) Median Min, Max	42.787 (13.42) 42.787 33.3, 52.274	0.05492 (0.02467) 0.0518 0.031947, 0.081	0.2072 (0.1296) 0.2072 0.1344, 0.28	2.176 (0.5317) 2.176 1.8, 2.552
	120mcg/Kg	Mean (SD) Median Min, Max	44.3547 (31.0107) 52.098 10.206, 70.76	0.01665 (0.01733) 0.011582 0.00242, 0.03596	0.2847 (0.14264) 0.3666 0.12, 0.3675	0.52813 (0.4661) 0.44 0.1124, 1.032
	180mcg/Kg	Mean (SD) Median Min, Max	28.114 (16.470) 24.472 13.77, 46.101	0.04971 (0.05528 0.04971 0.01063, 0.0888	0.26184 (0.2152) 0.223 0.0687, 0.4938	0.5376 (0.3296) 0.5278 0.213, 0.872

Table 5. 2: Sub-study results of naïve (CD45RA+CCR7+) CD4+ cells

A dose dependent effect was seen on the reduction of thymic reconstitution by palifermin. Naïve CD4+ T-cells at 6 months were lower in the 90mcg/Kg arm of the sub-study compared to placebo. Naïve CD4+ counts continued to reduce in the three patients on 120mcg/Kg and 180mcg/Kg of palifermin. The data shows either cell subset count or percentage of the total CD4+ T cell population. The data shows

mean (SD), median, minimum and maximum values. Adjusted with permission from Joanne Jones, principal investigator of the CAMTHY trial.

5.4 DISCUSSION

Palifermin at doses of up to 180 mcg/kg/day over consecutive days, the first time this dose regimen has been used in humans, was tolerable. However there was a significant burden of side effects experienced with the drug described above in the results section. However none of these side effects were deemed serious. It should be taken into account that the side effects mainly affected the integument and were therefore visible, which could potentially act as a significant deterrent to patients. For example 4/9 patients treated with palifermin suffered with hair loss. In at least one patient the hair loss was significant.

Palifermin exacerbated alemtuzumab's negative effect on thymopoiesis of T lymphocytes. In this tolerability sub-study this demonstrated an apparent dose effect of impaired thymic function after palifermin and alemtuzumab co-administration. This was a novel finding and a wholly unexpected result, particularly the increased thymopoiesis seen with palifermin administration in animal studies (Seggewiss et al., 2007). This paper had given 250mcg/Kg/day for 3 days to macaques, which had been well tolerated with effects lasting up to 12 months on thymopoiesis. Therefore it could be argued that a greater dose of palifermin was required in humans, however with the evidence from the sub-study demonstrating a dose effect this undermines this line of argument.

Previous studies of palifermin in humans in preventing graft vs host disease following allogeneic HSCT (Rizwan et al., 2011) and in HIV patients with a CD4+ lymphopenia despite HAART (Jacobson et al., 2014) demonstrated that at 90 mcg/kg/day palifermin did not improve thymopoiesis. The aim of the CAMTHY trial and the tolerability sub-study was to test the hypothesis that suboptimal dosing was the cause of the failure of an improvement in thymic function with previous use of palifermin in humans. The unexpected negative effect of palifermin at doses of 180 mcg/kg/day when co-administered with alemtuzumab may be due to upregulation of CD52 on thymic epithelial cells by palifermin, with subsequent greater depletion of these cells by alemtuzumab.

This trial has failed to answer the critical question of whether the thymus can be successfully manipulated post alemtuzumab, to increase thymopoiesis of T lymphocytes, leading to a decrease in the secondary autoimmune side effects encountered post alemtuzumab. Future strategies could include use other agents

such as IL-7 and IL-15 (Rizwan et al., 2011), either alone or in tandem to boost the role of the thymus in lymphocyte reconstitution following use of alemtuzumab.

CHAPTER 6 - LYMPHOCTYE RECOVERY AFTER ALEMTUZUMAB DOES NOT PREDICT MULTIPLE SCLEROSIS DISEASE ACTIVITY

This section of my thesis has been modified from my paper published in the American academy of neurology journal 'Neurology'. This section of my PhD enabled an important clinical question on prediction of autoimmunity based upon lymphocyte reconstitution to be answered. IL-7 has an integral role in the homeostatic proliferation of lymphocytes after alemtuzumab, and this part of my PhD enabled a deeper understanding of the behaviour of lymphocytes following alemtuzumab administration.

6.1 INTRODUCTION

Alemtuzumab has proven efficacy as a treatment for relapsing remitting multiple sclerosis. In a phase-2 trial, compared with interferon beta-1a, alemtuzumab reduced the risk of relapse and sustained accumulation of disability by more than 70% at three years, with sustained efficacy at five years (Coles et al., 2008) (Cohen et al., 2012). Two phase-3 trials (CARE-MS I and CARE-MS II) have confirmed efficacy in treatment-naïve patients, and established superiority over interferon beta-1a in patients who continue to relapse despite first-line therapy (Cohen et al., 2012) (Coles et al., 2012b). Alemtuzumab was licensed by the European Medicine Agency (EMA) (Genzyme, 2013) and is entering routine clinical practice, as a treatment for active multiple sclerosis.

Alemtuzumab is a lymphocyte depleting anti-CD52 monoclonal antibody. Each cycle causes profound pan-lymphocyte depletion, but the relatively infrequent dosing regimen (one treatment a year for two years followed by further treatments when there is breakthrough disease activity) allows reconstitution to occur. The rate and degree of recovery varies with cell type: B cells recover rapidly, whereas T cell lymphopenia is prolonged with CD4+ and CD8+ cells taking 35 and 20 months, respectively, to reach the lower limit of normal (Hill-Cawthorne et al., 2012). During this period of immune reconstitution, 30% of individuals experience thyroid autoimmunity, and 1% develop immune thrombocytopenic purpura (ITP); with rare cases of Goodpasture's syndrome, autoimmune haemolytic anaemia and autoimmune neutropenia also being reported (Coles et al., 2012a). It has been shown that the risk of developing autoimmunity after alemtuzumab is unrelated to

rate of T cell reconstitution (but rather reflects the degree to which recovery occurs by expansion of cells that have escaped depletion, rather than thymopoiesis) (Jones et al., 2013).

However, a report published in 2013 (Cossburn et al., 2013), has suggested that peripheral CD4+ recovery can be used to predict multiple sclerosis disease activity after treatment, with counts greater than 388.5 $x10^6$ cells per mL at 12 months following therapy identifying patients who are likely to have recurrent disease activity and who may therefore benefit from further treatment. Given the clear clinical implications of this claim, we re-assessed this finding in the 'Cambridge cohort' - a larger group of patients in whom the role of alemtuzumab in relapsing-remitting multiple sclerosis was originally evaluated and which therefore provides the most prolonged duration of follow-up in multiple sclerosis patients who have received alemtuzumab.

6.2 METHODS

I designed and conducted the study, analysed data in the study with the statistician Mr Richard Parker. I interpreted the data in the study, drafted and revised the manuscript. I am grateful to Dr Joanne Jones who also helped with the study design, data analysis and revision of the manuscript prior to publication.

6.2.1 PATIENTS AND PROCEDURES

All patients had relapsing-remitting multiple sclerosis (RRMS) and had participated in: CAMMS223 (a Phase 2 randomised control trial) and CAMMS 224 or SM3 (both investigator-led, open label studies). CAMMS223 key eligibility criteria were: disease onset within 3 years, at least two clinical relapses during the previous 2 years and a score of 3 or less on the Expanded Disability Status Scale (EDSS). Patients were included in CAMMS 224 and SM3 if they had at least 1 relapse in the previous year, an EDSS score of 6.0 or less, with disease duration of less than ten years. Subsequently all patients entered either CAMSAFE (an investigator led long-term observational study), or the extension phase of the CAMMS223 trial. The first patient from this cohort was treated on 22 November 1999 with the date for final collection of data on 1 January 2013.

All studies were approved by a regional ethics board and institutional research committee. All patients gave written informed consent.

6.2.2 CLINICAL TREATMENT AND FOLLOW-UP PROTOCOL

All patients received at least 2 elective cycles of alemtuzumab given annually, with the potential for further cycles if there was clinical or radiological evidence for on-going disease activity. Patients were reviewed at 1 and 3 months and then quarterly for the first two years after each treatment cycle. For the following two years, they were seen biannually and then at least annually thereafter. Patients were also seen whenever a relapse was suspected.

6.2.3 OUTCOME ASSESSMENTS

For participants in CAMMS223 study, EDSS scores were determined quarterly in a blinded fashion by a neurologist who also adjudicated possible relapses. The same assessor measured the EDSS of patients in the CAMMS224 and SM3 studies, albeit less frequently. Sustained accumulation of disability was defined as an increase of 1.5 EDSS points from a baseline of 0, or an increase of ≥1.0 if the baseline was ≥1.0 confirmed over 6 months. A relapse was defined as new neurological symptoms attributable to multiple sclerosis, lasting >48 hours with an objective change in neurological examination.

Peripheral blood mononuclear cell phenotyping was performed at baseline and then quarterly for the first 36 months and then at least annually (including total lymphocyte count, CD4+, CD8+, CD19+, CD56-NK, and monocyte counts).

Brain MRI scans were performed in most patients with a suspicion of active disease prior to re-treatment with alemtuzumab. Monthly MRI scans were performed in a subset of patients from the SM3 study. A number of clinically inactive patients had interval MRI scans to look for subclinical activity, and to provide a means for comparison in case of future disease activity.

6.2.4 STATISTICAL ANALYSIS

Statistical analysis was done in consultation with Mr Richard Parker, a statistician at the University of Cambridge.

Median time for recovery to the lower limit of normal (LLN) was calculated for each cell subset. All data was categorised depending on the cycle of alemtuzumab treatment. Patients were placed into ‘active’ or ‘non-active’ groups independent of when an event took place within a particular treatment cycle. Therefore, within each cycle, patients were defined as being ‘relapse-free’ or ‘relapsing’, ‘disability-free’ or having ‘accumulated disability’, or having reached a ‘positive composite endpoint’ (defined as: having relapsed, and/or accumulated disability, and/or having had an ‘active’ MRI scan); or with a ‘negative composite endpoint’ based on all three negative outcomes. A subgroup of patients (n=91), scanned after treatment, were classified as MRI ‘active’ or ‘non-active’.

To assess differential lymphocyte reconstitution between groups, a linear mixed effects regression method was undertaken with CD4+/CD8+/CD19+/CD56+/ monocytes or total lymphocyte count as the outcome variable, and 'relapse/ disability/ active MRI/ composite score' and time point as explanatory variables.

This method was used as it was the optimum way to explore the relationship between the outcome variable (e.g. CD4+ count) and the different explanatory variables (e.g. time point and relapse), which needed to be accounted for. A quadratic term (time point squared) was also included due to the observed relationship between time point and outcome. A separate linear mixed effects model was fitted within each cycle. A continuous autoregressive (order 1) correlation structure was assumed for all models. Model coefficients are presented with 95% confidence intervals and p-values.

A Fisher exact test was used to assess whether a CD4+ count of 388.5 x 10^6/mL or greater at 12 months predicts disease activity - either clinically or radiologically. A Fisher exact test was used, as it was the optimum way to compare categorical data and to see if there is a relationship between those values.

The standard 5% significance level was used throughout, and no adjustment made for multiple testing in order to avoid inflating the Type II error rate. The linear mixed effects regression method was implemented in R software using the 'nlme' package (Pinheiro et al., 2013). R software was also used to compute the Fisher's exact tests. All other analyses were performed in GraphPad PRISM (version 5.00 for Windows; www.graphpad.com).

6.3 RESULTS

6.3.1 STUDY POPULATION CHARACTERISTICS

Data was derived from 108 patients of whom 73 (67.6%) were female. The median follow-up from first treatment was 99 months (inter-quartile range [IQR] 74.75-117.25). The mean age of patients at first treatment with alemtuzumab was 32.8 years (SD 7.99). The median EDSS at baseline was 3.0 (IQR 1.5-4.75). Mean relapse frequency prior to treatment was 1.7 relapses per annum (SD 0.81).

6.3.2 LYMPHOCYTE RECONSTITUTION

As previously reported (Cossburn et al., 2013) (Coles et al., 2006) (Hill-Cawthorne et al., 2012), treatment with alemtuzumab led to profound pan-lymphocyte depletion, followed by differential recovery. CD19+ lymphocytes reached the LLN most rapidly, with a median recovery time of 3 months (IQR 3-6). The intervals for CD8+ and CD4+ lymphocytes were median times of 19.5 (IQR 10-34.5) and 32 (IQR 21.75-41) months, respectively.

6.3.3 CLINICAL OUTCOMES

The total number of patients who experienced at least 1 relapse during the follow-up period was 56 (51.85%). The mean relapse frequency post-treatment was 0.17 relapses per annum, equating to an 89.8% reduction in the annualised relapse rate compared to pre-treatment.

28 patients (25.9%) met the definition for sustained accumulation of disability.

91 patients had an MRI scan: 16 individuals (17.6%) had an ‘active’ scan (new T2/enhancing lesions) at some point during the follow-up period (please refer to Table 6.2 for a detailed breakdown of each alemtuzumab cycle).

6.3.4 ASSOCIATION OF PERIPHERAL MONONUCLEAR CELL SUBSETS WITH DISEASE ACTIVITY

Relapse:

Within each treatment cycle, there was no difference in the number of CD4+ T cells, CD8+ T cells, CD19+ B cells, CD56+ NK cells or monocytes between those with and without clinically defined relapses (Figure 6.1 and Table 6.1 for cycles 1-4; data not shown for NK cells and monocytes).

Using Fisher's exact test, we found no association between a CD4+ count of >388.5 cells x 10^6/mL at 12 months and risk of relapse (p=0.28). Given the possibility that relapses within the first few months of treatment may be due to lymphocytes that have already entered the central nervous system (Coles et al., 1999), we looked at timing of relapses following cycle 2 (chosen as it is the most informative cycle in terms of patient numbers, number of relapses and length of follow up). Only 3 out of 106 patients relapsed within 2 months of treatment, and of these, 2 went on to have additional relapses within cycle 2, leaving only one patient who was potentially misclassified using our method, increasing the confidence in our conclusion.

MRI activity:

Within each treatment cycle, there was no difference in the number of CD19+ B cells, CD56+ NK cells or monocytes between those with and without active MRI scans (Figure 6.2 and Table 6.1 for cycles 1-4; data not shown for NK cells and monocytes). CD4+ cells (p=0.016) and CD8+ cells (p=0.008) were found to be higher (on average by 0.146 and 0.125 x10^9/mL cells respectively) in the 'active MRI group' (n=5) vs. the inactive group (n=28) within treatment cycle 3. No difference was found in any other treatment cycle; indeed in cycles 1 and 2 (the most informative periods numerically), the trend was in the opposite direction (Figure 6.2, Table 6.1).

Using Fisher's exact test, we found an association between a CD4+ cell count of >388.5 x 10^6/mL at 12 months and the risk of having an 'active' MRI scan (overall p=0.02). However, further analysis of this result demonstrated that the difference was driven by patients within cycle 3 (p<0.0001), with no difference observed within cycles 1 (p= 1.0), cycle 2 (p= 0.91) or cycle 4 (p= 0.05).

Disability Accumulation:

Within each treatment cycle, there was no difference in the number of CD8+ T cells, CD19+ B cells, CD56+ NK cells or monocytes between those with and without accumulation of disability (Figure 6.3 for cycles 1-3 and Table 6.1). CD4+ T cells were found to be lower in patients who accumulated disability in cycle 1 (adjusted mean difference across the cycle 0.063 $x10^9$; p=0.002). No difference was found in any other treatment cycle, although the trend was in the same direction (Figure 6.3, Table 6.1).

Composite:

Within each treatment cycle, there was no difference in the number of CD8+ T cells, CD19+ B cells, CD56+ NK cells or monocytes between those who did and did not reach the composite end point (Table 6.1; data not shown for non-lymphocyte cell populations). Within cycle 4, CD4+ T cells were found to be lower in patients who met the composite end point compared to those who did not. No difference was found in any other treatment cycle (Table 6.1).

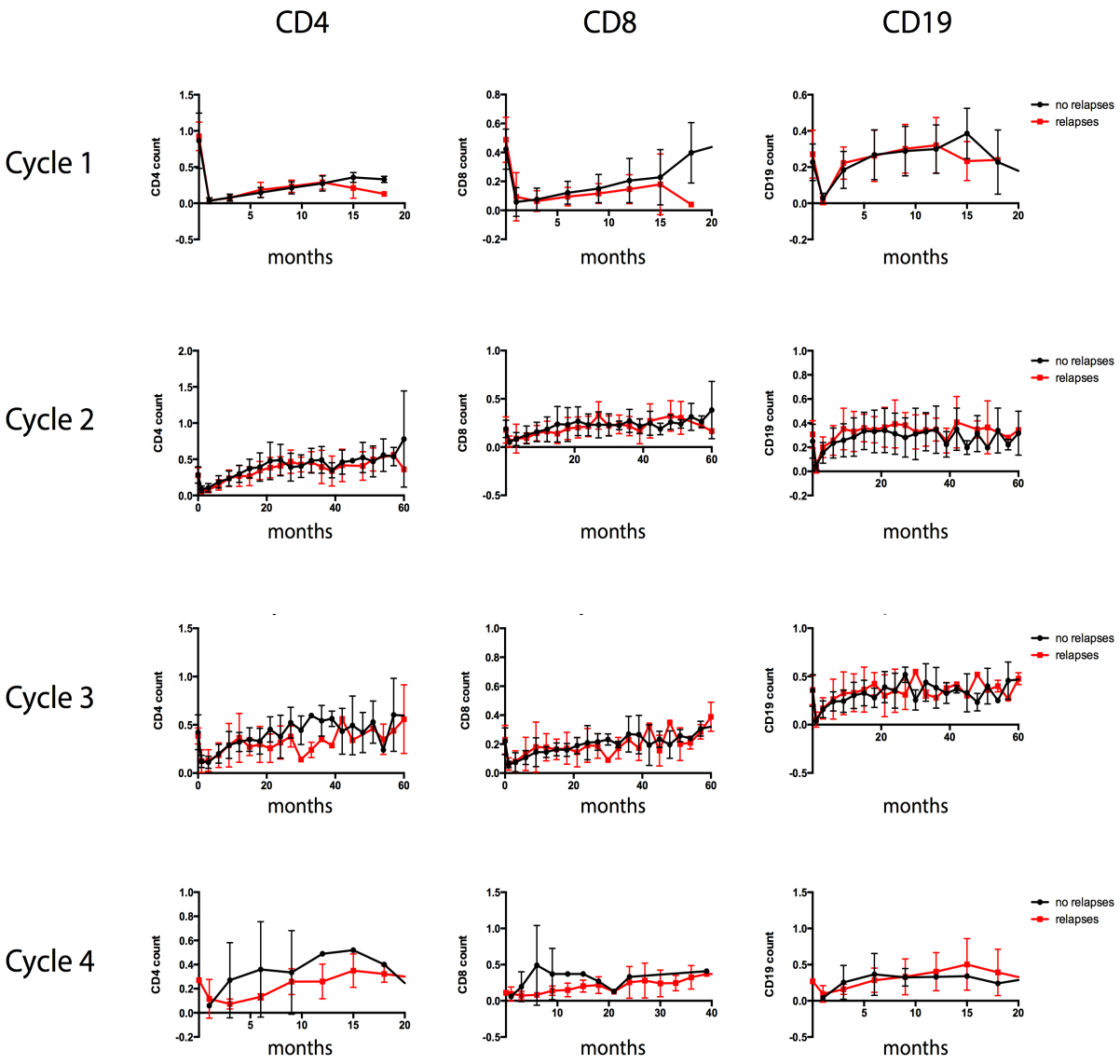

Figure 6. 1: Comparison CD4+, CD8+ and CD19+ cell counts after each cycle of alemtuzumab in patients with and without on-study relapses.

Patients were defined as having relapsed (shown in red) if they developed neurological symptoms attributable to multiple sclerosis, lasting >48 hours with an objective change in neurological examination in the absence of infection. Cell units are $x10^9$/L. Error bars indicate standard deviation.

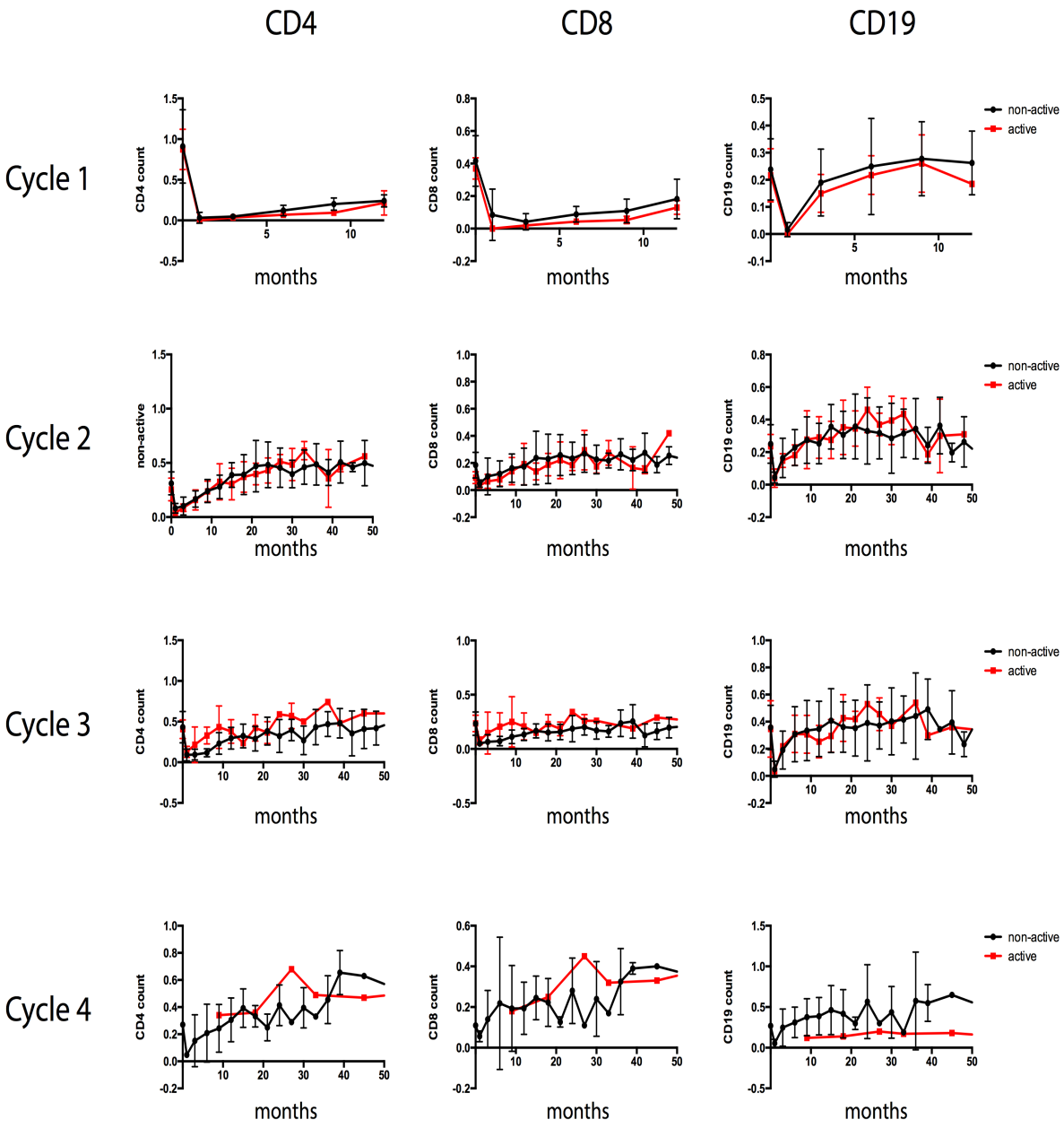

Figure 6. 2: Comparison of CD4+, CD8+ and CD19+ cell counts after each cycle of alemtuzumab in patients with and without on-study active MRI scans.

Patients were defined as having an active MRI scan (shown in red) if they had acquired new T2 lesions, or enhancing lesions. Cell units are $x10^9$/L. Error bars indicate standard deviation.

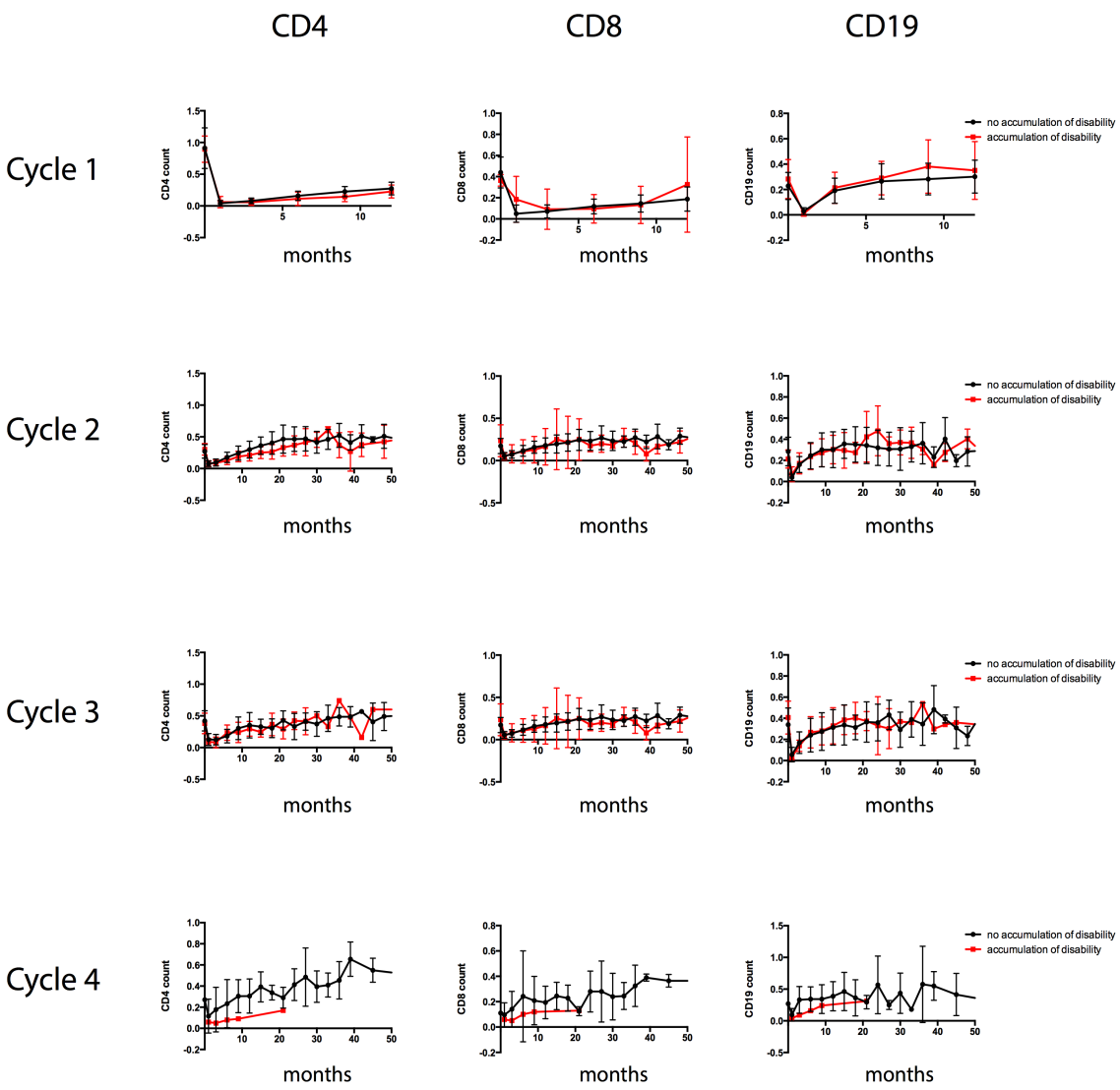

Figure 6. 3: Comparison of CD4+, CD8+, CD19+ cell counts after each cycle of alemtuzumab in patients with and without acquisition of disability.

Patients were deemed to have met the definition of sustained accumulation of disability if their EDSS increased by 1.5 points from a baseline of 0, or by 1.0 point from a baseline of 1.0. Patients meeting this definition are shown in red. Cell units are $x10^9$/L. Error bars indicate standard deviation.

		CD4			CD8			CD19		
	Cycle	Coefficient	95% CI	P value	Coefficient	95% CI	P value	Coefficient	95% CI	P value
Relapse	1	0.003	0.028 to 0.034	0.859	-0.020	-0.069 to 0.030	0.438	0.025	-0.026 to 0.077	0.330
	2	-0.029	-0.075 to 0.017	0.209	-0.011	-0.051 to 0.028	0.577	0.040	-0.007 to 0.087	0.095
	3	-0.009	-0.083 to 0.065	0.806	0.037	-0.017 to 0.090	0.173	0.005	-0.069 to 0.079	0.901
	4	-0.042	-0.333 to 0.248	0.741	-0.046	-0.255 to 0.163	0.620	-0.031	-0.376 to 0.314	0.839
MRI	1	-0.053	-0.117 to 0.012	0.104	-0.071	-0.168 to 0.027	0.144	0.011	-0.124 to 0.146	0.865
	2	-0.007	-0.094 to 0.080	0.873	-0.026	-0.116 to 0.063	0.558	0.010	-0.086 to 0.106	0.831
	3	0.146	0.029 to 0.264	0.016 *	0.125	0.035 to 0.214	0.008 *	0.029	-0.101 to 0.159	0.653
	4	0.068	-0.239 to 0.375	0.606	0.107	-0.134 to 0.348	0.319	-0.228	-0.588 to 0.131	0.171
Disability	1	-0.063	-0.103 to -0.024	0.002*	-0.008	-0.076 to 0.060	0.813	0.042	-0.027 to 0.111	0.226
	2	-0.041	-0.099 to 0.016	0.154	-0.009	-0.057 to 0.039	0.709	0.005	-0.055 to 0.065	0.861
	3	-0.028	-0.114 to 0.058	0.522	-0.022	-0.084 to 0.040	0.473	-0.001	-0.087 to 0.084	0.977
	4	-0.204	-0.546 to 0.139	0.203	-0.104	-0.374 to 0.166	0.392	-0.054	-0.508 to 0.399	0.784
Composite	1	-0.019	-0.047 to 0.009	0.179	-0.004	-0.050 to 0.041	0.860	0.013	-0.033 to 0.060	0.571
	2	-0.028	-0.074 to 0.018	0.233	0.002	-0.035 to 0.039	0.923	0.021	-0.025 to 0.068	0.367
	3	-0.022	-0.095 to 0.051	0.550	0.021	-0.032 to 0.074	0.432	0.004	-0.069 to 0.077	0.915
	4	-0.285	-0.548 to -0.023	0.037*	-0.184	-0.390 to 0.022	0.073	-0.107	-0.548 to 0.335	0.587

Table 6. 1: Comparison of CD4+, CD8+ and CD19+ counts after each cycle of alemtuzumab in those with and without active disease.

For each cycle patients were defined as active or not based on: clinical relapse, MRI activity, disability acquisition and the composite endpoint. A linear mixed effects regression method was used with CD4+ CD8+ CD19+ CD56+NK cells or monocytes as the outcome variable, and with relapse, disability, MRI activity or composite score and time point as explanatory variables. A quadratic term (time point squared) was also included due to the observed relationship between time point and outcome. A separate linear mixed effects model was fitted within each cycle. A continuous autoregressive (order 1) correlation structure was assumed for all models. Model coefficients are presented with 95% confidence intervals and p-values are shown for CD4+ CD8+ and CD19+ cells (other subpopulations are reported in the text). * p <0.05.

A)

Cycle	Total number of patients per cycle	Number who relapsed per cycle	Number who accumulated disability per cycle	Number who met the composite endpoint per cycle
1	108	22	10	28
2	106	40	19	50
3	52	20	12	24
4	11	7	2	9

B)

Cycle	Number of who had an MRI scan per cycle.	Number with an "active" MRI per cycle	Number with a "non-active" scan per cycle.
1	19	4	15
2	59	7	52
3	33	5	28
4	10	4	6

Table 6. 2: Breakdown of patient numbers by treatment cycle.

A) Shows the number of patients following each cycle of alemtuzumab who had a clinical relapse, met the definition for sustained accumulation of disability or the definition of a 'positive composite endpoint'

B) Shows the number of patients who had an MRI scan following each cycle of alemtuzumab. Patients were considered to have an active scan if they had acquired new T2 lesions or if they had enhancing lesions.

6.4 DISCUSSION

Using a much larger cohort and more prolonged follow-up, we fail to confirm the claim that accelerated CD4+ T cell recovery after treatment is a biomarker for recurrent multiple sclerosis disease activity following lymphocyte depletion with alemtuzumab. We also find no evidence that a CD4+ T cell count of greater than $388.5x10^6$ cells per mL at 12 months has utility in selecting a group of patients who may benefit from more intensive monitoring or perhaps even prophylactic repeat dosing.

There are a number of differences between our work and the previous report (Cossburn et al., 2013). Firstly, our cohort is larger (108 versus 56 patients) with a longer duration of follow up (median follow up of 99 months IQR 74.75-117.25 vs. 55 months IQR 24–115). Although both studies selected patients with active RRMS, baseline MS disease activity was somewhat higher in the previous study (ARR of 2.6 SD 0.9 vs. 1.7 SD 0.8), however, very few of their patients experienced disease activity post alemtuzumab (probably reflecting their shorter follow-up): only 8/56 experienced a clinical relapse, with a further 4 patients showing MRI disease activity alone; this small number of data points makes the study susceptible to extreme outliers. Unlike the previous study, we did not perform routine MRI brain scans at month 24. As a consequence analysis of MRI outcome is based on data from fewer patients (19 for cycle 1, 59 for cycle 2, 33 for cycle 3 and 10 for cycle 4); this is a limitation of our study.

However, we do not believe these differences explain why our two studies have reached opposite conclusions; we believe this is best explained by weaknesses in their statistical methods. Firstly, they did not account for repeated treatments only cell counts from the most recent alemtuzumab dose were analysed: post-cycle 2 CD4+ counts for those who remain in remission were compared to post-cycle 3 counts for those with active disease prompting re-dosing; and CD4+ counts post-cycle 3 were then correlated with disease activity prior to cycle 3, and used to "predict" an event that had already occurred. This method assumes that reconstitution is identical after each round of treatment, representing, in our opinion, a major limitation of their study. We controlled for this bias by looking at reconstitution and disease activity after and within each treatment cycle. Due to the

complexity of the analysis, the timing of the event within each cycle is still not accounted for. Secondly, the previous report compared mean cell counts at multiple time points using Student T tests, or Mann-Whitney U when non-normally distributed, without taking into account multiple non-independent observations per patient (an individual's CD4+ count at month 12 is not independent of their month 9 count, and so on). Furthermore they did not correct their p-values for multiple comparisons, of which there were many; so it is likely that some of their statistically significant results occurred by chance; when we repeated their analysis using our data; no p-value survived correction (data not shown).

The wish to identify a biomarker for recurrent disease activity after alemtuzumab is to be welcomed. This would reduce the need to monitor patients at low risk of relapse, and allow the pre-emptive treatment of high-risk patients. Although CD4+ counts may be an attractive candidate - they are readily measurable and T cells are undoubtedly involved in disease pathogenesis, given the complex nature of the immune system it is not surprising that peripheral CD4+ counts alone do not predict CNS inflammation. Indeed, it is known that selective anti-CD4+ depleting therapies do not suppress disease activity in multiple sclerosis (van Oosten et al., 1997). Also, after alemtuzumab treatment, composition of the circulating immune repertoire is radically altered. For example, for at least 6 months following each cycle the CD4+ T cell pool is dominated by memory cells, particularly those with a regulatory phenotype (CD4+CD45RA-CD35hiFoxP3+IL-7Rlo) (Cox et al., 2005). Self evidently, investing confidence in a single measure of a major cellular constituent of peripheral blood disregards the complexity of the immunopathogenesis of multiple sclerosis and is misplaced.

Arguably our data does not prove or disprove whether long-term disease stability is associated with lower CD4+ counts, as patients with clinical or radiological evidence of disease activity are automatically retreated. However this data demonstrates that peripheral CD4+ counts have no utility in predicting multiple sclerosis disease activity after alemtuzumab, and we strongly advise neurologists against using them to personalise treatment protocols. In particular, CD4+ counts should not be used as a marker of the need for pre-emptive re-treatment, thereby exposing patients to potential risk (Coles et al., 2008) (Cohen et al., 2012) (Coles et al., 2012b). Our cautionary message, refuting the claims of the previous report (Cossburn et al., 2013), is timely since alemtuzumab has now entered the clinic as a treatment for active relapsing remitting multiple sclerosis.

CHAPTER 7 – SUMMARY

This thesis investigates the role of IL-7Rα in multiple sclerosis. It first looks at antagonism of IL-7Rα in a first time in human trial. Although this trial was curtailed prior to the investigation into MS patients, as part of a translational medicine PhD this gave me a good insight into phase I trials. The original observations made by Gregory on increased soluble IL-7Rα in those with the at risk genotype for rs6897932 (Gregory et al., 2007) was made in Cambridge and it was this interest in IL-7Rα that persuaded GSK to have the trial in Cambridge. Despite the eventual curtailing of the trial it was a good example of translational medicine in action. However with the early termination of the trial the fundamental question of what effect (good or bad) the drug would have in MS patients remains unanswered.

The trial enabled *in vitro* investigation of antagonism of IL-7Rα. This enabled further investigation into how IL-7Rα mitigates its effect on MS risk between genotypes. This is the first time in the literature it has been demonstrated that IL-7Rα genotype has caused differences in downstream signaling from IL-7Rα. This led to further investigation into negative feedback mechanisms associated with IL-7Rα.

In the Lundstrom paper (Lundstrom et al., 2013) there had been investigation into downregulation of IL-7Rα and the Fas Ligand receptor (CD95), in which there had been demonstration of alteration of negative feedback of cell associated IL-7Rα with increased levels of soluble IL-7Rα, which is the consequence of the at risk SNP rs6897932 for MS. Therefore in the *in vitro* stimulation of IL-7Rα I focused on these two pathways with the first investigation into IL-7Rα using autologous serum from patients stratified according to IL-7Rα genotype. I was unable to reproduce the effects of soluble IL-7Rα on downregulation of IL-7Rα and CD95 that had been demonstrated in the Lundstrom paper. The study may have been limited by a lack of power. However, further investigation into the level of IL-7 in secondary lymphoid organs where it is produced, may lead to a different molar ratio that could produce effects on cell associated IL-7Rα from increased soluble IL-7Rα. Indeed although the Lundstrom paper quoted a soluble IL-7Rα:IL-7 molar ratio of 500:1, in vivo this ratio is dynamic as IL-7 is consumed and the molar ratio observation has not been replicated in other studies (Cote et al., 2015). Further investigation using different techniques such as PCR for alternative methods of negative feedback such as

suppressor of cytokine signaling 1 (SOCS-1) or Gfi-1 (involved in IL-7Rα downregulation) may provide further insights into negative feedback of IL-7Rα.

I was able to draw on the extensive experience of my laboratory group in Cambridge on alemtuzumab, a highly efficacious therapy for MS. This therapy depletes lymphocytes causing lymphocytes to repopulate in the periphery, driven by IL-7 and self-peptide-MHC interactions, which leads to a high incidence of secondary autoimmunity. IL-7 is fundamental to homeostatic proliferation, the process by which lymphocytes repopulate from lymphopenic conditions in the periphery, which propagates autoimmunity, examples of which also include post autologous stem cell therapy (Bakhuraysah et al., 2016) and post HAART with HIV treatment (G. Zandman-Goddard, 2002).

In chapter 5 with the CAMTHY trial I was heavily involved in the dose escalation sub-study of the trial. Palifermin was used to investigate if repopulation of lymphocytes could be driven from increasing thymic lymphopoiesis rather than from homeostatic proliferation in the periphery. The sub-study demonstrated the regime of Palifermin used was tolerable, but unfortunately when given with alemtuzumab the opposite effect to what was predicted was seen with a reduction in thymic lymphopoiesis. This was possibly driven by Palifermin causing increased expression of thymic CD52, which would lead to increased action of alemtuzumab on the thymus. Therefore the question of whether re-direction of lymphocyte proliferation in a lymphopenic environment more centrally to the thymus could lead to a reduction in secondary autoimmunity remains unanswered. My clinical work on the CAMTHY trial gave good exposure to how a clinical trial is run and the administration associated with running a clinical trial. The interaction I had with patients enabled me to see all of the side effects from Palifermin and although it was tolerable with no severe side effects, some of the symptoms experienced by patients were significant at the time. Therefore following the outcome of the CAMTHY trial I think palifermin will not be used outside of its current indication for mucositis associated with chemotherapy, particularly for use in preventing autoimmunity by offsetting the effects of homeostatic proliferation (e.g. for example in the treated HIV population). Future investigation may include the use of other products, which increase thymic lymphopoiesis such as IL-7.

In chapter 6 I investigated reconstitution of lymphocytes, which as described above is driven by IL-7, following treatment with alemtuzumab. I particularly investigated if

CD4+ T cells could be used as a biomarker for disease activity. The use of biomarkers for MS should be encouraged as it can lead to better utilization of the treatments that are currently available. However following the publication of a paper suggesting CD4+ T cells could act as a biomarker post alemtuzumab (Cossburn et al., 2013) I investigated this and found that in our Cambridge cohort this was not the case. This contributed to the practical knowledge on how to monitor alemtuzumab at a time when there were unanswered questions about how the drug would be used in clinical practice as it had just been licensed. Although this work did not lead to the discovery of a new biomarker for MS disease activity, future work into the possibility of IL-7Rα as a potential biomarker, as described in other autoimmune conditions such as lupus nephritis (Badot et al., 2013), could possibly lead to an improvement in patient care.

In summary this thesis investigates the role of IL-7 and its receptor in MS both in terms of the mechanism by which it exerts its risk, but also how it propagates some of the side effects experienced by patients post treatment with alemtuzumab. It also investigated homeostatic proliferation of lymphocytes post alemtuzumab with the potential use of biomarkers for MS treatment. It investigated antagonism of IL-7Rα in a phase 1 trial. It was able to shed some light on aspects of IL-7Rα physiology, particularly highlighting how negative feedback of IL-7Rα may mitigate the risk between individuals based on IL-7Rα genotype.

CHAPTER 8 - REFERENCES

ABRAHAMSSON, S. V., ANGELINI, D. F., DUBINSKY, A. N., MOREL, E., OH, U., JONES, J. L., CARASSITI, D., REYNOLDS, R., SALVETTI, M., CALABRESI, P. A., COLES, A. J., BATTISTINI, L., MARTIN, R., BURT, R. K. & MURARO, P. A. 2013. Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis. *Brain*.

ACHESON, E., BACHRACH, C. & WRIGHT, F. 1960. Some comments on the relationship of the distribution of multiple sclerosis to latitude, solar radiation, and other variables. *Acta Psychiatrica Scandinavica,* 35**,** 132-147.

ADMINISTRATION, F. A. D. 2013. *FDA Drug Safety Communication: FDA investigating rare brain infection in patient taking Gilenya (fingolimod)* [Online]. Available:http://www.fda.gov/Drugs/DrugSafety/ucm366529.htm.

ALASTAIR COMPSTON, ALASDAIR COLES. 2008. Multiple Sclerosis. *Lancet,* 372**,** 1502-17.

ALBRECHT, P., BOUCHACHIA, I., GOEBELS, N., HENKE, N., HOFSTETTER, H. H., ISSBERNER, A., KOVACS, Z., LEWERENZ, J., LISAK, D. & MAHER, P. 2012. Effects of dimethyl fumarate on neuroprotection and immunomodulation. *Journal of neuroinflammation,* 9**,** 163.

AMES, A. 2000. CNS energy metabolism as related to function. *Brain Research Reviews,* 34**,** 42-68.

ANDERSON, C. A., BOUCHER, G., LEES, C. W., FRANKE, A., D'AMATO, M., TAYLOR, K. D., LEE, J. C., GOYETTE, P., IMIELINSKI, M., LATIANO, A., LAGACE, C., SCOTT, R., AMININEJAD, L., BUMPSTEAD, S., BAIDOO, L., BALDASSANO, R. N., BARCLAY, M., BAYLESS, T. M., BRAND, S., BUNING, C., COLOMBEL, J. F., DENSON, L. A., DE VOS, M., DUBINSKY, M., EDWARDS, C., ELLINGHAUS, D., FEHRMANN, R. S., FLOYD, J. A., FLORIN, T., FRANCHIMONT, D., FRANKE, L., GEORGES, M., GLAS, J., GLAZER, N. L., GUTHERY, S. L., HARITUNIANS, T., HAYWARD, N. K., HUGOT, J. P., JOBIN, G., LAUKENS, D., LAWRANCE, I., LEMANN, M., LEVINE, A., LIBIOULLE, C., LOUIS, E., MCGOVERN, D. P., MILLA, M., MONTGOMERY, G. W., MORLEY, K. I., MOWAT, C., NG, A., NEWMAN, W., OPHOFF, R. A., PAPI, L., PALMIERI, O., PEYRIN-BIROULET, L., PANES, J., PHILLIPS, A., PRESCOTT, N. J., PROCTOR, D. D., ROBERTS, R., RUSSELL, R., RUTGEERTS, P., SANDERSON, J., SANS, M., SCHUMM, P., SEIBOLD, F., SHARMA, Y., SIMMS, L. A., SEIELSTAD, M., STEINHART, A. H., TARGAN, S. R., VAN DEN BERG, L. H., VATN, M., VERSPAGET, H., WALTERS, T., WIJMENGA, C., WILSON, D. C., WESTRA, H. J., XAVIER, R. J., ZHAO, Z. Z., PONSIOEN, C. Y., ANDERSEN, V., TORKVIST, L., GAZOULI, M., ANAGNOU, N. P., KARLSEN, T. H., KUPCINSKAS, L., SVENTORAITYTE, J., MANSFIELD, J. C., KUGATHASAN, S., SILVERBERG, M. S., HALFVARSON, J., ROTTER, J. I., MATHEW, C. G., GRIFFITHS, A. M., GEARRY, R., AHMAD, T., BRANT, S. R., CHAMAILLARD, M., et al. 2011. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet,* 43**,** 246-52.

ANDO, D. G., CLAYTON, J., KONO, D., URBAN, J. L. & SERCARZ, E. E. 1989. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell Immunol,* 124**,** 132-43.

ANNETTE OTURAI, F. L., LARS P. RYDER, HANS O. MADSEN, JAN HILLERT, STEN FREDRIKSON, MAGNHILD SANDBERG-WOLLHEIM, MIKKO LAAKSONEN, NILS KOCH-HENRIKSEN, STEPHEN SAWCER, LARS FUGGER, PER S. SORENSEN, AND ARNE SVEJGAARD 1999. Linkage and association analysis of susceptibility regions on chromosomes 5 and 6 in 106 Scandinavian sibling pair families with multiple sclerosis. *Ann Neurol,* 46**,** 612–616.

ARBELAEZ, C. A., GLATIGNY, S., DUHEN, R., EBERL, G., OUKKA, M. & BETTELLI, E. 2015. IL-7/IL-7 Receptor Signaling Differentially Affects Effector CD4+ T Cell Subsets Involved in Experimental Autoimmune Encephalomyelitis. *J Immunol,* 195**,** 1974-83.

ASCHERIO, A. & MUNGER, K. L. 2007. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Annals of neurology,* 61**,** 288-299.

ASCHERIO, A., MUNGER, K. L., WHITE, R., KÖCHERT, K., SIMON, K. C., POLMAN, C. H., FREEDMAN, M. S., HARTUNG, H.-P., MILLER, D. H. & MONTALBÁN, X. 2014. Vitamin D as an early predictor of multiple sclerosis activity and progression. *JAMA neurology,* 71**,** 306-314.

ASHBAUGH, J. J., BRAMBILLA, R., KARMALLY, S. A., CABELLO, C., MALEK, T. R. & BETHEA, J. R. 2013. IL7Ralpha Contributes to Experimental Autoimmune Encephalomyelitis through Altered T Cell Responses and Nonhematopoietic Cell Lineages. *J Immunol*.

AUBIN, F. 2003. Mechanisms involved in ultraviolet light‐induced immunosuppression. *European journal of dermatology,* 13**,** 515-523.

B.W. VAN OOSTEN, M. M. L., MD; S. HODGKINSON, MD; F. BARKHOF, MD; D.H. MILLER, MD; I.F. MOSELEY, MD; A.J. THOMPSON, MD; P. RUDGE, MD; A. MCDOUGALL, MD; J.G. MCLEOD, MD; H.J. AD&R,PHD; AND C.H. POLMAN, MD 1997. Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody cM-T412: Results of a randomized,double-blind,placebo-controlled, MR-monitored phase II trial. *NEUROLOGY,* 49**,** 351-357.

BACCALA, R. & THEOFILOPOULOS, A. N. 2005. The new paradigm of T-cell homeostatic proliferation-induced autoimmunity. *Trends Immunol,* 26**,** 5-8.

BADOT, V., DUREZ, P., VAN DEN EYNDE, B. J., NZEUSSEU-TOUKAP, A., HOUSSIAU, F. A. & LAUWERYS, B. R. 2011. Rheumatoid arthritis synovial fibroblasts produce a soluble form of the interleukin-7 receptor in response to pro-inflammatory cytokines. *J Cell Mol Med,* 15**,** 2335-42.

BADOT, V., LUIJTEN, R. K., VAN ROON, J. A., DEPRESSEUX, G., AYDIN, S., VAN DEN EYNDE, B. J., HOUSSIAU, F. A. & LAUWERYS, B. R. 2013. Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. *Ann Rheum Dis,* 72**,** 453-6.

BAECHER-ALLAN C, B. J., FREEMAN GJ, HAFLER DA 2001. CD4 CD25high Regulatory Cells in Human Peripheral Blood. *J Immunol,* 167**,** 1245-53.

BAHLO, M., BOOTH, D. R., BROADLEY, S. A., BROWN, M. A., FOOTE, S. J., GRIFFITHS, L. R., KILPATRICK, T. J., LECHNER-SCOTT, J., MOSCATO, P., PERREAU, V. M., RUBIO, J. P., SCOTT, R. J., STANKOVICH, J., STEWART, G. J.,

TAYLOR, B. V., WILEY, J., CLARKE, G., COX, M. B., CSURHES, P. A., DANOY, P., DRYSDALE, K., FIELD, J., GREER, J. M., HADLER, J., MCMORRAN, B. J., JENSEN, C. J., JOHNSON, L. J., MCCALLUM, R., MERRIMAN, M., MERRIMAN, T., PRYCE, K., TAJOURI, L., WILKINS, E. J., BROWNING, B. L., BROWNING, S. R., PERERA, D., BUTZKUEVEN, H., CARROLL, W. M., CHAPMAN, C., KERMODE, A. G., MARRIOTT, M., MASON, D., HEARD, R. N., PENDER, M. P., SLEE, M., TUBRIDY, N., WILLOUGHBY, E., AUSTRALIA & NEW ZEALAND MULTIPLE, S. 2009. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nature Genetics,* 41**,** 824-U84.

BAILEY, O. T., PAPPENHEIMER, A. M., CHEEVER, F. S. & DANIELS, J. B. 1949. A MURINE VIRUS (JHM) CAUSING DISSEMINATED ENCEPHALOMYELITIS WITH EXTENSIVE DESTRUCTION OF MYELIN : II. PATHOLOGY. *J Exp Med,* 90**,** 195-212.

BAKHURAYSAH, M. M., SIATSKAS, C. & PETRATOS, S. 2016. Hematopoietic stem cell transplantation for multiple sclerosis: is it a clinical reality? *Stem Cell Res Ther,* 7**,** 12.

BAN, M., CAILLIER, S., MERO, I. L., MYHR, K. M., CELIUS, E. G., AARSETH, J., TORKILDSEN, O., HARBO, H. F., OKSENBERG, J., HAUSER, S. L., SAWCER, S. & COMPSTON, A. 2013. No evidence of association between mutant alleles of the CYP27B1 gene and multiple sclerosis. *Ann Neurol,* 73**,** 430-2.

BARANZINI, S. E., WANG, J., GIBSON, R. A., GALWEY, N., NAEGELIN, Y., BARKHOF, F., RADUE, E. W., LINDBERG, R. L. P., UITDEHAAG, B. M. G., JOHNSON, M. R., ANGELAKOPOULOU, A., HALL, L., RICHARDSON, J. C., PRINJHA, R. K., GASS, A., GEURTS, J. J. G., KRAGT, J., SOMBEKKE, M., VRENKEN, H., QUALLEY, P., LINCOLN, R. R., GOMEZ, R., CAILLIER, S. J., GEORGE, M. F., MOUSAVI, H., GUERRERO, R., OKUDA, D. T., CREE, B. A. C., GREEN, A. J., WAUBANT, E., GOODIN, D. S., PELLETIER, D., MATTHEWS, P. M., HAUSER, S. L., KAPPOS, L., POLMAN, C. H. & OKSENBERG, J. R. 2009. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. *Human Molecular Genetics,* 18**,** 767-778.

BARCELLOS, L. F., SAWCER, S., RAMSAY, P. P., BARANZINI, S. E., THOMSON, G., BRIGGS, F., CREE, B. C., BEGOVICH, A. B., VILLOSLADA, P. & MONTALBAN, X. 2006. Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. *Human molecular genetics,* 15**,** 2813-2824.

BARIZZONE, N., PAUWELS, I., LUCIANO, B., FRANCKAERT, D., GUERINI, F. R., COSEMANS, L., HILVEN, K., SALVIATI, A., DOOLEY, J. & DANSO‐ABEAM, D. 2013. No evidence for a role of rare CYP27B1 functional variations in multiple sclerosis. *Annals of neurology,* 73**,** 433-437.

BARKHOF, F., BRÜCK, W., DE GROOT, C. J., BERGERS, E., HULSHOF, S., GEURTS, J., POLMAN, C. H. & VAN DER VALK, P. 2003. Remyelinated lesions in multiple sclerosis: magnetic resonance image appearance. *Archives of neurology,* 60**,** 1073-1081.

BARTHOLOMÄUS, I., KAWAKAMI, N., ODOARDI, F., SCHLÄGER, C., MILJKOVIC, D., ELLWART, J. W., KLINKERT, W. E., FLÜGEL-KOCH, C., ISSEKUTZ, T. B. & WEKERLE, H. 2009. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature,* 462**,** 94-98.

BAXTER, A. G. & HODGKIN, P. D. 2002. Activation rules: the two-signal theories of immune activation. *Nature Reviews Immunology,* 2**,** 439-446.

BAYES, H. K., WEIR, C. J. & O'LEARY, C. 2010. Timing of birth and risk of multiple sclerosis in the Scottish population. *European neurology,* 63**,** 36-40.

BEART, P., ROBINSON, M., RATTRAY, M. & MARAGAKIS, N. J. 2017. *Neurodegenerative Diseases: Pathology, Mechanisms, and Potential Therapeutic Targets*, Springer.

BEBO, B. F., JR., SCHUSTER, J. C., ADLARD, K., VANDENBARK, A. A. & OFFNER, H. 2000. Interleukin 7 is a potent co-stimulator of myelin specific T cells that enhances the adoptive transfer of experimental autoimmune encephalomyelitis. *Cytokine,* 12**,** 324-31.

BEN-NUN, A., WEKERLE, H. & COHEN, I. R. 2017. Pillars Article: The Rapid Isolation of Clonable Antigen-specific T Lymphocyte Lines Capable of Mediating Autoimmune Encephalomyelitis. Eur J Immunol. 1981.11: 195-199. *J Immunol,* 198**,** 3384-3388.

BENDER, S. J. & WEISS, S. R. 2010. Pathogenesis of murine coronavirus in the central nervous system. *Journal of Neuroimmune Pharmacology,* 5**,** 336-354.

BERNARD, C. 1976. EXPERIMENTAL AUTOIMMUNE ENCEPHALO‐MYELITIS IN MICE: GENETIC CONTROL OF SUSCEPTIBILITY. *International Journal of Immunogenetics,* 3**,** 263-274.

BETTELLI, E., SULLIVAN, B., SZABO, S. J., SOBEL, R. A., GLIMCHER, L. H. & KUCHROO, V. K. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med,* 200**,** 79-87.

BIELEKOVA, B., CATALFAMO, M., REICHERT-SCRIVNER, S., PACKER, A., CERNA, M., WALDMANN, T. A., MCFARLAND, H., HENKART, P. A. & MARTIN, R. 2006. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. *Proc Natl Acad Sci U S A,* 103**,** 5941-6.

BIELEKOVA, B., GOODWIN, B., RICHERT, N., CORTESE, I., KONDO, T., AFSHAR, G., GRAN, B., EATON, J., ANTEL, J., FRANK, J. A., MCFARLAND, H. F. & MARTIN, R. 2000. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med,* 6**,** 1167-75.

BIELEKOVA, B., MURARO, P. A., GOLESTANEH, L., PASCAL, J., MCFARLAND, H. F. & MARTIN, R. 1999. Preferential expansion of autoreactive T lymphocytes from the memory T-cell pool by IL-7. *J Neuroimmunol,* 100**,** 115-23.

BJARTMAR, C., WUJEK, J. & TRAPP, B. 2003. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *Journal of the neurological sciences,* 206**,** 165-171.

BLAKEMORE, W. 1982. Ethidium bromide induced demyelination in the spinal cord of the cat. *Neuropathology and applied neurobiology,* 8**,** 365-375.

BLANC, C. A., ROSEN, H. & LANE, T. E. 2014. FTY720 (fingolimod) modulates the severity of viral-induced encephalomyelitis and demyelination. *Journal of neuroinflammation,* 11**,** 138.

BLAZAR, B. R., WEISDORF, D. J., DEFOR, T., GOLDMAN, A., BRAUN, T., SILVER, S. & FERRARA, J. L. M. 2006. Phase 1/2 randomized, placebo-control trial of palifermin to prevent graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). *Blood,* 108**,** 3216-3222.

BOHRA, C., SOKOL, L. & DALIA, S. 2017. Progressive Multifocal Leukoencephalopathy and Monoclonal Antibodies: A Review. *Cancer Control,* 24**,** 1073274817729901.

BOYMAN, O., RAMSEY, C., KIM, D. M., SPRENT, J. & SURH, C. D. 2008. IL-7/anti-IL-7 mAb complexes restore T cell development and induce homeostatic T Cell expansion without lymphopenia. *J Immunol,* 180**,** 7265-75.

BRAMOW, S., FRISCHER, J. M., LASSMANN, H., KOCH-HENRIKSEN, N., LUCCHINETTI, C. F., SØRENSEN, P. S. & LAURSEN, H. 2010. Demyelination versus remyelination in progressive multiple sclerosis. *Brain,* 133**,** 2983-2998.

BRÄNDLE, S. M., OBERMEIER, B., SENEL, M., BRUDER, J., MENTELE, R., KHADEMI, M., OLSSON, T., TUMANI, H., KRISTOFERITSCH, W. & LOTTSPEICH, F. 2016. Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins. *Proceedings of the National Academy of Sciences,* 113**,** 7864-7869.

BRENNAN, K. M., GALBAN-HORCAJO, F., RINALDI, S., O'LEARY, C. P., GOODYEAR, C. S., KALNA, G., ARTHUR, A., ELLIOT, C., BARNETT, S. & LININGTON, C. 2011. Lipid arrays identify myelin-derived lipids and lipid complexes as prominent targets for oligoclonal band antibodies in multiple sclerosis. *Journal of neuroimmunology,* 238**,** 87-95.

BRØNNUM‐HANSEN, H., KOCH‐HENRIKSEN, N. & STENAGER, E. 2004. Trends in survival and cause of death in Danish patients with multiple sclerosis. *Brain,* 127**,** 844-850.

BROWNLEE, W. J. & MILLER, D. H. 2014. Clinically isolated syndromes and the relationship to multiple sclerosis. *J Clin Neurosci,* 21**,** 2065-71.

BRÜCK, W., PORADA, P., POSER, S., RIECKMANN, P., HANEFELD, F., KRETZSCHMARCH, H. A. & LASSMANN, H. 1995. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Annals of neurology,* 38**,** 788-796.

BURMAN, J., IACOBAEUS, E., SVENNINGSSON, A., LYCKE, J., GUNNARSSON, M., NILSSON, P., VRETHEM, M., FREDRIKSON, S., MARTIN, C., SANDSTEDT, A., UGGLA, B., LENHOFF, S., JOHANSSON, J. E., ISAKSSON, C., HAGGLUND, H., CARLSON, K. & FAGIUS, J. 2014. Autologous haematopoietic stem cell transplantation for aggressive multiple sclerosis: the Swedish experience. *J Neurol Neurosurg Psychiatry,* 85**,** 1116-21.

BURT, R. K., BALABANOV, R., HAN, X., SHARRACK, B., MORGAN, A., QUIGLEY, K., YAUNG, K., HELENOWSKI, I. B., JOVANOVIC, B., SPAHOVIC, D., ARNAUTOVIC, I., LEE, D. C., BENEFIELD, B. C., FUTTERER, S., OLIVEIRA, M. C. & BURMAN, J. 2015. Association of nonmyeloablative hematopoietic stem cell transplantation with neurological disability in patients with relapsing-remitting multiple sclerosis. *JAMA,* 313**,** 275-84.

BYRAVAN, S., FOSTER, L. M., PHAN, T., VERITY, A. N. & CAMPAGNONI, A. T. 1994. Murine oligodendroglial cells express nerve growth factor. *Proc Natl Acad Sci U S A,* 91**,** 8812-6.

CALABRESE, M., FILIPPI, M. & GALLO, P. 2010. Cortical lesions in multiple sclerosis. *Nat Rev Neurol,* 6**,** 438-44.

CAMPBELL, G. & MAHAD, D. J. 2018. Mitochondrial dysfunction and axon degeneration in progressive multiple sclerosis. *FEBS Lett*.

CANTORNA, M. T., HAYES, C. E. & DELUCA, H. F. 1996. 1, 25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proceedings of the National Academy of Sciences,* 93**,** 7861-7864.

CARLSON, C. S., EMERSON, R. O., SHERWOOD, A. M., DESMARAIS, C., CHUNG, M. W., PARSONS, J. M., STEEN, M. S., LAMADRID-HERRMANNSFELDT, M. A., WILLIAMSON, D. W., LIVINGSTON, R. J., WU, D., WOOD, B. L., RIEDER, M. J. & ROBINS, H. 2013. Using synthetic templates to design an unbiased multiplex PCR assay. *Nat Commun,* 4**,** 2680.

CARRETTE, F. & SURH, C. D. 2012. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin Immunol,* 24**,** 209-17.

CARSON, K. R., EVENS, A. M., RICHEY, E. A., HABERMANN, T. M., FOCOSI, D., SEYMOUR, J. F., LAUBACH, J., BAWN, S. D., GORDON, L. I. & WINTER, J. N. 2009. Progressive multifocal leukoencephalopathy after rituximab therapy in HIV-negative patients: a report of 57 cases from the Research on Adverse Drug Events and Reports project. *Blood,* 113**,** 4834-4840.

CASTILLO-TRIVINO, T., BRAITHWAITE, D., BACCHETTI, P. & WAUBANT, E. 2013. Rituximab in relapsing and progressive forms of multiple sclerosis: a systematic review. *PloS one,* 8**,** e66308.

CEPOK, S., ZHOU, D., SRIVASTAVA, R., NESSLER, S., STEI, S., BUSSOW, K., SOMMER, N. & HEMMER, B. 2005. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest,* 115**,** 1352-60.

CHANDELE, A., JOSHI, N. S., ZHU, J., PAUL, W. E., LEONARD, W. J. & KAECH, S. M. 2008. Formation of IL-7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1. *J Immunol,* 180**,** 5309-19.

CHOI, S. R., HOWELL, O. W., CARASSITI, D., MAGLIOZZI, R., GVERIC, D., MURARO, P. A., NICHOLAS, R., RONCAROLI, F. & REYNOLDS, R. 2012. Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. *Brain,* 135**,** 2925-2937.

CLARKE, C. 2016. *Neurology: a queen square textbook*, John Wiley & Sons.

CLAUSSEN, M. C. & KORN, T. 2012. Immune mechanisms of new therapeutic strategies in MS—Teriflunomide. *Clinical immunology,* 142**,** 49-56.

CLEMENTS, J. L., JOHN, S. A. & GARRETT-SINHA, L. A. 2006. Impaired generation of CD8+ thymocytes in Ets-1-deficient mice. *J Immunol,* 177**,** 905-12.

CLIFFORD, D. B., ANCES, B., COSTELLO, C., ROSEN-SCHMIDT, S., ANDERSSON, M., PARKS, D., PERRY, A., YERRA, R., SCHMIDT, R. & ALVAREZ, E. 2011. Rituximab-associated progressive multifocal leukoencephalopathy in rheumatoid arthritis. *Archives of neurology,* 68**,** 1156-1164.

COHEN, J. A., BARKHOF, F., COMI, G., HARTUNG, H. P., KHATRI, B. O., MONTALBAN, X., PELLETIER, J., CAPRA, R., GALLO, P., IZQUIERDO, G., TIEL-WILCK, K., DE VERA, A., JIN, J., STITES, T., WU, S., ARADHYE, S., KAPPOS, L., COHEN, J., BARKHOF, F., COMI, G., HARTUNG, H. P., KAPPOS, L., KHATRI, B., MONTALBAN, X., PELLETIER, J., EASTON, J. D., CALANDRA, T., DIMARCO, J., HUDSON, L., KESSELRING, J., LAUPACIS, A., TEMKIN, N., WEINSHENKER, B., ZARBIN, M., BARKHOF, F., POPPE, P., LUETIC, G., CRISTIANO, E., CACERES, F., GARCEA, O., CORREALE, J., BALLARIO, C., PIEDRABUENA, R., POLLARD, J., BERAN, R., HODGKINSON, S., SCHWARTZ,

R., HEARD, R., KING, J., BUTZKUEVEN, H., MAIDA, E. M., VASS, K., FRANTA-ELMER, C., BERGER, T., AICHNER, F., LADURNER, G., BISSAY, V., SINDIC, C., D'HOOGHE, M., MULLENERS, E., DAMASCENO, B., BARREIRA, A., NAYLOR, R., ALVARENGA, R., BACELLAR, A., HAUSSEN, S., DUQUETTE, P., ANTEL, J., LAMONTAGNE, A., GRAND'MAISON, F., FREEDMAN, M., CHRISTIE, S., O'CONNOR, P., VOROBEYCHIK, G., DEVONSHIRE, V., RAMADAN, M., HAMDY, S., REDA, E., HASHEM, S., FOUAD, M., LEBRUN-FRENAY, C., PELLETIER, J., CLANET, M., BROCHET, B., DEBOUVERIE, M., HEINZLEF, O., ZIEMSSEN, T., KOEHLER, W., TIEL-WILCK, K., BACHUS, R., ALTMANN, N., FAISS, J., BAUM, K., DRESSEL, A., LUCKNER, K., EBKE, M., STANGEL, M., HARTUNG, H. P., DIENER, H. C., BETHKE, F., et al. 2010. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N. Engl. J. Med.,* 362**,** 402-415.

COHEN, J. A., COLES, A. J., ARNOLD, D. L., CONFAVREUX, C., FOX, E. J., HARTUNG, H. P., HAVRDOVA, E., SELMAJ, K. W., WEINER, H. L., FISHER, E., BRINAR, V. V., GIOVANNONI, G., STOJANOVIC, M., ERTIK, B. I., LAKE, S. L., MARGOLIN, D. H., PANZARA, M. A. & COMPSTON, D. A. 2012. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet,* 380**,** 1819-28.

COLES A J, M. G. W., PAUL MOLYNEUX ANDREA PAOLILLO CHARLIE M. DAVIE GEOFF HALE DAVID MILLER HERMANN WALDMANN & COMPSTON, A. 1999. Monoclonal Antibody Treatment Exposes Three Mechanisms Underlying the Clinical Course of Multiple Sclerosis. *Annals of Neurology,* 46**,** 296-304.

COLES, A. J. 2013. Alemtuzumab therapy for multiple sclerosis. *Neurotherapeutics,* 10**,** 29-33.

COLES, A. J., AZZOPARDI, L., KOUSIN-EZEWU, O., MULLAY, H. K., THOMPSON, S. A., JARVIS, L., DAVIES, J., HOWLETT, S., RAINBOW, D., BABAR, J., SADLER, T. J., BROWN, J. W. L., NEEDHAM, E., MAY, K., GEORGIEVA, Z. G., HANDEL, A. E., MAIO, S., DEADMAN, M., ROTA, I., HOLLANDER, G., DAWSON, S., JAYNE, D., SEGGEWISS-BERNHARDT, R., DOUEK, D. C., ISAACS, J. D. & JONES, J. L. 2019. Keratinocyte growth factor impairs human thymic recovery from lymphopenia. *JCI Insight,* 5.

COLES, A. J., COMPSTON, D. A., SELMAJ, K. W., LAKE, S. L., MORAN, S., MARGOLIN, D. H., NORRIS, K., TANDON, P. K., PANITCH, H., ANAISSIE, E., CINES, D., DEGROOT, L., DORSEY, F., PHILLIPS, T., SIMON, J., BRINAR, V., DEMARIN, V., JANCULJAK, D., RUDEZ, J., VLADIC, A., CZLONKOWSKA, A., MIROWSKA-GUZEL, D., KOZUBSKI, W., KWIECINSKI, H., SELMAJ, K., STELMASIAK, Z., SZCZUDLIK, A., BOYKO, A., GUSEV, E. I., SKOROMETS, A., STOLYAROV, I., YAKHNO, N., ZAVALISHIN, I., COLES, A. J., COMPSTON, D. A., SHAWCROSS, J., JONES, J., COX, A. L., BASS, A., WENZEL, D., BITON, V., CASCIONE, M., CLEEREMANS, B., COOPER, J., COYLE, P. K., MADIGAN, D., CUTLER, B., FOX, E. J., MAYER, L., TYER, R., GAZDA, S., GLYMAN, S., GUPTA, A., HARNEY, J., HUTTON, G., JACOBS, D., KHAN, O., LISAK, R., TSELIS, A., KLAPPER, J., LISS, J., MEYER, D., RAE-GRANT, A., ROSSMAN, H., BOUDOURIS, W., BELKIN, M., PIERCE, R., ROYAL, W., SHUBIN, R., SIDER, D., STEIN, M., STEINGO, B., SULLIVAN, H., TWYMAN, C., WEBB, R., WEINSHENKER, B., KEEGAN, B. M., RAUCHWARTER, D., WINGERCHUK, D., CARTER, J., WRAY, S., WYNN, D.,

ALLEN, N., NAGAR, C., O'BRIEN, D., PASKAVITZ, J., SCHAEFER, P., DEGROOT, L., VALENTE, W., BEARDSLEY, D., BUSSEL, J., CINES, D., GOLDBERG, M., MACMILLAN, R., SCADDEN, D., D'AGOSTINO, R. B., PENCINA, M., GONZALES, G., WALDMANN, H. & HALE, G. 2008. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. *N. Engl. J. Med.,* 359**,** 1786-1801.

COLES, A. J., COX, A., LE PAGE, E., JONES, J., TRIP, S. A., DEANS, J., SEAMAN, S., MILLER, D. H., HALE, G., WALDMANN, H. & COMPSTON, D. A. 2006. The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy. *J. Neurol.,* 253**,** 98-108.

COLES, A. J., FOX, E., VLADIC, A., GAZDA, S. K., BRINAR, V., SELMAJ, K. W., SKOROMETS, A., STOLYAROV, I., BASS, A., SULLIVAN, H., MARGOLIN, D. H., LAKE, S. L., MORAN, S., PALMER, J., SMITH, M. S. & COMPSTON, D. A. 2012a. Alemtuzumab more effective than interferon beta-1a at 5-year follow-up of CAMMS223 clinical trial. *Neurology,* 78**,** 1069-78.

COLES, A. J., TWYMAN, C. L., ARNOLD, D. L., COHEN, J. A., CONFAVREUX, C., FOX, E. J., HARTUNG, H. P., HAVRDOVA, E., SELMAJ, K. W., WEINER, H. L., MILLER, T., FISHER, E., SANDBRINK, R., LAKE, S. L., MARGOLIN, D. H., OYUELA, P., PANZARA, M. A. & COMPSTON, D. A. 2012b. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet,* 380**,** 1829-39.

COMPSTON, D., BATCHELOR, J. & MCDONALD, W. 1976. B-lymphocyte alloantigens associated with multiple sclerosis. *The Lancet,* 308**,** 1261-1265.

CONFAVREUX, C. & VUKUSIC, S. 2006. Natural history of multiple sclerosis: a unifying concept. *Brain,* 129**,** 606-616.

CONFAVREUX, C., VUKUSIC, S. & ADELEINE, P. 2003. Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process. *Brain,* 126**,** 770-782.

CONFAVREUX, C., VUKUSIC, S., MOREAU, T. & ADELEINE, P. 2000. Relapses and progression of disability in multiple sclerosis. *New England Journal of Medicine,* 343**,** 1430-1438.

CONSTANTINESCU, C. S., HILLIARD, B., VENTURA, E., WYSOCKA, M., SHOWE, L., LAVI, E., FUJIOKA, T., SCOTT, P., TRINCHIERI, G. & ROSTAMI, A. 2001. Modulation of susceptibility and resistance to an autoimmune model of multiple sclerosis in prototypically susceptible and resistant strains by neutralization of interleukin-12 and interleukin-4, respectively. *Clin Immunol,* 98**,** 23-30.

CORDER, E., SAUNDERS, A., STRITTMATTER, W., SCHMECHEL, D., GASKELL, P., SMALL, G. A., ROSES, A., HAINES, J. & PERICAK-VANCE, M. A. 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science,* 261**,** 921-923.

CORTES, A. & BROWN, M. A. 2011. Promise and pitfalls of the Immunochip. *Arthritis research & therapy,* 13**,** 101.

COSSBURN, M. D., HARDING, K., INGRAM, G., EL-SHANAWANY, T., HEAPS, A., PICKERSGILL, T. P., JOLLES, S. & ROBERTSON, N. P. 2013. Clinical relevance of differential lymphocyte recovery after alemtuzumab therapy for multiple sclerosis. *Neurology,* 80**,** 55-61.

COTE, S., MATTE, J., SAD, S., ANGEL, J. B. & CRAWLEY, A. M. 2015. Complexed soluble IL-7 receptor alpha and IL-7 increase IL-7-mediated proliferation and viability of CD8 T-cells in vitro. *Cell Immunol,* 293**,** 122-125.

COUTURIER, N., BUCCIARELLI, F., NURTDINOV, R. N., DEBOUVERIE, M., LEBRUN-FRENAY, C., DEFER, G., MOREAU, T., CONFAVREUX, C., VUKUSIC, S. & COURNU-REBEIX, I. 2011. Tyrosine kinase 2 variant influences T lymphocyte polarization and multiple sclerosis susceptibility. *Brain,* 134**,** 693-703.

COX, A. L., THOMPSON, S. A., JONES, J. L., ROBERTSON, V. H., HALE, G., WALDMANN, H., COMPSTON, D. A. & COLES, A. J. 2005. Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis. *Eur. J. Immunol.,* 35**,** 3332-3342.

COZZO, C., LARKIN, J., 3RD & CATON, A. J. 2003. Cutting edge: self-peptides drive the peripheral expansion of CD4+CD25+ regulatory T cells. *J Immunol,* 171**,** 5678-82.

CRAWLEY, A. M., FAUCHER, S. & ANGEL, J. B. 2010. Soluble IL-7R alpha (sCD127) inhibits IL-7 activity and is increased in HIV infection. *J Immunol,* 184**,** 4679-87.

CUA, D. J., SHERLOCK, J., CHEN, Y., MURPHY, C. A., JOYCE, B., SEYMOUR, B., LUCIAN, L., TO, W., KWAN, S., CHURAKOVA, T., ZURAWSKI, S., WIEKOWSKI, M., LIRA, S. A., GORMAN, D., KASTELEIN, R. A. & SEDGWICK, J. D. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature,* 421**,** 744-8.

CUA, D. J. & TATO, C. M. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol,* 10**,** 479-89.

D. WYNN, D. L. A., J.A. COHEN, A.J. COLES, E.J. FOX, H.-P.. HARTUNG, E. HAVRDOVA, K.W.SELMAJ, H.L. WEINER, J. PALMER, D.H. MARGOLIN, P. OYUELA, M.A. PANZARA, DAS COMPSTON 2013. Detection, incidence and management of glomerulonephritis in the alemtuzumab clinical development programme. *Mult Scler,* 19**,** 74-558.

DAI, X., QU, P. & DREYFUS, C. F. 2001. Neuronal signals regulate neurotrophin expression in oligodendrocytes of the basal forebrain. *Glia,* 34**,** 234-9.

DATTA, S. & SARVETNICK, N. 2009. Lymphocyte proliferation in immune-mediated diseases. *Trends in Immunology,* 30**,** 430-438.

DE JAGER, P., BAECHER-ALLAN, C., MAIER, L., ARTHUR, A., OTTOBONI, L., BARCELLOS, L., MCCAULEY, J., SAWCER, S., GORIS, A. & SAARELA, J. The role of the CD58 locus in multiple sclerosis. Proc Natl Acad Sci USA. *Proc Natl Acad Sci USA*.

DE JAGER, P. L., JIA, X. M., WANG, J., DE BAKKER, P. I. W., OTTOBONI, L., AGGARWAL, N. T., PICCIO, L., RAYCHAUDHURI, S., TRAN, D., AUBIN, C., BRISKIN, R., ROMANO, S., BARANZINI, S. E., MCCAULEY, J. L., PERICAK-VANCE, M. A., HAINES, J. L., GIBSON, R. A., NAEGLIN, Y., UITDEHAAG, B., MATTHEWS, P. M., KAPPOS, L., POLMAN, C., MCARDLE, W. L., STRACHAN, D. P., EVANS, D., CROSS, A. H., DALY, M. J., COMPSTON, A., SAWCER, S. J., WEINER, H. L., HAUSER, S. L., HAFLER, D. A., OKSENBERG, J. R. & INT, M. S. G. C. 2009. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nature Genetics,* 41**,** 776-U26.

DELUCA, G. C., WILLIAMS, K., EVANGELOU, N., EBERS, G. C. & ESIRI, M. M. 2006. The contribution of demyelination to axonal loss in multiple sclerosis. *Brain,* 129**,** 1507-16.

DENIC, A., JOHNSON, A. J., BIEBER, A. J., WARRINGTON, A. E., RODRIGUEZ, M. & PIRKO, I. 2011. The relevance of animal models in multiple sclerosis research. *Pathophysiology,* 18**,** 21-29.

DOBSON, R., GIOVANNONI, G. & RAMAGOPALAN, S. 2013. The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude. *J Neurol Neurosurg Psychiatry,* 84**,** 427-432.

DOUCETTE, J. R., JIAO, R. & NAZARALI, A. J. 2010. Age-related and cuprizone-induced changes in myelin and transcription factor gene expression and in oligodendrocyte cell densities in the rostral corpus callosum of mice. *Cellular and molecular neurobiology,* 30**,** 607-629.

DOUEK, D. C., MCFARLAND, R. D., KEISER, P. H., GAGE, E. A., MASSEY, J. M., HAYNES, B. F., POLIS, M. A., HAASE, A. T., FEINBERG, M. B., SULLIVAN, J. L., JAMIESON, B. D., ZACK, J. A., PICKER, L. J. & KOUP, R. A. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature,* 396**,** 690-5.

DOUGHERTY, K. D., DREYFUS, C. F. & BLACK, I. B. 2000. Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. *Neurobiol Dis,* 7**,** 574-85.

DUTTA, R. & TRAPP, B. D. 2011. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. *Progress in neurobiology,* 93**,** 1-12.

EBERS GC, K. K., BULMAN DE, SADOVNICK AD, RICE G, ANDERSON C, ARMSTRONG H, COUSIN K, BELL RB, HADER W, PATY DW, HASHIMOTO S, OGER J, DUQUETTE P, WARREN S, GRAY T, O'CONNOR P, NATH A, AUTY A, METZ L, FRANCIS G, PAULSETH JE, MURRAY TJ, PRYSE-PHILLIPS W, NELSON R, FREEDMAN M, BRUNET D, BOUCHARD JP, HINDS D, RISCH N 1996. A full genome search in multiple sclerosis. . *Nat Genet* 13**,** 472-6.

EBERS, G. C., TRABOULSEE, A., LI, D., LANGDON, D., REDER, A. T., GOODIN, D. S., BOGUMIL, T., BECKMANN, K., WOLF, C., KONIECZNY, A. & INVESTIGATORS OF THE 16-YEAR LONG-TERM FOLLOW-UP, S. 2010. Analysis of clinical outcomes according to original treatment groups 16 years after the pivotal IFNB-1b trial. *J Neurol Neurosurg Psychiatry,* 81**,** 907-12.

ELLIS, J., VAN MAURIK, A., FORTUNATO, L., GISBERT, S., CHEN, K., SCHWARTZ, A., MCHUGH, S., WANT, A., SANTOS FRANCO, S., OLIVEIRA, J. J., PRICE, J., COLES, A., BROWN, K., SU, D., CRAIGEN, J. L., YANG, J., BRETT, S., DAVIS, B., CHERIYAN, J., KOUSIN-EZEWU, O., GRAY, F., THOMPSON, P. W. & FERNANDO, D. 2019. Anti-IL-7 receptor alpha monoclonal antibody (GSK2618960) in healthy subjects - a randomized, double-blind, placebo-controlled study. *Br J Clin Pharmacol,* 85**,** 304-315.

ERMIS, U., WEIS, J. & SCHULZ, J. B. 2013. PML in a patient treated with fumaric acid. *New England Journal of Medicine,* 368**,** 1657-1658.

EUROPEAN MEDICINES AGENCY, P. R. A. C. P. 2017. *PRAC recommends further restrictions for multiple sclerosis medicine Zinbryta due to risk of liver damage.[*Online].Available: http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2017/10/news_detail_002837.jsp&mid=WC0b01ac058004d5c1.

FANCY, S. P., KOTTER, M. R., HARRINGTON, E. P., HUANG, J. K., ZHAO, C., ROWITCH, D. H. & FRANKLIN, R. J. 2010. Overcoming remyelination failure in multiple sclerosis and other myelin disorders. *Exp Neurol,* 225**,** 18-23.

FAWCETT, J. W. & ASHER, R. A. 1999. The glial scar and central nervous system repair. *Brain Res Bull,* 49**,** 377-91.

FERBER, I. A., BROCKE, S., TAYLOR-EDWARDS, C., RIDGWAY, W., DINISCO, C., STEINMAN, L., DALTON, D. & FATHMAN, C. G. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol,* 156**,** 5-7.

FERNALD, G. H., YEH, R.-F., HAUSER, S. L., OKSENBERG, J. R. & BARANZINI, S. E. 2005. Mapping gene activity in complex disorders: Integration of expression and genomic scans for multiple sclerosis. *Journal of neuroimmunology,* 167**,** 157-169.

FERNANDEZ, O., RODRIGUEZ-ANTIGUEDAD, A., OLASCOAGA, J., OREJA-GUEVARA, C., PRIETO, J. M., MENDIBE-BILBAO, M. M., GARCIA-MERINO, J. A., RAMIO-TORRENTA, L., GINESTAL, R., MECA-LALLANA, J. E., ROMERO-PINEL, L., MUNOZ, D., SAIZ, A., CALLES-HERNANDEZ, M. C., IZQUIERDO, G., VILLAR, L. M., OLIVA-NACARINO, P., ARNAL-GARCIA, C., COMABELLA, M., BRIEVA, L., ARROYO, R. & MONTALBAN, X. 2016. Review of the novelties from the 31st ECTRIMS Congress, 2015, presented at the 8th Post-ECTRIMS meeting. *Rev Neurol,* 62**,** 559-569.

FERREIRA, R. C., FREITAG, D. F., CUTLER, A. J., HOWSON, J. M., RAINBOW, D. B., SMYTH, D. J., KAPTOGE, S., CLARKE, P., BOREHAM, C., COULSON, R. M., PEKALSKI, M. L., CHEN, W. M., ONENGUT-GUMUSCU, S., RICH, S. S., BUTTERWORTH, A. S., MALARSTIG, A., DANESH, J. & TODD, J. A. 2013. Functional IL6R 358Ala allele impairs classical IL-6 receptor signaling and influences risk of diverse inflammatory diseases. *PLoS Genet,* 9**,** e1003444.

FIDDES, B., WASON, J., KEMPPINEN, A., BAN, M., COMPSTON, A. & SAWCER, S. 2013. Confounding underlies the apparent month of birth effect in multiple sclerosis. *Annals of neurology,* 73**,** 714-720.

FIERCEBIOTECH. 2013. *GSK statement regarding 2010 Nature Medicine study* [Online]. FierceBiotech. Available: http://www.fiercebiotech.com/press-releases/gsk-statement-regarding-2010-nature-medicine-study [Accessed 10th June 2013].

FILIPPI, M., ROCCA, M. A., BARKHOF, F., BRUCK, W., CHEN, J. T., COMI, G., DELUCA, G., DE STEFANO, N., ERICKSON, B. J., EVANGELOU, N., FAZEKAS, F., GEURTS, J. J., LUCCHINETTI, C., MILLER, D. H., PELLETIER, D., POPESCU, B. F. & LASSMANN, H. 2012. Association between pathological and MRI findings in multiple sclerosis. *Lancet Neurol,* 11**,** 349-60.

FILIPPI, M., ROVARIS, M., GASPERINI, C., CAPRA, R., BASTIANELLO, S., KUHNE, I. & YOUSRY, T. A. 1998. A preliminary study comparing the sensitivity of serial monthly enhanced MRI after standard and triple dose gadolinium-DTPA for monitoring disease activity in primary progressive multiple sclerosis. *J Neuroimaging,* 8**,** 88-93.

FISCHER, M. T., SHARMA, R., LIM, J. L., HAIDER, L., FRISCHER, J. M., DREXHAGE, J., MAHAD, D., BRADL, M., VAN HORSSEN, J. & LASSMANN, H. 2012. NADPH

oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury. *Brain,* 135**,** 886-899.

FISNIKU, L. K., BREX, P. A., ALTMANN, D. R., MISZKIEL, K. A., BENTON, C. E., LANYON, R., THOMPSON, A. J. & MILLER, D. H. 2008. Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain,* 131**,** 808-17.

FITZGERALD, K. C., MUNGER, K. L., KÖCHERT, K., ARNASON, B. G., COMI, G., COOK, S., GOODIN, D. S., FILIPPI, M., HARTUNG, H.-P. & JEFFERY, D. R. 2015. Association of vitamin D levels with multiple sclerosis activity and progression in patients receiving interferon beta-1b. *JAMA neurology,* 72**,** 1458-1465.

FLACH, A.-C., LITKE, T., STRAUSS, J., HABERL, M., GÓMEZ, C. C., REINDL, M., SAIZ, A., FEHLING, H.-J., WIENANDS, J. & ODOARDI, F. 2016. Autoantibody-boosted T-cell reactivation in the target organ triggers manifestation of autoimmune CNS disease. *Proceedings of the National Academy of Sciences,* 113**,** 3323-3328.

FLETCHER, J. M., LONERGAN, R., COSTELLOE, L., KINSELLA, K., MORAN, B., O'FARRELLY, C., TUBRIDY, N. & MILLS, K. H. 2009. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol,* 183**,** 7602-10.

FOX, R. J., MILLER, D. H., PHILLIPS, J. T., HUTCHINSON, M., HAVRDOVA, E., KITA, M., YANG, M., RAGHUPATHI, K., NOVAS, M., SWEETSER, M. T., VIGLIETTA, V. & DAWSON, K. T. 2012. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. *N Engl J Med,* 367**,** 1087-97.

FOXWELL, B. M., BEADLING, C., GUSCHIN, D., KERR, I. & CANTRELL, D. 1995. Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *Eur J Immunol,* 25**,** 3041-6.

FRANKLIN, R. J., ZHAO, C. & SIM, F. J. 2002. Ageing and CNS remyelination. *Neuroreport,* 13**,** 923-928.

FREEDMAN, M. S., THOMPSON, E. J., DEISENHAMMER, F., GIOVANNONI, G., GRIMSLEY, G., KEIR, G., ÖHMAN, S., RACKE, M. K., SHARIEF, M. & SINDIC, C. J. 2005. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. *Archives of neurology,* 62**,** 865-870.

FRISCHER, J. M., BRAMOW, S., DAL-BIANCO, A., LUCCHINETTI, C. F., RAUSCHKA, H., SCHMIDBAUER, M., LAURSEN, H., SORENSEN, P. S. & LASSMANN, H. 2009. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain,* 132**,** 1175-1189.

FRY, T. J. & MACKALL, C. L. 2005. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol,* 174**,** 6571-6.

G. ZANDMAN-GODDARD, Y. S. 2002. HIV and autoimmunity. *Autoimmunity reviews***,** 329-337.

GENZYME. 2013. *European Commission Approves Genzyme's Multiple Sclerosis Treatment LemtradaTM (alemtuzumab)* [Online]. Genzyme, a Sanofi Company.Available: http://www.colescambridge.org.uk/index_htm_files/2013 Sep Sanofi press release.pdf [Accessed 22 November 2013 2013].

GEURTS, J. J. & BARKHOF, F. 2008. Grey matter pathology in multiple sclerosis. *The Lancet Neurology,* 7**,** 841-851.

GHADIRIAN, P., DADGOSTAR, B., AZANI, R. & MAISONNEUVE, P. 2001. A case-control study of the association between socio-demographic, lifestyle and medical history factors and multiple sclerosis. *Canadian journal of public health,* 92**,** 281.

GILQUIN, J., VIARD, J.-P., JUBAULT, V., SERT, C. & KAZATCHKINE, M. D. 1998. Delayed occurrence of Graves' disease after immune restoration with HAART. *The Lancet,* 352**,** 1907-1908.

GIOVANNONI, G. 2017. PML and fingolimod. Available from: http://multiple-sclerosis-research.blogspot.com/2017/07/newsspeak-clinicspeak-pml-and-fingolimod.html.

GLAXOSMITHKLINE. 2013. *GSK statement regarding 2010 Nature Medicine study* [Online]. GlaxoSmithKline. Available: http://www.gsk.com/media/press-releases/2013/gsk-statement-regarding-2010-nature-medicine-study.html [Accessed 10th June 2013].

GOLD, R., GIOVANNONI, G., SELMAJ, K., HAVRDOVA, E., MONTALBAN, X., RADUE, E.-W., STEFOSKI, D., ROBINSON, R., RIESTER, K. & RANA, J. 2013. Daclizumab high-yield process in relapsing-remitting multiple sclerosis (SELECT): a randomised, double-blind, placebo-controlled trial. *The Lancet,* 381**,** 2167-2175.

GOLD, R., KAPPOS, L., ARNOLD, D. L., BAR-OR, A., GIOVANNONI, G., SELMAJ, K., TORNATORE, C., SWEETSER, M. T., YANG, M., SHEIKH, S. I. & DAWSON, K. T. 2012. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N Engl J Med,* 367**,** 1098-107.

GONZALEZ-QUINTIAL, R., LAWSON, B. R., SCATIZZI, J. C., CRAFT, J., KONO, D. H., BACCALA, R. & THEOFILOPOULOS, A. N. 2011. Systemic autoimmunity and lymphoproliferation are associated with excess IL-7 and inhibited by IL-7Ralpha blockade. *PLoS One,* 6**,** e27528.

GOODWIN, R. G. & NAMEN, A. E. 1989. The cloning and characterization of interleukin-7. *Year Immunol,* 6**,** 127-39.

GREGORY, A. P., DENDROU, C. A., ATTFIELD, K. E., HAGHIKIA, A., XIFARA, D. K., BUTTER, F., POSCHMANN, G., KAUR, G., LAMBERT, L., LEACH, O. A., PROMEL, S., PUNWANI, D., FELCE, J. H., DAVIS, S. J., GOLD, R., NIELSEN, F. C., SIEGEL, R. M., MANN, M., BELL, J. I., MCVEAN, G. & FUGGER, L. 2012. TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis. *Nature,* 488**,** 508-11.

GREGORY, S. G., SCHMIDT, S., SETH, P., OKSENBERG, J. R., HART, J., PROKOP, A., CAILLIER, S. J., BAN, M., GORIS, A., BARCELLOS, L. F., LINCOLN, R., MCCAULEY, J. L., SAWCER, S. J., COMPSTON, D. A., DUBOIS, B., HAUSER, S. L., GARCIA-BLANCO, M. A., PERICAK-VANCE, M. A. & HAINES, J. L. 2007. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet,* 39**,** 1083-91.

GRENNINGLOH, R., TAI, T. S., FRAHM, N., HONGO, T. C., CHICOINE, A. T., BRANDER, C., KAUFMANN, D. E. & HO, I. C. 2011. Ets-1 maintains IL-7 receptor expression in peripheral T cells. *J Immunol,* 186**,** 969-76.

GRIFFITHS, I., KLUGMANN, M., ANDERSON, T., YOOL, D., THOMSON, C., SCHWAB, M. H., SCHNEIDER, A., ZIMMERMANN, F., MCCULLOCH, M. & NADON, N. 1998. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science,* 280**,** 1610-1613.

INTERFERON BETA MULTIPLE SCLEROSIS STUDY GROUP. 1993. Interferon beta‐1b is effective in relapsing‐remitting multiple sclerosis I. Clinical results of a multicenter, randomized, double‐blind, placebo-controlled trial. *Neurology,* 43**,** 655-655.

LENERCEPT MULTIPLE SCLEROSIS STUDY GROUP. 1999. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology,* 53**,** 457-65.

GUDI, V., GINGELE, S., SKRIPULETZ, T. & STANGEL, M. 2014. Glial response during cuprizone-induced de-and remyelination in the CNS: lessons learned. *Frontiers in cellular neuroscience,* 8.

GUIMOND, M., VEENSTRA, R. G., GRINDLER, D. J., ZHANG, H., CUI, Y., MURPHY, R. D., KIM, S. Y., NA, R., HENNIGHAUSEN, L., KURTULUS, S., ERMAN, B., MATZINGER, P., MERCHANT, M. S. & MACKALL, C. L. 2009. Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. *Nat Immunol,* 10**,** 149-57.

HAAHR, S. & HOLLSBERG, P. 2006. Multiple sclerosis is linked to Epstein-Barr virus infection. *Rev Med Virol,* 16**,** 297-310.

HAAS, J., FRITZSCHING, B., TRUBSWETTER, P., KORPORAL, M., MILKOVA, L., FRITZ, B., VOBIS, D., KRAMMER, P. H., SURI-PAYER, E. & WILDEMANN, B. 2007. Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol,* 179**,** 1322-30.

HAAS, J., HUG, A., VIEHOVER, A., FRITZSCHING, B., FALK, C. S., FILSER, A., VETTER, T., MILKOVA, L., KORPORAL, M., FRITZ, B., STORCH-HAGENLOCHER, B., KRAMMER, P. H., SURI-PAYER, E. & WILDEMANN, B. 2005. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol,* 35**,** 3343-52.

HAAS, J., KORPORAL, M., SCHWARZ, A., BALINT, B. & WILDEMANN, B. 2011. The interleukin-7 receptor alpha chain contributes to altered homeostasis of regulatory T cells in multiple sclerosis. *Eur J Immunol,* 41**,** 845-53.

HABEK, M., ARNOLD, D., COHEN, J., COLES, A., CONFAVREUX, C., FOX, E., HARTUNG, H., HAVRDOVA, E., SELMAJ, K. & WEINER, H. Thyroid autoimmunity in comparison of Alemtuzumab and Rebif (R) efficacy in multiple sclerosis studies I and II. Journal of Neurology, 2012. SPRINGER HEIDELBERG TIERGARTENSTRASSE 17, D-69121 HEIDELBERG, GERMANY, S66-S66.

HAFLER, D. A., COMPSTON, A., SAWCER, S., LANDER, E. S., DALY, M. J., DE JAGER, P. L., DE BAKKER, P. I. W., GABRIEL, S. B., MIREL, D. B., IVINSON, A. J., PERICAK-VANCE, M. A., GREGORY, S. G., RIOUX, J. D., MCCAULEY, J. L., HAINES, J. L., BARCELLOS, L. F., CREE, B., OKSENBERG, J. R., HAUSER, S. L. & INT MULTIPLE SCLEROSIS GENETICS, C. 2007. Risk alleles for multiple sclerosis identified by a genomewide study. *New England Journal of Medicine,* 357**,** 851-862.

HALL, S. M. 1972. The effect of injections of lysophosphatidyl choline into white matter of the adult mouse spinal cord. *Journal of cell science,* 10**,** 535-546.

HANDEL, A. E., WILLIAMSON, A. J., DISANTO, G., DOBSON, R., GIOVANNONI, G. & RAMAGOPALAN, S. V. 2011. Smoking and multiple sclerosis: an updated meta-analysis. *PloS one,* 6**,** e16149.

HANDEL, A. E., WILLIAMSON, A. J., DISANTO, G., HANDUNNETTHI, L., GIOVANNONI, G. & RAMAGOPALAN, S. V. 2010. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PloS one,* 5**,** e12496.

HAUSER, S. L., BAR-OR, A., COMI, G., GIOVANNONI, G., HARTUNG, H.-P., HEMMER, B., LUBLIN, F., MONTALBAN, X., RAMMOHAN, K. W. & SELMAJ, K. 2017. Ocrelizumab versus interferon beta-1a in relapsing multiple sclerosis. *New England Journal of Medicine,* 376**,** 221-234.

HAUSER, S. L., WAUBANT, E., ARNOLD, D. L., VOLLMER, T., ANTEL, J., FOX, R. J., BAR-OR, A., PANZARA, M., SARKAR, N., AGARWAL, S., LANGER-GOULD, A. & SMITH, C. H. 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med,* 358**,** 676-88.

HAUSER, S. L., WEINER, H. L., CHE, M., SHAPIRO, M. E., GILLES, F. & LETVIN, N. 1984. Prevention of experimental allergic encephalomyelitis (EAE) in the SJL/J mouse by whole body ultraviolet irradiation. *The Journal of Immunology,* 132**,** 1276-1281.

HAVRDOVA, E., ARNOLD, D. L., COHEN, J. A., HARTUNG, H. P., FOX, E. J., GIOVANNONI, G., SCHIPPLING, S., SELMAJ, K. W., TRABOULSEE, A., COMPSTON, D. A. S., MARGOLIN, D. H., THANGAVELU, K., RODRIGUEZ, C. E., JODY, D., HOGAN, R. J., XENOPOULOS, P., PANZARA, M. A. & COLES, A. J. 2017. Alemtuzumab CARE-MS I 5-year follow-up: Durable efficacy in the absence of continuous MS therapy. *Neurology,* 89**,** 1107-1116.

HAWKES, C. H. 2007. Smoking is a risk factor for multiple sclerosis: a metanalysis. *Multiple Sclerosis Journal,* 13**,** 610-615.

HEDSTRÖM, A., ALFREDSSON, L., LUNDKVIST RYNER, M., FOGDELL-HAHN, A., HILLERT, J. & OLSSON, T. 2014a. Smokers run increased risk of developing anti-natalizumab antibodies. *Multiple Sclerosis Journal,* 20**,** 1081-1085.

HEDSTRÖM, A., BÄÄRNHIELM, M., OLSSON, T. & ALFREDSSON, L. 2011a. Exposure to environmental tobacco smoke is associated with increased risk for multiple sclerosis. *Multiple Sclerosis Journal,* 17**,** 788-793.

HEDSTRÖM, A. K., BÄÄRNHIELM, M., OLSSON, T. & ALFREDSSON, L. 2009. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology,* 73**,** 696-701.

HEDSTRÖM, A. K., RYNER, M., FINK, K., FOGDELL-HAHN, A., ALFREDSSON, L., OLSSON, T. & HILLERT, J. 2014b. Smoking and risk of treatment-induced neutralizing antibodies to interferon β-1a. *Multiple Sclerosis Journal,* 20**,** 445-450.

HEDSTRÖM, A. K., SUNDQVIST, E., BÄÄRNHIELM, M., NORDIN, N., HILLERT, J., KOCKUM, I., OLSSON, T. & ALFREDSSON, L. 2011b. Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis. *Brain,* 134**,** 653-664.

HENRIQUES, C. M., RINO, J., NIBBS, R. J., GRAHAM, G. J. & BARATA, J. T. 2010. IL-7 induces rapid clathrin-mediated internalization and JAK3-dependent degradation of IL-7Ralpha in T cells. *Blood,* 115**,** 3269-77.

HENSIEK, A., SAWCER, S., FEAKES, R., DEANS, J., MANDER, A., AKESSON, E., ROXBURGH, R., CORADDU, F., SMITH, S. & COMPSTON, D. 2002. Hla-dr 15 is associated with female sex and younger age at diagnosis in multiple sclerosis. *Journal of Neurology, Neurosurgery, & Psychiatry,* 72**,** 184-187.

HILL-CAWTHORNE, G. A., BUTTON, T., TUOHY, O., JONES, J. L., MAY, K., SOMERFIELD, J., GREEN, A., GIOVANNONI, G., COMPSTON, D. A., FAHEY, M. T. & COLES, A. J. 2012. Long term lymphocyte reconstitution after alemtuzumab treatment of multiple sclerosis. *J Neurol Neurosurg Psychiatry,* 83**,** 298-304.

HILL-CAWTHORNE, G. A., BUTTON, T., TUOHY, O., JONES, J. L., MAY, K., SOMERFIELD, J., GREEN, A., GIOVANNONI, G., COMPSTON, D. A. S., FAHEY, M. T. & COLES, A. J. 2011. Long term lymphocyte reconstitution after alemtuzumab treatment of multiple sclerosis. *Journal of Neurology, Neurosurgery & Psychiatry*.

HIREMATH, M., SAITO, Y., KNAPP, G., TING, J.-Y., SUZUKI, K. & MATSUSHIMA, G. 1998. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. *Journal of neuroimmunology,* 92**,** 38-49.

HO, T., MCKHANN, G. & GRIFFIN, J. 1998. Human autoimmune neuropathies. *Annual review of neuroscience,* 21**,** 187-226.

HOE, E., MCKAY, F. C., SCHIBECI, S. D., GANDHI, K., HEARD, R. N., STEWART, G. J. & BOOTH, D. R. 2010. Functionally significant differences in expression of disease-associated IL-7 receptor alpha haplotypes in CD4 T cells and dendritic cells. *J Immunol,* 184**,** 2512-7.

HOHLFELD, R. 1997. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain: a journal of neurology,* 120**,** 865-916.

HOHLFELD, R. & MEINL, E. 2017. Ocrelizumab in multiple sclerosis: markers and mechanisms. *The Lancet Neurology,* 16**,** 259-261.

HOLLENBACH, J. A. & OKSENBERG, J. R. 2015. The immunogenetics of multiple sclerosis: a comprehensive review. *Journal of autoimmunity,* 64**,** 13-25.

HOLMOY, T. & VARTDAL, F. 2004. Cerebrospinal fluid T cells from multiple sclerosis patients recognize autologous Epstein-Barr virus-transformed B cells. *J Neurovirol,* 10**,** 52-6.

HORI, S., NOMURA, T. & SAKAGUCHI, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**,** 1057-61.

HOWELL, O. W., REEVES, C. A., NICHOLAS, R., CARASSITI, D., RADOTRA, B., GENTLEMAN, S. M., SERAFINI, B., ALOISI, F., RONCAROLI, F. & MAGLIOZZI, R. 2011. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain,* 134**,** 2755-2771.

HSIAO, L., LIU, J., YEN, C., WANG, W., FAN, F., CHIOU, T. & CHEN, P. 2001. Case report: relapse of Graves' disease after successful allogeneic bone marrow transplantation. *Bone marrow transplantation,* 28**,** 1151-1153.

HUAN, J., CULBERTSON, N., SPENCER, L., BARTHOLOMEW, R., BURROWS, G. G., CHOU, Y. K., BOURDETTE, D., ZIEGLER, S. F., OFFNER, H. & VANDENBARK, A. A. 2005. Decreased FOXP3 levels in multiple sclerosis patients. *J Neurosci Res,* 81**,** 45-52.

HUBER, M., HEINK, S., PAGENSTECHER, A., REINHARD, K., RITTER, J., VISEKRUNA, A., GURALNIK, A., BOLLIG, N., JELTSCH, K., HEINEMANN, C., WITTMANN, E., BUCH, T., PRAZERES DA COSTA, O., BRUSTLE, A.,

BRENNER, D., MAK, T. W., MITTRUCKER, H. W., TACKENBERG, B., KAMRADT, T. & LOHOFF, M. 2013. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J Clin Invest,* 123**,** 247-60.

HUG, A., KORPORAL, M., SCHRODER, I., HAAS, J., GLATZ, K., STORCH-HAGENLOCHER, B. & WILDEMANN, B. 2003. Thymic export function and T cell homeostasis in patients with relapsing remitting multiple sclerosis. *J Immunol,* 171**,** 432-7.

HUSEBY, E. S., LIGGITT, D., BRABB, T., SCHNABEL, B., OHLEN, C. & GOVERMAN, J. 2001. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med,* 194**,** 669-76.

IKOTA, H., IWASAKI, A., KAWARAI, M. & NAKAZATO, Y. 2010. Neuromyelitis optica with intraspinal expansion of Schwann cell remyelination. *Neuropathology,* 30**,** 427-433.

INTERNATIONAL MULTIPLE SCLEROSIS GENETICS, C., BEECHAM, A. H., PATSOPOULOS, N. A., XIFARA, D. K., DAVIS, M. F., KEMPPINEN, A., COTSAPAS, C., SHAH, T. S., SPENCER, C., BOOTH, D., GORIS, A., OTURAI, A., SAARELA, J., FONTAINE, B., HEMMER, B., MARTIN, C., ZIPP, F., D'ALFONSO, S., MARTINELLI-BONESCHI, F., TAYLOR, B., HARBO, H. F., KOCKUM, I., HILLERT, J., OLSSON, T., BAN, M., OKSENBERG, J. R., HINTZEN, R., BARCELLOS, L. F., WELLCOME TRUST CASE CONTROL, C., INTERNATIONAL, I. B. D. G. C., AGLIARDI, C., ALFREDSSON, L., ALIZADEH, M., ANDERSON, C., ANDREWS, R., SONDERGAARD, H. B., BAKER, A., BAND, G., BARANZINI, S. E., BARIZZONE, N., BARRETT, J., BELLENGUEZ, C., BERGAMASCHI, L., BERNARDINELLI, L., BERTHELE, A., BIBERACHER, V., BINDER, T. M., BLACKBURN, H., BOMFIM, I. L., BRAMBILLA, P., BROADLEY, S., BROCHET, B., BRUNDIN, L., BUCK, D., BUTZKUEVEN, H., CAILLIER, S. J., CAMU, W., CARPENTIER, W., CAVALLA, P., CELIUS, E. G., COMAN, I., COMI, G., CORRADO, L., COSEMANS, L., COURNU-REBEIX, I., CREE, B. A., CUSI, D., DAMOTTE, V., DEFER, G., DELGADO, S. R., DELOUKAS, P., DI SAPIO, A., DILTHEY, A. T., DONNELLY, P., DUBOIS, B., DUDDY, M., EDKINS, S., ELOVAARA, I., ESPOSITO, F., EVANGELOU, N., FIDDES, B., FIELD, J., FRANKE, A., FREEMAN, C., FROHLICH, I. Y., GALIMBERTI, D., GIEGER, C., GOURRAUD, P. A., GRAETZ, C., GRAHAM, A., GRUMMEL, V., GUASCHINO, C., HADJIXENOFONTOS, A., HAKONARSON, H., HALFPENNY, C., HALL, G., HALL, P., HAMSTEN, A., HARLEY, J., HARROWER, T., et al. 2013. Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet*.

INTERNATIONAL MULTIPLE SCLEROSIS GENETICS, C., WELLCOME TRUST CASE CONTROL, C., SAWCER, S., HELLENTHAL, G., PIRINEN, M., SPENCER, C. C., PATSOPOULOS, N. A., MOUTSIANAS, L., DILTHEY, A., SU, Z., FREEMAN, C., HUNT, S. E., EDKINS, S., GRAY, E., BOOTH, D. R., POTTER, S. C., GORIS, A., BAND, G., OTURAI, A. B., STRANGE, A., SAARELA, J., BELLENGUEZ, C., FONTAINE, B., GILLMAN, M., HEMMER, B., GWILLIAM, R., ZIPP, F., JAYAKUMAR, A., MARTIN, R., LESLIE, S., HAWKINS, S., GIANNOULATOU, E., D'ALFONSO, S., BLACKBURN, H., MARTINELLI BONESCHI, F., LIDDLE, J., HARBO, H. F., PEREZ, M. L., SPURKLAND, A., WALLER, M. J., MYCKO, M. P., RICKETTS, M., COMABELLA, M., HAMMOND, N., KOCKUM, I., MCCANN, O. T., BAN, M., WHITTAKER, P., KEMPPINEN, A., WESTON, P., HAWKINS, C.,

WIDAA, S., ZAJICEK, J., DRONOV, S., ROBERTSON, N., BUMPSTEAD, S. J., BARCELLOS, L. F., RAVINDRARAJAH, R., ABRAHAM, R., ALFREDSSON, L., ARDLIE, K., AUBIN, C., BAKER, A., BAKER, K., BARANZINI, S. E., BERGAMASCHI, L., BERGAMASCHI, R., BERNSTEIN, A., BERTHELE, A., BOGGILD, M., BRADFIELD, J. P., BRASSAT, D., BROADLEY, S. A., BUCK, D., BUTZKUEVEN, H., CAPRA, R., CARROLL, W. M., CAVALLA, P., CELIUS, E. G., CEPOK, S., CHIAVACCI, R., CLERGET-DARPOUX, F., CLYSTERS, K., COMI, G., COSSBURN, M., COURNU-REBEIX, I., COX, M. B., COZEN, W., CREE, B. A., CROSS, A. H., CUSI, D., DALY, M. J., DAVIS, E., DE BAKKER, P. I., DEBOUVERIE, M., D'HOOGHE M, B., DIXON, K., DOBOSI, R., DUBOIS, B., ELLINGHAUS, D., et al. 2011. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature,* 476**,** 214-9.

ISLAM, T., GAUDERMAN, W. J., COZEN, W. & MACK, T. M. 2007. Childhood sun exposure influences risk of multiple sclerosis in monozygotic twins. *Neurology,* 69**,** 381-388.

JACOBSON, J. M., WANG, H., BORDI, R., ZHENG, L., GROSS, B. H., LANDAY, A. L., SPRITZLER, J., ROUTY, J. P., BENSON, C., ABERG, J., TEBAS, P., HAAS, D. W., TIU, J., COUGHLIN, K., PURDUE, L. & SEKALY, R. P. 2014. A randomized controlled trial of palifermin (recombinant human keratinocyte growth factor) for the treatment of inadequate CD4+ T-lymphocyte recovery in patients with HIV-1 infection on antiretroviral therapy. *J Acquir Immune Defic Syndr,* 66**,** 399-406.

JAGESSAR, S. A., HEIJMANS, N., BLEZER, E. L., BAUER, J., WEISSERT, R. & A'T HART, B. 2015. Immune profile of an atypical EAE model in marmoset monkeys immunized with recombinant human myelin oligodendrocyte glycoprotein in incomplete Freund's adjuvant. *Journal of neuroinflammation,* 12**,** 169.

JAMESON, S. C. 2002. Maintaining the norm: T-cell homeostasis. *Nat Rev Immunol,* 2**,** 547-56.

JANA, M., MONDAL, S., JANA, A. & PAHAN, K. 2014. Interleukin‐12 (IL‐12), but not IL‐23, induces the expression of IL‐7 in microglia and macrophages: implications for multiple sclerosis. *Immunology,* 141**,** 549-563.

JEFFERY, N. & BLAKEMORE, W. 1995. Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. *Journal of neurocytology,* 24**,** 775-781.

JIANG, Q., LI, W. Q., AIELLO, F. B., MAZZUCCHELLI, R., ASEFA, B., KHALED, A. R. & DURUM, S. K. 2005. Cell biology of IL-7, a key lymphotrophin. *Cytokine Growth Factor Rev,* 16**,** 513-33.

JOHNSON, K. P., BROOKS, B. R., COHEN, J. A., FORD, C. C., GOLDSTEIN, J., LISAK, R. P., MYERS, L. W., PANITCH, H. S., ROSE, J. W. & SCHIFFER, R. B. 1995. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. *Neurology,* 45**,** 1268-1276.

JONES, J. L. & COLES, A. J. 2010. New treatment strategies in multiple sclerosis. *Exp Neurol,* 225**,** 34-9.

JONES, J. L., PHUAH, C. L., COX, A. L., THOMPSON, S. A., BAN, M., SHAWCROSS, J., WALTON, A., SAWCER, S. J., COMPSTON, A. & COLES, A. J. 2009. IL-21

drives secondary autoimmunity in patients with multiple sclerosis, following therapeutic lymphocyte depletion with alemtuzumab (Campath-1H). *J. Clin. Invest.,* 119**,** 2052-2061.

JONES, J. L., THOMPSON, S. A., LOH, P., DAVIES, J. L., TUOHY, O. C., CURRY, A. J., AZZOPARDI, L., HILL-CAWTHORNE, G., FAHEY, M. T., COMPSTON, A. & COLES, A. J. 2013. Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. *Proc Natl Acad Sci U S A,* 110**,** 20200-5.

JOSHI, S., PANTALENA, L., LIU, X., GAFFEN, S., LIU, H. & ROHOWSKY-KOCHAN, C. 2011. 188 Ichiyama K, Yoshimura A, Steinman L, Christakos S, and Youssef S. 1, 25-189 dihydroxyvitamin D (3) ameliorates Th17 autoimmunity via transcriptional 190 modulation of interleukin-17A. *Molecular and Cellular Biology,* 31**,** 3653-3669.

KAPPOS, L., HARTUNG, H.-P., FREEDMAN, M. S., BOYKO, A., RADÜ, E. W., MIKOL, D. D., LAMARINE, M., HYVERT, Y., FREUDENSPRUNG, U. & PLITZ, T. 2014. Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial. *The Lancet Neurology,* 13**,** 353-363.

KAPPOS, L., WIENDL, H., SELMAJ, K., ARNOLD, D. L., HAVRDOVA, E., BOYKO, A., KAUFMAN, M., ROSE, J., GREENBERG, S., SWEETSER, M., RIESTER, K., O'NEILL, G. & ELKINS, J. 2015. Daclizumab HYP versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med,* 373**,** 1418-28.

KAVANAUGH, A. & MATTESON, E. 2008. Hotline: rituximab and progressive multifocal leukoencephalopathy. *American College of Rheumatology*.

KEBIR, H., IFERGAN, I., ALVAREZ, J. I., BERNARD, M., POIRIER, J., ARBOUR, N., DUQUETTE, P. & PRAT, A. 2009. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol,* 66**,** 390-402.

KERN, B., LI, W., BONO, C., LEE, L. F. & KRAYNOV, E. 2016. Receptor occupancy and blocking of STAT5 signaling by an anti-IL-7 receptor alpha antibody in cynomolgus monkeys. *Cytometry B Clin Cytom,* 90**,** 191-8.

KHORUTS, A. & FRASER, J. 2005. A causal link between lymphopenia and autoimmunity. *Immunology Letters,* 98**,** 23-31.

KIM, S.-M., WOODHALL, M. R., KIM, J.-S., KIM, S.-J., PARK, K. S., VINCENT, A., LEE, K.-W. & WATERS, P. 2015. Antibodies to MOG in adults with inflammatory demyelinating disease of the CNS. *Neurology-Neuroimmunology Neuroinflammation,* 2**,** e163.

KING, C., ILIC, A., KOELSCH, K. & SARVETNICK, N. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell,* 117**,** 265-277.

KLARESKOG, L., CATRINA, A. I. & PAGET, S. 2009. Rheumatoid arthritis. *Lancet,* 373**,** 659-72.

KLEINSCHMIDT-DEMASTERS, B. K. & TYLER, K. L. 2005. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med,* 353**,** 369-74.

KLUGMANN, M., SCHWAB, M. H., PÜHLHOFER, A., SCHNEIDER, A., ZIMMERMANN, F., GRIFFITHS, I. R. & NAVE, K.-A. 1997. Assembly of CNS myelin in the absence of proteolipid protein. *Neuron,* 18**,** 59-70.

KOUSIN-EZEWU, O., AZZOPARDI, L., PARKER, R. A., TUOHY, O., COMPSTON, A., COLES, A. & JONES, J. 2014. Accelerated lymphocyte recovery after

alemtuzumab does not predict multiple sclerosis activity. *Neurology,* 82**,** 2158-64.
KOUSIN-EZEWU, O. & COLES, A. 2013. Alemtuzumab in multiple sclerosis: latest evidence and clinical prospects. *Therapeutic Advances in Chronic Disease*.
KREMLEV, S. G., GAURNIER-HAUSSER, A. L., DEL VALLE, L., PEREZ-LIZ, G., DIMITROV, S. & TUSZYNSKI, G. 2008. Angiocidin promotes pro-inflammatory cytokine production and antigen presentation in multiple sclerosis. *Journal of neuroimmunology,* 194**,** 132-142.
KRISHNAMOORTHY, G., LASSMANN, H., WEKERLE, H. & HOLZ, A. 2006. Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation. *J Clin Invest,* 116**,** 2385-92.
KRUPICA JR, T., FRY, T. & MACKALL, C. 2006. Autoimmunity during lymphopenia: A two-hit model. *Clinical Immunology,* 120**,** 121-128.
KURTZKE, J. F. 1967. On the fine structure of the distribution of multiple sclerosis. *Acta Neurol Scand,* 43**,** 257-82.
KURTZKE, J. F., BEEBE, G. W. & NORMAN JR, J. 1985. Epidemiology of multiple sclerosis in US veterans: III. Migration and the risk of MS. *Neurology,* 35**,** 672-678.
KUTZELNIGG, A. & LASSMANN, H. 2006. Cortical demyelination in multiple sclerosis: a substrate for cognitive deficits? *Journal of the neurological sciences,* 245**,** 123-126.
KUTZELNIGG, A., LUCCHINETTI, C. F., STADELMANN, C., BRÜCK, W., RAUSCHKA, H., BERGMANN, M., SCHMIDBAUER, M., PARISI, J. E. & LASSMANN, H. 2005. Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain,* 128**,** 2705-2712.
LANGER-GOULD, A., ATLAS, S. W., GREEN, A. J., BOLLEN, A. W. & PELLETIER, D. 2005. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med,* 353**,** 375-81.
LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *Journal of Experimental Medicine,* 201**,** 233-240.
LASSMANN, H. & BRADL, M. 2017. Multiple sclerosis: experimental models and reality. *Acta neuropathologica,* 133**,** 223-244.
LASSMANN, H., NIEDOBITEK, G., ALOISI, F. & MIDDELDORP, J. M. 2011. Epstein–Barr virus in the multiple sclerosis brain: a controversial issue—report on a focused workshop held in the Centre for Brain Research of the Medical University of Vienna, Austria. *Brain,* 134**,** 2772-2786.
LASSMANN, H. & WISNIEWSKI, H. M. 1979. Chronic relapsing experimental allergic encephalomyelitis: clinicopathological comparison with multiple sclerosis. *Archives of neurology,* 36**,** 490-497.
LE CAMPION, A., GAGNERAULT, M.-C., AUFFRAY, C., BÉCOURT, C., POITRASSON-RIVIÈRE, M., LALLEMAND, E., BIENVENU, B., MARTIN, B., LEPAULT, F. & LUCAS, B. 2009. Lymphopenia-induced spontaneous T-cell proliferation as a cofactor for autoimmune disease development. *Blood,* 114**,** 1784-1793.
LEE, L. F., AXTELL, R., TU, G. H., LOGRONIO, K., DILLEY, J., YU, J., RICKERT, M., HAN, B., EVERING, W., WALKER, M. G., SHI, J., DE JONG, B. A., KILLESTEIN,

J., POLMAN, C. H., STEINMAN, L. & LIN, J. C. 2011. IL-7 promotes T(H)1 development and serum IL-7 predicts clinical response to interferon-beta in multiple sclerosis. *Sci Transl Med,* 3**,** 93ra68.

LEIBOWITZ, U., SHARON, D. & ALTER, M. 1967. Geographical considerations in multiple sclerosis. *Brain,* 90**,** 871-886.

LEMIRE, J. M. & ARCHER, D. C. 1991. 1, 25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *Journal of Clinical Investigation,* 87**,** 1103.

LEVIN, L. I., MUNGER, K. L., O'REILLY, E. J., FALK, K. I. & ASCHERIO, A. 2010. Primary infection with the epstein‐barr virus and risk of multiple sclerosis. *Annals of neurology,* 67**,** 824-830.

LININGTON, C., BRADL, M., LASSMANN, H., BRUNNER, C. & VASS, K. 1988. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *The American journal of pathology,* 130**,** 443.

LIU, X., LEUNG, S., WANG, C., TAN, Z., WANG, J., GUO, T. B., FANG, L., ZHAO, Y., WAN, B., QIN, X., LU, L., LI, R., PAN, H., SONG, M., LIU, A., HONG, J., LU, H. & ZHANG, J. Z. 2010. Crucial role of interleukin-7 in T helper type 17 survival and expansion in autoimmune disease. *Nat Med,* 16**,** 191-7.

LIU, X., LEUNG, S., WANG, C., TAN, Z., WANG, J., GUO, T. B., FANG, L., ZHAO, Y., WAN, B., QIN, X., LU, L., LI, R., PAN, H., SONG, M., LIU, A., HONG, J., LU, H. & ZHANG, J. Z. 2013. Crucial role of interleukin-7 in T helper type 17 survival and expansion in autoimmune disease. *Nat Med,* 19**,** 1673.

LOCK, C., HERMANS, G., PEDOTTI, R., BRENDOLAN, A., SCHADT, E., GARREN, H., LANGER-GOULD, A., STROBER, S., CANNELLA, B., ALLARD, J., KLONOWSKI, P., AUSTIN, A., LAD, N., KAMINSKI, N., GALLI, S. J., OKSENBERG, J. R., RAINE, C. S., HELLER, R. & STEINMAN, L. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med,* 8**,** 500-8.

LONGBRAKE, E. E. & RACKE, M. K. 2009. Why did IL-12/IL-23 antibody therapy fail in multiple sclerosis? *Expert Rev Neurother,* 9**,** 319-21.

LORENZI, A. R., PATTERSON, A. M., PRATT, A., JEFFERSON, M., CHAPMAN, C. E., PONCHEL, F. & ISAACS, J. D. 2008. Determination of thymic function directly from peripheral blood: a validated modification to an established method. *J Immunol Methods,* 339**,** 185-94.

LOVETT-RACKE, A. E., YANG, Y. & RACKE, M. K. 2011. Th1 versus Th17: are T cell cytokines relevant in multiple sclerosis? *Biochim Biophys Acta,* 1812**,** 246-51.

LUBLIN, F. D. & REINGOLD, S. C. 1996. Defining the clinical course of multiple sclerosis results of an international survey. *Neurology,* 46**,** 907-911.

LUBLIN, F. D., REINGOLD, S. C., COHEN, J. A., CUTTER, G. R., SØRENSEN, P. S., THOMPSON, A. J., WOLINSKY, J. S., BALCER, L. J., BANWELL, B. & BARKHOF, F. 2014. Defining the clinical course of multiple sclerosis The 2013 revisions. *Neurology,* 83**,** 278-286.

LUCCHINETTI, C., BRUCK, W., PARISI, J., SCHEITHAUER, B., RODRIGUEZ, M. & LASSMAN, H. 2000. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Annals of neurology,* 47**,** 707-717.

LUCCHINETTI, C. F., POPESCU, B. F., BUNYAN, R. F., MOLL, N. M., ROEMER, S. F., LASSMANN, H., BRÜCK, W., PARISI, J. E., SCHEITHAUER, B. W. & GIANNINI, C. 2011. Inflammatory cortical demyelination in early multiple sclerosis. *New England Journal of Medicine,* 365**,** 2188-2197.

LUDWIG KAPPOS, M. D., ERNST-WILHELM RADUE, M.D., PAUL O'CONNOR, M.D., CHRIS POLMAN, M.D., REINHARD HOHLFELD, M.D., PETER CALABRESI, M.D., KRZYSZTOF SELMAJ, M.D., CATHERINE AGOROPOULOU, PH.D., MALGORZATA LEYK, PH.D., LIXIN ZHANG-AUBERSON, M.D., PH.D., AND PASCALE BURTIN, M.D., PH.D., & THE FREEDOMS STUDY GROUP. 2010. A Placebo-Controlled Trial of Oral Fingolimod in Relapsing Multiple Sclerosis. *The New England Journal of Medicine,* 362**,** 387-401.

LUNDMARK, F., DUVEFELT, K., IACOBAEUS, E., KOCKUM, I., WALLSTROM, E., KHADEMI, M., OTURAI, A., RYDER, L. P., SAARELA, J., HARBO, H. F., CELIUS, E. G., SALTER, H., OLSSON, T. & HILLERT, J. 2007. Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat Genet,* 39**,** 1108-13.

LUNDSTROM, W., FEWKES, N. M. & MACKALL, C. L. 2012. IL-7 in human health and disease. *Semin Immunol,* 24**,** 218-24.

LUNDSTROM, W., HIGHFILL, S., WALSH, S. T., BEQ, S., MORSE, E., KOCKUM, I., ALFREDSSON, L., OLSSON, T., HILLERT, J. & MACKALL, C. L. 2013. Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity. *Proc Natl Acad Sci U S A*.

LUNEMANN, J. D., EDWARDS, N., MURARO, P. A., HAYASHI, S., COHEN, J. I., MUNZ, C. & MARTIN, R. 2006. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. *Brain,* 129**,** 1493-506.

LUNEMANN, J. D., RUCKERT, S., KERN, F., WENDLING, U., VAN DER ZEE, R., VOLK, H. D. & ZIPP, F. 2004. Cross-sectional and longitudinal analysis of myelin-reactive T cells in patients with multiple sclerosis. *J Neurol,* 251**,** 1111-20.

M HABEK, D. A., J COHEN, AJ COLES, C CONFAVREUX, EJ FOX, HP HARTUNG, E HAVRDOVA, K SELMAJ, H WEINER, V BRINAR, C TWYMAN, P OYUELA, SL LAKE, DH MARGOLIN, M PANZARA, AND DAS COMPSTON FOR THE CARE-MS INVESTIGATORS 2012. Thyroid autoimmunity in comparison of alemtuzumab and Rebif efficacy in multiple sclerosis studies I and II. . *J Neurol* 259**,** S236.

MACKALL, C. L., FRY, T. J. & GRESS, R. E. 2011. Harnessing the biology of IL-7 for therapeutic application. *Nat Rev Immunol,* 11**,** 330-42.

MAGLIOZZI, R., HOWELL, O., VORA, A., SERAFINI, B., NICHOLAS, R., PUOPOLO, M., REYNOLDS, R. & ALOISI, F. 2007. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain,* 130**,** 1089-1104.

MAIER, L. M., ANDERSON, D. E., SEVERSON, C. A., BAECHER-ALLAN, C., HEALY, B., LIU, D. V., WITTRUP, K. D., DE JAGER, P. L. & HAFLER, D. A. 2009. Soluble IL-2RA levels in multiple sclerosis subjects and the effect of soluble IL-2RA on immune responses. *The Journal of Immunology,* 182**,** 1541-1547.

MALASPINA, A., MOIR, S., HO, J., WANG, W., HOWELL, M. L., O'SHEA, M. A., ROBY, G. A., REHM, C. A., MICAN, J. M., CHUN, T. W. & FAUCI, A. S. 2006. Appearance of immature/transitional B cells in HIV-infected individuals

with advanced disease: correlation with increased IL-7. *Proc Natl Acad Sci U S A,* 103**,** 2262-7.

MANCARDI, G. & SACCARDI, R. 2008. Autologous haematopoietic stem-cell transplantation in multiple sclerosis. *Lancet Neurol,* 7**,** 626-36.

MANOUCHEHRINIA, A., TENCH, C. R., MAXTED, J., BIBANI, R. H., BRITTON, J. & CONSTANTINESCU, C. S. 2013. Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain,* 136**,** 2298-2304.

MANRIQUE‐HOYOS, N., JÜRGENS, T., GRØNBORG, M., KREUTZFELDT, M., SCHEDENSACK, M., KUHLMANN, T., SCHRICK, C., BRÜCK, W., URLAUB, H. & SIMONS, M. 2012. Late motor decline after accomplished remyelination: impact for progressive multiple sclerosis. *Annals of neurology,* 71**,** 227-244.

MARTIN, B., HIROTA, K., CUA, D. J., STOCKINGER, B. & VELDHOEN, M. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity,* 31**,** 321-30.

MARTYN, C. N., CRUDDAS, M. & COMPSTON, D. A. 1993. Symptomatic Epstein-Barr virus infection and multiple sclerosis. *J Neurol Neurosurg Psychiatry,* 56**,** 167-8.

MATSUSHIMA, G. K. & MORELL, P. 2001. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain pathology,* 11**,** 107-116.

MATUSEVICIUS, D., KIVISAKK, P., HE, B., KOSTULAS, N., OZENCI, V., FREDRIKSON, S. & LINK, H. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler,* 5**,** 101-4.

MAZZUCCHELLI, R. I., RIVA, A. & DURUM, S. K. 2012. The human IL-7 receptor gene: deletions, polymorphisms and mutations. *Semin Immunol,* 24**,** 225-30.

MCBRIDE, R. 2013a. *GlaxoSmithKline investigates alleged data fraud involving R&D lab staffers in China*[Online]. FierceBiotech. Available: http://www.fiercebiotech.com/story/glaxosmithkline-investigates-alleged-data-fraud-involving-rd-lab-staffers-c/2013-06-06 [Accessed 6th June 2013].

MCBRIDE, R. 2013b. *UPDATED: GlaxoSmithKline slams brakes on trial of MS drug linked to data scandal* [Online]. FierceBiotech. Available: http://www.fiercebiotech.com/story/glaxosmithkline-slams-brakes-trial-ms-drug-linked-data-scandal/2013-06-13 [Accessed 13th June 2013].

MCGEACHY, M. J., CHEN, Y., TATO, C. M., LAURENCE, A., JOYCE-SHAIKH, B., BLUMENSCHEIN, W. M., MCCLANAHAN, T. K., O'SHEA, J. J. & CUA, D. J. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17–producing effector T helper cells in vivo. *Nature immunology,* 10**,** 314-324.

MCLEOD, I. X., ZHOU, X., LI, Q. J., WANG, F. & HE, Y. W. 2011. The class III kinase Vps34 promotes T lymphocyte survival through regulating IL-7Ralpha surface expression. *J Immunol,* 187**,** 5051-61.

MEHLING, M., LINDBERG, R., RAULF, F., KUHLE, J., HESS, C., KAPPOS, L. & BRINKMANN, V. 2010. Th17 central memory T cells are reduced by FTY720 in patients with multiple sclerosis. *Neurology,* 75**,** 403-10.

MENDEL, I., KERLERO DE ROSBO, N. & BEN-NUN, A. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. *Eur J Immunol,* 25**,** 1951-9.

MILLER, D., BARKHOF, F., MONTALBAN, X., THOMPSON, A. & FILIPPI, M. 2005. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *The Lancet Neurology,* 4**,** 281-288.

MIN, D., TAYLOR, P. A., PANOSKALTSIS-MORTARI, A., CHUNG, B., DANILENKO, D. M., FARRELL, C., LACEY, D. L., BLAZAR, B. R. & WEINBERG, K. I. 2002. Protection from thymic epithelial cell injury by keratinocyte growth factor: a new approach to improve thymic and peripheral T-cell reconstitution after bone marrow transplantation. *Blood,* 99**,** 4592-4600.

MOHAN, N., EDWARDS, E. T., CUPPS, T. R., OLIVERIO, P. J., SANDBERG, G., CRAYTON, H., RICHERT, J. R. & SIEGEL, J. N. 2001. Demyelination occurring during anti-tumor necrosis factor alpha therapy for inflammatory arthritides. *Arthritis Rheum,* 44**,** 2862-9.

MONTALBAN, X., HAUSER, S. L., KAPPOS, L., ARNOLD, D. L., BAR-OR, A., COMI, G., DE SEZE, J., GIOVANNONI, G., HARTUNG, H. P., HEMMER, B., LUBLIN, F., RAMMOHAN, K. W., SELMAJ, K., TRABOULSEE, A., SAUTER, A., MASTERMAN, D., FONTOURA, P., BELACHEW, S., GARREN, H., MAIRON, N., CHIN, P., WOLINSKY, J. S. & INVESTIGATORS, O. C. 2017. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med,* 376**,** 209-220.

MONTALBAN, X., HEMMER, B., RAMMOHAN, K., GIOVANNONI, G., DE SEZE, J., BAR-OR, A., ARNOLD, D., SAUTER, A., MASTERMAN, D. & FONTOURA, P. 2016. Efficacy and safety of ocrelizumab in primary progressive multiple sclerosis: results of the Phase III double-blind, placebo-controlled ORATORIO Study (S49. 001). *Neurology,* 86**,** S49. 001.

MONTES, M., ZHANG, X., BERTHELOT, L., LAPLAUD, D. A., BROUARD, S., JIN, J., ROGAN, S., ARMAO, D., JEWELLS, V., SOULILLOU, J. P. & MARKOVIC-PLESE, S. 2009. Oligoclonal myelin-reactive T-cell infiltrates derived from multiple sclerosis lesions are enriched in Th17 cells. *Clin Immunol,* 130**,** 133-44.

MORTIER, E., QUEMENER, A., VUSIO, P., LORENZEN, I., BOUBLIK, Y., GROTZINGER, J., PLET, A. & JACQUES, Y. 2006. Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins. *J Biol Chem,* 281**,** 1612-9.

MOSMANN, T. R., CHERWINSKI, H., BOND, M. W., GIEDLIN, M. A. & COFFMAN, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136**,** 2348-57.

MOWRY, E. M. & MCARTHUR, J. C. 2017. PML in natalizumab-treated multiple sclerosis Modeling errors and risk miscalculations. AAN Enterprises.

MULLEN, A. C., HIGH, F. A., HUTCHINS, A. S., LEE, H. W., VILLARINO, A. V., LIVINGSTON, D. M., KUNG, A. L., CEREB, N., YAO, T.-P. & YANG, S. Y. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science,* 292**,** 1907-1910.

MUNGER, K. L., LEVIN, L. I., HOLLIS, B. W., HOWARD, N. S. & ASCHERIO, A. 2006. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *Jama,* 296**,** 2832-2838.

NAMEN, A. E., SCHMIERER, A. E., MARCH, C. J., OVERELL, R. W., PARK, L. S., URDAL, D. L. & MOCHIZUKI, D. Y. 1988. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J Exp Med,* 167**,** 988-1002.

NASH, R. A., HUTTON, G. J., RACKE, M. K., POPAT, U., DEVINE, S. M., GRIFFITH, L. M., MURARO, P. A., OPENSHAW, H., SAYRE, P. H., STUVE, O., ARNOLD, D. L., SPYCHALA, M. E., MCCONVILLE, K. C., HARRIS, K. M., PHIPPARD, D., GEORGES, G. E., WUNDES, A., KRAFT, G. H. & BOWEN, J. D. 2015. High-dose immunosuppressive therapy and autologous hematopoietic cell transplantation for relapsing-remitting multiple sclerosis (HALT-MS): a 3-year interim report. *JAMA Neurol,* 72**,** 159-69.

NAVE, K.-A. 1996. Myelin-specific genes and their mutations in the mouse. *Glial Cell Development. Basic Principles and Clinical Relevance. Oxford: Bios***,** 141-164.

NAVE, K.-A. 2010. Myelination and the trophic support of long axons. *Nature Reviews Neuroscience,* 11**,** 275-283.

NAVE, K.-A. & TRAPP, B. D. 2008. Axon-glial signaling and the glial support of axon function. *Annu. Rev. Neurosci.,* 31**,** 535-561.

NEWPORT, M., SIRUGO, G., LYONS, E., VANNBERG, F., HILL, A. V. S., BRADBURY, L. A., FARRAR, C., POINTON, J. J., WORDSWORTH, P., BROWN, M. A., FRANKLYN, J. A., HEWARD, J. M., SIMMONDS, M. J., GOUGH, S. C., SEAL, S., STRATTON, M. R., RAHMAN, N., BAN, M., GORIS, A., SAWCER, S. J., COMPSTON, A., CONWAY, D., JALLOW, M., ROCKETT, K. A., KWIATKOWSKI, D. P., BUMPSTEAD, S. J., CHANEY, A., DOWNES, K., GHORI, M. J., GWILLIAM, R., HUNT, S. E., INOUYE, M., KENIRY, A., KING, E., MCGINNIS, R., POTTER, S., RAVINDRARAJAH, R., WHITTAKER, P., WIDDEN, C., WITHERS, D., DELOUKAS, P., LEUNG, H. T., NUTLAND, S., STEVENS, H. E., WALKER, N. M., TODD, J. A., EASTON, D., CLAYTON, D. G., BURTON, P. R., TOBIN, M. D., BARRETT, J. C., EVANS, D. M., MORRIS, A. P., CARDON, L. R., CARDIN, N. J., DAVISON, D., FERREIRA, T., PEREIRA-GALE, J., HALLGRIMSDOTTIR, I. B., HOWIE, B. N., MARCHINI, J. L., SPENCER, C. C., SU, Z., TEO, Y. Y., VUKCEVIC, D., DONNELLY, P., BENTLEY, D., CAULFIELD, M., CRADDOCK, N., FARRALL, M., BARTON, A., BRUCE, I. N., DONOVAN, H., EYRE, S., GILBERT, P. D., HILDER, S. L., HINKS, A. M., JOHN, S. L., POTTER, C., SILMAN, A. J., SYMMONS, D. P. M., THOMSON, W., WORTHINGTON, J., HALL, A. S., HATTERSLEY, A. T., MATTHEW, C. G., MCCARTHY, M. I., OUWEHAND, W. H., PARKES, M., PEMBREY, M., SAMANI, N. J., MITCHELL, S. L., NEWBY, P. R., BRAND, O. J., CARR-SMITH, J., PEARCE, S. H. S., GOUGH, S. C. L., REVEILLE, J. D., ZHOU, X., SIMS, A. M., et al. 2007. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nature Genetics,* 39**,** 1329-1337.

O'CONNOR, P., WOLINSKY, J. S., CONFAVREUX, C., COMI, G., KAPPOS, L., OLSSON, T. P., BENZERDJEB, H., TRUFFINET, P., WANG, L., MILLER, A. & FREEDMAN, M. S. 2011. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *N Engl J Med,* 365**,** 1293-303.

OBERMEIER, B., MENTELE, R., MALOTKA, J., KELLERMANN, J., KÜMPFEL, T., WEKERLE, H., LOTTSPEICH, F., HOHLFELD, R. & DORNMAIR, K. 2008. Matching of oligoclonal immunoglobulin transcriptomes and proteomes of cerebrospinal fluid in multiple sclerosis. *Nature medicine,* 14**,** 688-693.

ODOARDI, F., SIE, C., STREYL, K., ULAGANATHAN, V. K., SCHLÄGER, C., LODYGIN, D., HECKELSMILLER, K., NIETFELD, W., ELLWART, J. & KLINKERT, W. E. 2012. T cells become licensed in the lung to enter the central nervous system. *Nature,* 488**,** 675-679.

OGURA, Y., BONEN, D. K., INOHARA, N. & NICOLAE, D. L. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature,* 411**,** 603.

OKSENBERG, J. R., BARANZINI, S. E., SAWCER, S. & HAUSER, S. L. 2008. The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. *Nature Reviews Genetics,* 9**,** 516-526.

OLSSON, T., BARCELLOS, L. F. & ALFREDSSON, L. 2017. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nature Reviews Neurology,* 13**,** 25-36.

ORTON, S.-M., HERRERA, B. M., YEE, I. M., VALDAR, W., RAMAGOPALAN, S. V., SADOVNICK, A. D., EBERS, G. C. & GROUP, C. C. S. 2006. Sex ratio of multiple sclerosis in Canada: a longitudinal study. *The Lancet Neurology,* 5**,** 932-936.

PALACE, J., DUDDY, M., BREGENZER, T., LAWTON, M., ZHU, F., BOGGILD, M., PISKE, B., ROBERTSON, N. P., OGER, J., TREMLETT, H., TILLING, K., BEN-SHLOMO, Y. & DOBSON, C. 2015. Effectiveness and cost-effectiveness of interferon beta and glatiramer acetate in the UK Multiple Sclerosis Risk Sharing Scheme at 6 years: a clinical cohort study with natural history comparator. *Lancet Neurol,* 14**,** 497-505.

PALANICHAMY, A., JAHN, S., NICKLES, D., DERSTINE, M., ABOUNASR, A., HAUSER, S. L., BARANZINI, S. E., LEPPERT, D. & VON BÜDINGEN, H.-C. 2014. Rituximab efficiently depletes increased CD20-expressing T cells in multiple sclerosis patients. *The Journal of Immunology,* 193**,** 580-586.

PALMER, M. J., MAHAJAN, V. S., CHEN, J., IRVINE, D. J. & LAUFFENBURGER, D. A. 2011. Signaling thresholds govern heterogeneity in IL-7-receptor-mediated responses of naive CD8(+) T cells. *Immunol Cell Biol,* 89**,** 581-94.

PANDEY, A., OZAKI, K., BAUMANN, H., LEVIN, S. D., PUEL, A., FARR, A. G., ZIEGLER, S. F., LEONARD, W. J. & LODISH, H. F. 2000. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nature immunology,* 1**,** 59-64.

PANITCH, H. S., HIRSCH, R. L., HALEY, A. S. & JOHNSON, K. P. 1987. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet,* 1**,** 893-5.

PARK, H., LI, Z., YANG, X. O., CHANG, S. H., NURIEVA, R., WANG, Y. H., WANG, Y., HOOD, L., ZHU, Z., TIAN, Q. & DONG, C. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol,* 6**,** 1133-41.

PARK, J. H., YU, Q., ERMAN, B., APPELBAUM, J. S., MONTOYA-DURANGO, D., GRIMES, H. L. & SINGER, A. 2004. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity,* 21**,** 289-302.

PATANI, R., BALARATNAM, M., VORA, A. & REYNOLDS, R. 2007. Remyelination can be extensive in multiple sclerosis despite a long disease course. *Neuropathology and applied neurobiology,* 33**,** 277-287.

PATERSON, P. Y. 1960. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J Exp Med,* 111**,** 119-36.

PATY, D. W. & LI, D. K. 1993. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology,* 43**,** 662-667.

PEDERSEN, L. B., NASHOLD, F. E., SPACH, K. M. & HAYES, C. E. 2007. 1,25-dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by inhibiting chemokine synthesis and monocyte trafficking. *J Neurosci Res,* 85**,** 2480-90.

PEELEN, E., KNIPPENBERG, S., MURIS, A.-H., THEWISSEN, M., SMOLDERS, J., TERVAERT, J. W. C., HUPPERTS, R. & DAMOISEAUX, J. 2011. Effects of vitamin D on the peripheral adaptive immune system: a review. *Autoimmunity reviews,* 10**,** 733-743.

PENARANDA, C., KUSWANTO, W., HOFMANN, J., KENEFECK, R., NARENDRAN, P., WALKER, L. S., BLUESTONE, J. A., ABBAS, A. K. & DOOMS, H. 2012. IL-7 receptor blockade reverses autoimmune diabetes by promoting inhibition of effector/memory T cells. *Proc Natl Acad Sci U S A,* 109**,** 12668-73.

PETERS, M., JACOBS, S., EHLERS, M., VOLLMER, P., MÜLLBERG, J., WOLF, E., BREM, G., ZUM BÜSCHENFELDE, K. M. & ROSE-JOHN, S. 1996. The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6. *Journal of Experimental Medicine,* 183**,** 1399-1406.

PETERSON, J., KIDD, D. & TRAPP, B. D. 2005. Axonal degeneration in multiple sclerosis: the histopathological evidence. *Multiple sclerosis as a neuronal disease. Amsterdam: Elsevier***,** 165-84.

PETERSON, J. W., BÖ, L., MÖRK, S., CHANG, A. & TRAPP, B. D. 2001. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Annals of neurology,* 50**,** 389-400.

PINHEIRO, J., BATES, D., DEBROY, S., SARKAR, D., AUTHORS, E., HEISTERKAMP, S. & WILIGEN, B. V. 2013. Package 'nlme': Linear and Nonlinear Mixed Effects Models [computer program]. 1-109.

PIRKO, I., LUCCHINETTI, C. F., SRIRAM, S. & BAKSHI, R. 2007. Gray matter involvement in multiple sclerosis. *Neurology,* 68**,** 634-42.

PLAVINA, T., SUBRAMANYAM, M., BLOOMGREN, G., RICHMAN, S., PACE, A., LEE, S., SCHLAIN, B., CAMPAGNOLO, D., BELACHEW, S. & TICHO, B. 2014. Anti-JC virus antibody levels in serum or plasma further define risk of natalizumab-associated progressive multifocal leukoencephalopathy. *Ann Neurol,* 76**,** 802-12.

PLEIMAN, C. M., GIMPEL, S. D., PARK, L. S., HARADA, H., TANIGUCHI, T. & ZIEGLER, S. F. 1991. Organization of the murine and human interleukin-7

receptor genes: two mRNAs generated by differential splicing and presence of a type I-interferon-inducible promoter. *Mol Cell Biol,* 11**,** 3052-9.

POLLINGER, B., KRISHNAMOORTHY, G., BERER, K., LASSMANN, H., BOSL, M. R., DUNN, R., DOMINGUES, H. S., HOLZ, A., KURSCHUS, F. C. & WEKERLE, H. 2009. Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. *J Exp Med,* 206**,** 1303-16.

POLMAN, C. H., O'CONNOR, P. W., HAVRDOVA, E., HUTCHINSON, M., KAPPOS, L., MILLER, D. H., PHILLIPS, J. T., LUBLIN, F. D., GIOVANNONI, G., WAJGT, A., TOAL, M., LYNN, F., PANZARA, M. A. & SANDROCK, A. W. 2006. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.,* 354**,** 899-910.

POLMAN, C. H., REINGOLD, S. C., BANWELL, B., CLANET, M., COHEN, J. A., FILIPPI, M., FUJIHARA, K., HAVRDOVA, E., HUTCHINSON, M., KAPPOS, L., LUBLIN, F. D., MONTALBAN, X., O'CONNOR, P., SANDBERG-WOLLHEIM, M., THOMPSON, A. J., WAUBANT, E., WEINSHENKER, B. & WOLINSKY, J. S. 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol,* 69**,** 292-302.

POMEROY, I. M., MATTHEWS, P. M., FRANK, J. A., JORDAN, E. K. & ESIRI, M. M. 2005. Demyelinated neocortical lesions in marmoset autoimmune encephalomyelitis mimic those in multiple sclerosis. *Brain,* 128**,** 2713-2721.

POPESCU, B. F., PIRKO, I. & LUCCHINETTI, C. F. 2013. Pathology of multiple sclerosis: where do we stand? *Continuum (Minneap Minn),* 19**,** 901-21.

POPESCU, B. F. G. & LUCCHINETTI, C. F. 2012a. Meningeal and cortical grey matter pathology in multiple sclerosis. *BMC neurology,* 12**,** 11.

POPESCU, B. F. G. & LUCCHINETTI, C. F. 2012b. Pathology of demyelinating diseases. *Annual Review of Pathology: Mechanisms of Disease,* 7**,** 185-217.

POSER, C. M., PATY, D. W., SCHEINBERG, L., MCDONALD, W. I., DAVIS, F. A., EBERS, G. C., JOHNSON, K. P., SIBLEY, W. A., SILBERBERG, D. H. & TOURTELLOTTE, W. W. 1983. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol,* 13**,** 227-31.

PRAET, J., GUGLIELMETTI, C., BERNEMAN, Z., VAN DER LINDEN, A. & PONSAERTS, P. 2014. Cellular and molecular neuropathology of the cuprizone mouse model: clinical relevance for multiple sclerosis. *Neuroscience & Biobehavioral Reviews,* 47**,** 485-505.

PRINEAS, J. W., KWON, E. E., CHO, E. S., SHARER, L. R., BARNETT, M. H., OLESZAK, E. L., HOFFMAN, B. & MORGAN, B. P. 2001. Immunopathology of secondary‐progressive multiple sclerosis. *Annals of neurology,* 50**,** 646-657.

PRINEAS JW, M. W. 1997. *Demyelinating diseases. In Greenfield's neuropathology,* London, England, Arnold.

RAJASURIAR, R., BOOTH, D., SOLOMON, A., CHUA, K., SPELMAN, T., GOUILLOU, M., SCHLUB, T. E., DAVENPORT, M., CROWE, S., ELLIOTT, J., HOY, J., FAIRLEY, C., STEWART, G., CAMERON, P. & LEWIN, S. R. 2010. Biological determinants of immune reconstitution in HIV-infected patients receiving antiretroviral therapy: the role of interleukin 7 and interleukin 7 receptor alpha and microbial translocation. *J Infect Dis,* 202**,** 1254-64.

RAMAGOPALAN, S. V., DYMENT, D. A., CADER, M. Z., MORRISON, K. M., DISANTO, G., MORAHAN, J. M., BERLANGA‐TAYLOR, A. J., HANDEL, A., DE LUCA, G. C. & SADOVNICK, A. D. 2011. Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Annals of neurology,* 70**,** 881-886.

REBOLDI, A., COISNE, C., BAUMJOHANN, D., BENVENUTO, F., BOTTINELLI, D., LIRA, S., UCCELLI, A., LANZAVECCHIA, A., ENGELHARDT, B. & SALLUSTO, F. 2009. CC chemokine receptor 6–regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nature immunology,* 10**,** 514-523.

RENZI, P. & GINNS, L. C. 1987. Analysis of T cell subsets in normal adults. Comparison of whole blood lysis technique to Ficoll-Hypaque separation by flow cytometry. *J Immunol Methods,* 98**,** 53-6.

RIZWAN, R., LEVINE, J. E., DEFOR, T., FERARRA, J. L., WEISDORF, D. J., BLAZAR, B. R. & VERNERIS, M. R. 2011a. Peritransplant palifermin use and lymphocyte recovery after T-cell replete, matched related allogeneic hematopoietic cell transplantation. *Am J Hematol,* 86**,** 879-82.

ROBINS, H. S., CAMPREGHER, P. V., SRIVASTAVA, S. K., WACHER, A., TURTLE, C. J., KAHSAI, O., RIDDELL, S. R., WARREN, E. H. & CARLSON, C. S. 2009. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood,* 114**,** 4099-107.

RONCADOR, G., BROWN, P. J., MAESTRE, L., HUE, S., MARTINEZ-TORRECUADRADA, J. L., LING, K. L., PRATAP, S., TOMS, C., FOX, B. C., CERUNDOLO, V., POWRIE, F. & BANHAM, A. H. 2005. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol,* 35**,** 1681-91.

ROSSI, S., BLAZAR, B. R., FARRELL, C. L., DANILENKO, D. M., LACEY, D. L., WEINBERG, K. I., KRENGER, W. & HOLLANDER, G. A. 2002. Keratinocyte growth factor preserves normal thymopoiesis and thymic microenvironment during experimental graft-versus-host disease. *Blood,* 100**,** 682-691.

RUNMARKER, B. & ANDERSEN, O. 1993. Prognostic factors in a multiple sclerosis incidence cohort with twenty-five years of follow-up. *Brain,* 116**,** 117-134.

S LEUNG, P. S.-S., J PINER, P THOMPSON, S BARRON 2012. GSK2618960 Investigator's Brochure. GlaxoSmithKline.

SACCARDI, R., FREEDMAN, M. S., SORMANI, M. P., ATKINS, H., FARGE, D., GRIFFITH, L. M., KRAFT, G., MANCARDI, G. L., NASH, R., PASQUINI, M., MARTIN, R. & MURARO, P. A. 2012. A prospective, randomized, controlled trial of autologous haematopoietic stem cell transplantation for aggressive multiple sclerosis: a position paper. *Mult Scler,* 18**,** 825-34.

SAKAGUCHI S, F. K., KURIBAYASHI K, MASUDA T 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med,* 161**,** 72-87.

SAKAGUCHI, S., MIYARA, M., COSTANTINO, C. M. & HAFLER, D. A. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol,* 10**,** 490-500.

SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-

2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol,* 155**,** 1151-64.
SALZER, J., SVENNINGSSON, R., ALPING, P., NOVAKOVA, L., BJÖRCK, A., FINK, K., ISLAM-JAKOBSSON, P., MALMESTRÖM, C., AXELSSON, M. & VÅGBERG, M. 2016. Rituximab in multiple sclerosis A retrospective observational study on safety and efficacy. *Neurology,* 87**,** 2074-2081.
SANDBERG, L., BISTRÖM, M., SALZER, J., VÅGBERG, M., SVENNINGSSON, A. & SUNDSTRÖM, P. 2016. Vitamin D and axonal injury in multiple sclerosis. *Multiple Sclerosis Journal,* 22**,** 1027-1031.
SANNA, S., PITZALIS, M., ZOLEDZIEWSKA, M., ZARA, I., SIDORE, C., MURRU, R., WHALEN, M. B., BUSONERO, F., MASCHIO, A., COSTA, G., MELIS, M. C., DEIDDA, F., PODDIE, F., MORELLI, L., FARINA, G., LI, Y., DEI, M., LAI, S., MULAS, A., CUCCURU, G., PORCU, E., LIANG, L. M., ZAVATTARI, P., MOI, L., DERIU, E., URRU, M. F., BAJOREK, M., SATTA, M. A., COCCO, E., FERRIGNO, P., SOTGIU, S., PUGLIATTI, M., TRACCIS, S., ANGIUS, A., MELIS, M., ROSATI, G., ABECASIS, G. R., UDA, M., MARROSU, M. G., SCHLESSINGER, D. & CUCCA, F. 2010. Variants within the immunoregulatory CBLB gene are associated with multiple sclerosis. *Nature Genetics,* 42**,** 495-497.
SARAH C. SASSON, J. J. Z. A. A. D. K. 2006. The IL-7/IL-7 Receptor Axis: Understanding its Central Role in T-Cell Homeostasis and the Challenges Facing its Utilization as a Novel Therapy. *Current Drug Targets,* 7**,** 1571-1582.
SAWCER S, J. H., FEAKES R, GRAY J, SMALDON N, CHATAWAY J, ROBERTSON N, CLAYTON D, GOODFELLOW PN, COMPSTON A 1996. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. 1996 *Nat Genet,* Aug;13**,** 464-8.
SAXENA, A., BAUER, J., SCHEIKL, T., ZAPPULLA, J., AUDEBERT, M., DESBOIS, S., WAISMAN, A., LASSMANN, H., LIBLAU, R. S. & MARS, L. T. 2008. Cutting edge: Multiple sclerosis-like lesions induced by effector CD8 T cells recognizing a sequestered antigen on oligodendrocytes. *J Immunol,* 181**,** 1617-21.
SCALFARI, A., NEUHAUS, A., DEGENHARDT, A., RICE, G. P., MURARO, P. A., DAUMER, M. & EBERS, G. C. 2010. The natural history of multiple sclerosis: a geographically based study 10: relapses and long-term disability. *Brain,* 133**,** 1914-29.
SCANNEVIN, R. H., CHOLLATE, S., JUNG, M.-Y., SHACKETT, M., PATEL, H., BISTA, P., ZENG, W., RYAN, S., YAMAMOTO, M. & LUKASHEV, M. 2012. Fumarates promote cytoprotection of central nervous system cells against oxidative stress via the nuclear factor (erythroid-derived 2)-like 2 pathway. *Journal of Pharmacology and Experimental Therapeutics,* 341**,** 274-284.
SCHLUNS KS, K. W., JAMESON SC, LEFRANÇOIS L. 2000. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo.pdf. *Nat Immunol,* Nov;1(5)**,** 426-32.
SCHMIDT, H., WILLIAMSON, D. & ASHLEY-KOCH, A. 2007. HLA-DR15 haplotype and multiple sclerosis: a HuGE review. *Am J Epidemiol,* 165**,** 1097-109.
SCHUH, E., BERER, K., MULAZZANI, M., FEIL, K., MEINL, I., LAHM, H., KRANE, M., LANGE, R., PFANNES, K. & SUBKLEWE, M. 2016. Features of human CD3+ CD20+ T Cells. *The Journal of Immunology,* 197**,** 1111-1117.

SCOLDING, N., BARNES, D., CADER, S., CHATAWAY, J., CHAUDHURI, A., COLES, A., GIOVANNONI, G., MILLER, D., RASHID, W. & SCHMIERER, K. 2015. Association of British Neurologists: revised (2015) guidelines for prescribing disease-modifying treatments in multiple sclerosis. *Practical neurology,* 15**,** 273-279.

SEGAL, B. M., CONSTANTINESCU, C. S., RAYCHAUDHURI, A., KIM, L., FIDELUS-GORT, R. & KASPER, L. H. 2008. Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *Lancet Neurol,* 7**,** 796-804.

SEGGEWISS, R., LORE, K., GUENAGA, F. J., PITTALUGA, S., MATTAPALLIL, J., CHOW, C. K., KOUP, R. A., CAMPHAUSEN, K., NASON, M. C., MEIER-SCHELLERSHEIM, M., DONAHUE, R. E., BLAZAR, B. R., DUNBAR, C. E. & DOUEK, D. C. 2007. Keratinocyte growth factor augments immune reconstitution after autologous hematopoietic progenitor cell transplantation in rhesus macaques. *Blood,* 110**,** 441-9.

SEPÚLVEDA, M., ARMANGUE, T., MARTINEZ-HERNANDEZ, E., ARRAMBIDE, G., SOLA-VALLS, N., SABATER, L., TÉLLEZ, N., MIDAGLIA, L., ARIÑO, H. & PESCHL, P. 2016. Clinical spectrum associated with MOG autoimmunity in adults: significance of sharing rodent MOG epitopes. *Journal of neurology,* 263**,** 1349-1360.

SERAFINI, B., ROSICARELLI, B., FRANCIOTTA, D., MAGLIOZZI, R., REYNOLDS, R., CINQUE, P., ANDREONI, L., TRIVEDI, P., SALVETTI, M., FAGGIONI, A. & ALOISI, F. 2007. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J Exp Med,* 204**,** 2899-912.

SERAFINI, B., ROSICARELLI, B., MAGLIOZZI, R., STIGLIANO, E. & ALOISI, F. 2004. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol,* 14**,** 164-74.

SHEN, S., SANDOVAL, J., SWISS, V. A., LI, J., DUPREE, J., FRANKLIN, R. J. & CASACCIA-BONNEFIL, P. 2008. Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency. *Nature neuroscience,* 11**,** 1024-1034.

SHENG, W., YANG, F., ZHOU, Y., YANG, H., LOW, P. Y., KEMENY, D. M., TAN, P., MOH, A., KAPLAN, M. H. & ZHANG, Y. 2014. STAT5 programs a distinct subset of GM-CSF-producing T helper cells that is essential for autoimmune neuroinflammation. *Cell research,* 24**,** 1387-1402.

SHIBATA, K., YAMADA, H., HARA, H., KISHIHARA, K. & YOSHIKAI, Y. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol,* 178**,** 4466-72.

SHOCHAT, C., TAL, N., BANDAPALLI, O. R., PALMI, C., GANMORE, I., TE KRONNIE, G., CARIO, G., CAZZANIGA, G., KULOZIK, A. E., STANULLA, M., SCHRAPPE, M., BIONDI, A., BASSO, G., BERCOVICH, D., MUCKENTHALER, M. U. & IZRAELI, S. 2011. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. *J Exp Med,* 208**,** 901-8.

SMOLDERS, J., DAMOISEAUX, J., MENHEERE, P. & HUPPERTS, R. 2008. Vitamin D as an immune modulator in multiple sclerosis, a review. *J Neuroimmunol,* 194**,** 7-17.

SOUMELIS, V., RECHE, P. A., KANZLER, H., YUAN, W., EDWARD, G., HOMEY, B., GILLIET, M., HO, S., ANTONENKO, S. & LAUERMA, A. 2002. Human epithelial cells trigger dendritic cell–mediated allergic inflammation by producing TSLP. *Nature immunology,* 3**,** 673-680.

SPACH, K. M. & HAYES, C. E. 2005. Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. *The Journal of Immunology,* 175**,** 4119-4126.

SPANIER, J. A., NASHOLD, F. E., OLSON, J. K. & HAYES, C. E. 2012. The Ifng gene is essential for Vdr gene expression and vitamin D(3)-mediated reduction of the pathogenic T cell burden in the central nervous system in experimental autoimmune encephalomyelitis, a multiple sclerosis model. *J Immunol,* 189**,** 3188-97.

SPIELBERGER, R., STIFF, P., BENSINGER, W., GENTILE, T., WEISDORF, D., KEWALRAMANI, T., SHEA, T., YANOVICH, S., HANSEN, K., NOGA, S., MCCARTY, J., LEMAISTRE, C. F., SUNG, E. C., BLAZAR, B. R., ELHARDT, D., CHEN, M.-G. & EMMANOUILIDES, C. 2004. Palifermin for Oral Mucositis after Intensive Therapy for Hematologic Cancers. *New England Journal of Medicine,* 351**,** 2590-2598.

SPORTES, C., BABB, R. R., KRUMLAUF, M. C., HAKIM, F. T., STEINBERG, S. M., CHOW, C. K., BROWN, M. R., FLEISHER, T. A., NOEL, P., MARIC, I., STETLER-STEVENSON, M., ENGEL, J., BUFFET, R., MORRE, M., AMATO, R. J., PECORA, A., MACKALL, C. L. & GRESS, R. E. 2010. Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. *Clin Cancer Res,* 16**,** 727-35.

SPRENT, J. & SURH, C. D. 2012. Interleukin 7, maestro of the immune system. *Semin Immunol,* 24**,** 149-50.

STEURER, M., CLAUSEN, J., GOTWALD, T., GUNSILIUS, E., STOCKHAMMER, G., GASTL, G. & NACHBAUR, D. 2003. Progressive multifocal leukoencephalopathy after allogeneic stem cell transplantation and posttransplantation rituximab. *Transplantation,* 76**,** 435-436.

STOKLASEK, T. A., SCHLUNS, K. S. & LEFRANÇOIS, L. 2006. Combined IL-15/IL-15Rα immunotherapy maximizes IL-15 activity in vivo. *The Journal of Immunology,* 177**,** 6072-6080.

STORCH, M. K., STEFFERL, A., BREHM, U., WEISSERT, R., WALLSTRÖM, E., KERSCHENSTEINER, M., OLSSON, T., LININGTON, C. & LASSMANN, H. 1998. Autoimmunity to myelin oligodendrocyte glycoprotein in rats mimics the spectrum of multiple sclerosis pathology. *Brain Pathology,* 8**,** 681-694.

STROMNES, I. M., CERRETTI, L. M., LIGGITT, D., HARRIS, R. A. & GOVERMAN, J. M. 2008. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med,* 14**,** 337-42.

STUMHOFER, J. S., SILVER, J. S., LAURENCE, A., PORRETT, P. M., HARRIS, T. H., TURKA, L. A., ERNST, M., SARIS, C. J., O'SHEA, J. J. & HUNTER, C. A. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature immunology,* 8**,** 1363-1371.

SUMAYA, C. V., MYERS, L. W., ELLISON, G. W. & ENCH, Y. 1985. Increased prevalence and titer of Epstein-Barr virus antibodies in patients with multiple sclerosis. *Ann Neurol,* 17**,** 371-7.

SUN, Z., ZHONG, W., LU, X., SHI, B., ZHU, Y., CHEN, L., ZHANG, G. & ZHANG, X. 2008. Association of Graves' disease and prevalence of circulating IFN-gamma-producing CD28(-) T cells. *J Clin Immunol,* 28**,** 464-72.

SUNDSTRÖM, P. & NYSTRÖM, L. 2008. Smoking worsens the prognosis in multiple sclerosis. *Multiple Sclerosis Journal,* 14**,** 1031-1035.

SUZUKI, K., NAKAJIMA, H., SAITO, Y., SAITO, T., LEONARD, W. J. & IWAMOTO, I. 2000. Janus kinase 3 (Jak3) is essential for common cytokine receptor gamma chain (gamma(c))-dependent signaling: comparative analysis of gamma(c), Jak3, and gamma(c) and Jak3 double-deficient mice. *Int Immunol,* 12**,** 123-32.

SZABO, S. J., KIM, S. T., COSTA, G. L., ZHANG, X., FATHMAN, C. G. & GLIMCHER, L. H. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell,* 100**,** 655-669.

TAKATORI, H., KANNO, Y., WATFORD, W. T., TATO, C. M., WEISS, G., IVANOV, II, LITTMAN, D. R. & O'SHEA, J. J. 2009. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med,* 206**,** 35-41.

TAKEDA, S., RODEWALD, H. R., ARAKAWA, H., BLUETHMANN, H. & SHIMIZU, T. 1996. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity,* 5**,** 217-28.

TANCHOT, C., LEMONNIER, F. A., PERARNAU, B., FREITAS, A. A. & ROCHA, B. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science,* 276**,** 2057-62.

TECHNOLOGIES, S. C. 2018. Rosette sep T cell isolation.

TERASAKI, P. I. & MICKEY, M. R. 1976. A single mutation hypothesis for multiple sclerosis based on the HL-A system. *Neurology,* 26**,** 56-8.

TEUTSCH, S. M., BOOTH, D. R., BENNETTS, B. H., HEARD, R. N. & STEWART, G. J. 2003. Identification of 11 novel and common single nucleotide polymorphisms in the interleukin-7 receptor-alpha gene and their associations with multiple sclerosis. *Eur J Hum Genet,* 11**,** 509-15.

THOMPSON, A., POLMAN, C., MILLER, D., MCDONALD, W., BROCHET, B., FILIPPI M MONTALBAN, X. & DE SA, J. 1997. Primary progressive multiple sclerosis. *Brain: a journal of neurology,* 120**,** 1085-1096.

THOMPSON, S. A., JONES, J. L., COX, A. L., COMPSTON, D. A. & COLES, A. J. 2009. B-Cell Reconstitution and BAFF After Alemtuzumab (Campath-1H) Treatment of Multiple Sclerosis. *J Clin.Immunol.*

TRAGGIAI, E., BIAGIOLI, T., ROSATI, E., BALLERINI, C., MAZZANTI, B., BEN NUN, A., MASSACESI, L. & VERGELLI, M. 2001. IL-7-enhanced T-cell response to myelin proteins in multiple sclerosis. *J Neuroimmunol,* 121**,** 111-9.

TRAYNOR, K. 2006. Tysabri's return draws cautious optimism. ASHP.

TROTTI, A., COLEVAS, A. D., SETSER, A., RUSCH, V., JAQUES, D., BUDACH, V., LANGER, C., MURPHY, B., CUMBERLIN, R. & COLEMAN, C. N. CTCAE v3. 0: development of a comprehensive grading system for the adverse effects of cancer treatment. Seminars in radiation oncology, 2003. Elsevier, 176-181.

TSAI, S. & SANTAMARIA, P. 2013. MHC class II polymorphisms, autoreactive T-cells, and autoimmunity. *Frontiers in immunology,* 4.

TUOHY, O., COSTELLOE, L., HILL-CAWTHORNE, G., BJORNSON, I., HARDING, K., ROBERTSON, N., MAY, K., BUTTON, T., AZZOPARDI, L., KOUSIN-EZEWU, O., JONES, J., COMPSTON, D. A. & COLES, A. 2015. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. *J Neurol Neurosurg Psychiatry,* 86**,** 208-215.

TUOHY, O., COSTELLOE, L., HILL-CAWTHORNE, G., BJORNSON, I., HARDING, K., ROBERTSON, N., MAY, K., BUTTON, T., AZZOPARDI, L., KOUSIN-EZEWU, O., JONES, J., COMPSTON, D. A. S. & COLES, A. 2014. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. *Journal of Neurology, Neurosurgery & Psychiatry*.

TZARTOS, J. S., FRIESE, M. A., CRANER, M. J., PALACE, J., NEWCOMBE, J., ESIRI, M. M. & FUGGER, L. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol,* 172**,** 146-55.

VADHAN-RAJ, S., TRENT, J., PATEL, S., ZHOU, X., JOHNSON, M. M., ARAUJO, D., LUDWIG, J. A., O'ROARK, S., GILLENWATER, A. M., BUESO-RAMOS, C., EL-NAGGAR, A. K. & BENJAMIN, R. S. 2010. Single-dose palifermin prevents severe oral mucositis during multicycle chemotherapy in patients with cancer: a randomized trial. *Ann Intern Med,* 153**,** 358-67.

VAN DER MEI, I. A., PONSONBY, A.-L., BLIZZARD, L. & DWYER, T. 2001. Regional variation in multiple sclerosis prevalence in Australia and its association with ambient ultraviolet radiation. *Neuroepidemiology,* 20**,** 168-174.

VAN OOSTEN, B. W., KILLESTEIN, J., BARKHOF, F., POLMAN, C. H. & WATTJES, M. P. 2013. PML in a patient treated with dimethyl fumarate from a compounding pharmacy. *New England Journal of Medicine,* 368**,** 1658-1659.

VENKEN, K., HELLINGS, N., BROEKMANS, T., HENSEN, K., RUMMENS, J. L. & STINISSEN, P. 2008. Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol,* 180**,** 6411-20.

VIGLIETTA, V., BAECHER-ALLAN, C., WEINER, H. L. & HAFLER, D. A. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med,* 199**,** 971-9.

VOSKUHL, R. R., MARTIN, R., BERGMAN, C., DALAL, M., RUDDLE, N. H. & MCFARLAND, H. F. 1993. T helper 1 (Th1) functional phenotype of human myelin basic protein-specific T lymphocytes. *Autoimmunity,* 15**,** 137-43.

WALLINE, C. C., KANAKASABAI, S. & BRIGHT, J. J. 2011. IL-7Ralpha confers susceptibility to experimental autoimmune encephalomyelitis. *Genes Immun,* 12**,** 1-14.

WANG, W. Y., BARRATT, B. J., CLAYTON, D. G. & TODD, J. A. 2005. Genome-wide association studies: theoretical and practical concerns. *Nature reviews. Genetics,* 6**,** 109.

WEGNER, C., ESIRI, M., CHANCE, S., PALACE, J. & MATTHEWS, P. 2006. Neocortical neuronal, synaptic, and glial loss in multiple sclerosis. *Neurology,* 67**,** 960-967.

WEINSHENKER, B. G., BASS, B., RICE, G., NOSEWORTHY, J., CARRIERE, W., BASKERVILLE, J. & EBERS, G. 1989. The natural history of multiple sclerosis: a geographically based study: I. Clinical course and disability. *Brain,* 112**,** 133-146.

WEISSERT, R., WALLSTRÖM, E., STORCH, M. K., STEFFERL, A., LORENTZEN, J., LASSMANN, H., LININGTON, C. & OLSSON, T. 1998. MHC haplotype-dependent regulation of MOG-induced EAE in rats. *Journal of Clinical Investigation,* 102**,** 1265.

WEKERLE, H. 2017. B cells in multiple sclerosis. *Autoimmunity,* 50**,** 57-60.

WILDBAUM, G., YOUSSEF, S., GRABIE, N. & KARIN, N. 1998. Neutralizing antibodies to IFN-gamma-inducing factor prevent experimental autoimmune encephalomyelitis. *J Immunol,* 161**,** 6368-74.

WILKINS, A., CHANDRAN, S. & COMPSTON, A. 2001. A role for oligodendrocyte-derived IGF-1 in trophic support of cortical neurons. *Glia,* 36**,** 48-57.

WILKINS, A. & SCOLDING, N. 2008. Protecting axons in multiple sclerosis. *Mult Scler,* 14**,** 1013-25.

WILLER, C. J., DYMENT, D. A., SADOVNICK, A. D., ROTHWELL, P. M., MURRAY, T. J. & EBERS, G. C. 2005. Timing of birth and risk of multiple sclerosis: population based study. *Bmj,* 330**,** 120.

WILSON, H. C., SCOLDING, N. J. & RAINE, C. S. 2006. Co-expression of PDGF alpha receptor and NG2 by oligodendrocyte precursors in human CNS and multiple sclerosis lesions. *J Neuroimmunol,* 176**,** 162-73.

WINGERCHUK, D. M., LESAUX, J., RICE, G., KREMENCHUTZKY, M. & EBERS, G. 2005. A pilot study of oral calcitriol (1, 25-dihydroxyvitamin D3) for relapsing–remitting multiple sclerosis. *Journal of Neurology, Neurosurgery & Psychiatry,* 76**,** 1294-1296.

WUCHERPFENNIG, K. W., NEWCOMBE, J., LI, H., KEDDY, C., CUZNER, M. L. & HAFLER, D. A. 1992. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci U S A,* 89**,** 4588-92.

WYNN, D., KAUFMAN, M., MONTALBAN, X., VOLLMER, T., SIMON, J., ELKINS, J., O'NEILL, G., NEYER, L., SHERIDAN, J. & WANG, C. 2010. Daclizumab in active relapsing multiple sclerosis (CHOICE study): a phase 2, randomised, double-blind, placebo-controlled, add-on trial with interferon beta. *The Lancet Neurology,* 9**,** 381-390.

YAO, Z., PAINTER, S. L., FANSLOW, W. C., ULRICH, D., MACDUFF, B. M., SPRIGGS, M. K. & ARMITAGE, R. J. 1995. Human IL-17: a novel cytokine derived from T cells. *J Immunol,* 155**,** 5483-6.

YIN, X., CRAWFORD, T. O., GRIFFIN, J. W., TU, P.-H., LEE, V. M.-Y., LI, C., RODER, J. & TRAPP, B. D. 1998. Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. *Journal of Neuroscience,* 18**,** 1953-1962.

ZIEGLER, S. F. & LIU, Y. J. 2006. Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. *Nat Immunol,* 7**,** 709-14.

ZIVADINOV, R., WEINSTOCK-GUTTMAN, B., HASHMI, K., ABDELRAHMAN, N., STOSIC, M., DWYER, M., HUSSEIN, S., DURFEE, J. & RAMANATHAN, M. 2009. Smoking is associated with increased lesion volumes and brain atrophy in multiple sclerosis. *Neurology,* 73**,** 504-510.

CHAPTER 9 – APPENDICES

Appendix 9.1 is the Participant Information Sheet from the first time in human trial. I wrote this document as part of the Integrated Research Application System (IRAS) documentation that had to be submitted in order to gain ethical approval for the trial.

Appendix 9.2 is the paper written for the CAMTHY trial. It has been published in the Journal of Clinical Investigation Insight (Coles et al., 2019).

Appendix 9.3 is a paper written as a case report for a patient from the CAMTHY trial that suffered from anaphylaxis following administration with alemtuzumab. This has been published in the Journal of Neurology (Nye et al., 2019).

9.1 PARTICIPANT INFORMATION SHEET FOR ANTI-IL-7R TRIAL

PARTICIPANT INFORMATION SHEET

First in human study of GSK2618960, an anti-IL7R monoclonal antibody STUDY PART C

Study doctors:

Dr. Alasdair Coles,

Institution name: Cambridge University Hospitals Trust

Site address: Dept of Clinical Neurosciences, University of Cambridge, Box 165, Addenbrookes Hospital, Cambridge, CB2 2QQ

Phone number: 01223 216751

Dr Joseph Cheriyan

Institution name: Clinical Unit Cambridge, GlaxoSmithKline

Site address: ACCI, Box 128, Addenbrookes Hospital, Cambridge, CB2 0GG

Phone number: 01223 296001

Introduction

You have been provided the information booklet explaining clinical trials. This document is the Participant Information Sheet, accompanied with the Consent Form (together make the Informed Consent Form mentioned in the leaflet). It contains specific information about this clinical trial. To keep the information in this form simple we shall refer to a clinical trial as a "study".

This information sheet and the consent form have been reviewed and given a favourable opinion by an Ethics Committee (EC). This committee reviews research studies to protect the rights and well-being of the people taking part. Some of the information in this information sheet is required by law.

Why is this research study being done?

GlaxoSmithKline (GSK) is developing GSK2618960 as a potential medicine for treatment of Multiple sclerosis (MS), which is a disease where patients' own immune system cells, called lymphocytes, attack the brain and spinal cord causing damage.

GSK2618960 is a humanised monoclonal antibody that blocks a protein present in the body called Interleukin-7 receptor (IL7-R in

short). IL7R is involved in a pathway that is crucial to the lymphocytes, causing multiple sclerosis.

Study Part C is the third, final part of the first-in-human clinical trial of GSK2618960. The purpose of **Study Part C** is to check, for the first time in patients with multiple sclerosis,

- the safety and tolerability of different doses of a new medicine called GSK2618960,
- how the medicine works in your body and how long it blocks IL7R,
- how long the body takes to get rid of the medicine,

when GSK2618960 is given by intravenous injection (directly into the blood stream). GSK2618960 has been given to people only in Study Part A and Study Part B. In Study Part A, 24 healthy volunteers received up to 2 doses of GSK2618960 (at about 3 months apart), and in Study Part B, up to 24 healthy volunteers received up to 4 doses so that IL7R was blocked for up to 10 weeks. Before Study Part A, GSK2618960 had only been tested in animals and in the laboratory.

You have been asked to take part in the study because you have relapsing remitting multiple sclerosis.

How does the study work?

You are invited to take part in **Study Part C** only, which involves 20 multiple sclerosis patients. Study Part C will see how GSK2618960 affects multiple sclerosis patients and if it is safe – GSK2618960 is called 'study medication' or 'treatment' to keep the information simple.

All 20 patients will get the study medication. Each subject will receive an intravenous (injection into the vein) dose of study medication two, three or four times during Study Part C at several weeks interval. The dose and dosing interval is dependent on the information on the study medication we get from Parts A and B of the study, where the study medication is used in healthy volunteers.

All patients will have 3 MRI scans of the brain at various times before receiving study medication (at approximately 8 weeks before, 4 weeks before and just prior to receiving study medication). MRI scans will also occur at 6, 10, 14 and 18 weeks after receiving the study medication. The purpose of the scans is to see how the treatment may be affecting the inflammatory brain lesions causing your multiple sclerosis.

The study will take place in the Wellcome Trust Clinical Research Facility (CRF for short) and in the GSK Clinical Unit Cambridge (CUC for short), which are situated on Level 5 and on Level 2 respectively of the Addenbrooke's Centre for Clinical Investigation (ACCI for short). The MRI scans of the brain will be done in the MRIS Unit (Magnetic

Resonance Imaging and Spectroscopy). These 3 places are all in Addenbrooke's Hospital in Cambridge.

What am I expected to do in this study? How will being part of this study affect my lifestyle?

If you agree to participate in this study, you will be invited to a screening visit. During the screening visit, a study doctor will discuss the study with you and you will be asked to sign the Informed Consent Form. You will be given a copy of this form to keep.

We will then do a physical examination and ask details of your medical history, and take blood tests and other measurements, to verify whether you meet the eligibility criteria of the study. If you qualify for the study and wish to join, you will be asked to have a brain MRI scan, which will be similar to the MRI scan used in your diagnosis (This will be called the 'first baseline' MRI), within 28 days of the screening visit. You will have a second baseline MRI approximately 4 weeks after the first baseline MRI, and you will be asked to return to the clinic for the first treatment period approximately 4 weeks after the second baseline MRI. During the study, you will need to visit the clinic on schedule, and to tell the staff about any changes to your health. A list of the planned study visits is given in the table below, followed by descriptions of what happens during the study visits.
It is expected that the total participation time in this study will be approximately 7 months, from the initial screening visit to the final visit (the follow-up visit).
In total, you will need to come to the CUC on at least 14 to 20 separate occasions. Two to four of the visits are the treatment visits, during which you will be resident in the CUC for up to 6 days/5 nights (you may be discharged earlier than this at the discretion of the study doctors). The other ones are out-patient clinical visits or for MRI scans. You will also be asked to receive phone calls from the study staff, approximately 8 times, on the weeks when you do not have a visit, as shown below.

Study Visit		**Location**	**Time involved**
Screening	(Week -12)	Out-patient visit at WTCRF	Within 12 weeks of the first day of treatment (about 2 to 4 hours)
1st baseline MRI scan	(Week -8)	Out-patient MRI scan at MRIS Unit	1 day (MRI scan takes approximately 30 – 60 minutes)
2nd baseline MRI scan	(Week -4)	Out-patient MRI scan at MRIS Unit	1 day (MRI scan takes approximately 30 – 60 minutes)
Treatment Period 1	Day -1 to Day 5	MRI scan at MRIS Unit and then start	From Day -1 until discharge in the morning of Day 5, probably before noon after having been reviewed by the study

		in-patient stay at CUC	doctor. If the study doctor deems it necessary, they may ask you to stay another night in CUC. It may be possible that the in-patient stay will be shorter than 6 days/5 nights, based on the safety data during Part A and Part B. The study doctor will let you know.
	Days 8, 15, 29, 42, 56, 70, 84, 98 and 112 (approximately 1, 2, 4, 6* and 8 10*, 12, 14* and 16 weeks after 1st dose on Day 1)	Out-patient visit at CUC	Approximately 1 hour, up to 2 hours at most (*except MRI visits: 1 day, see below)
	Days 21, 35, 49, 63, 70, 77 105, 119, 133, 147, 154 and 161 (3, 5, 7, 9, 11, 13, 15, 17, weeks after 1st dose on Day 1)	At home	Phone call, up to approximately 15 minutes
Possibly other Treatment Periods	Depending on results of Study Part A and Study Part B, you may need to receive a total of 2, 3, or 4 doses. The study staff will give you the details of all the visits if you need to receive more than 1 dose. If you are due to receive several doses of GSK2618960, the doses will be given within a period of up to about 8 to 10 weeks, i.e. likely to be included in the visits above.		
Post treatment MRI visits visits for tests	Week 6 (after 1st dose on Day 1)	MRI scan at MRIS Unit	1 day (scan takes approximately 30-60 minutes)
	Week 10 (after 1st dose on Day 1)	MRI scan at MRIS Unit	1 day (scan takes approximately 30-60 minutes)
	Week 14 (after 1st dose on Day 1)	MRI scan at MRIS Unit	1 day (scan takes approximately 30-60 minutes)
Follow-up visit = end of study	Week 18 (after 1st dose on Day 1) (approximately 4 ½ months)	Out-patient visit at CUC	Approximately 1 day (includes an MRI scan of 30 to 60 minutes)

The duration of the study may be longer and/or additional visits may be required, for additional blood samples or safety tests if indicated by events during the study – we will let you know as soon as we know and these tests would not differ from those already specified.

Please keep in mind how the study tests and visits described here will affect your work and family schedules. Consider if you need transportation to and from the clinic. You may find that these tests and visits need some planning. Some tests may be uncomfortable. Ask the study doctor if you have any questions about the tests and procedures for the study.

You will need to be prepared for the phone calls from study staff.

You are required to comply with the following instructions and restrictions:

Adverse Events	You should report any adverse events to the study staff. An adverse event is when you feel unwell or different in any way. After you have left the CUC, you need to tell the study staff when any ongoing adverse events that you experienced during the study have finished.
Blood Donation	You should not have donated blood 3 months before the study start and you should not donate blood for 3 months after you have completed the study.
Participation in Other Studies	You should not have been dosed with another study drug within 30 days or longer before dosing in this study. You must inform the study doctor of any studies you have participated in so that he/she can advise you on your participation in this study. You should also not take part in another drug study for at least 30 days after you have completed this study If these timelines change, you will be informed by the study doctor. You should not have been dosed with more than 4 different study drugs within the 12 months before dosing in this study.
Recreational / Illegal drugs	The use of these drugs is forbidden from screening to final follow-up visit. We will perform a urine drug test at screening and before the dosing of study medication. If your urine drug screen is positive, you will be excluded from the study.
Smoking	Smokers must not smoke over 10 cigarettes a day during the course of the study. Smoking and use of nicotine-containing products (including nicotine patches) will not be permitted while you are in the CUC or during the out-patient visits.
Alcohol	You must not consume alcohol from 24 hours prior to dosing until discharge from the CUC on each treatment period. You must also refrain from alcohol for 24 hours prior to all

	outpatient visits. You should not consume more than 2 units of alcohol per day throughout the remainder of the study until after your last study visit. For guidance, 1 unit is equivalent to a half-pint (220mL) of beer or 1 measure (25mL) of spirits or 1 glass (125mL) of wine.
Medications	You should inform the study doctor or nurse if you are taking or are prescribed any medication during the study. If necessary, you may receive medications for treating adverse events during the study. Do not take other medicines unless you talk first to the study doctor. You must inform the study doctor during your screening visit of any medicines you have been taking so that he/she can advise you whether a wash-out period is needed before your intravenous dose. For example you are ineligible for the study if you have used first-line multiple sclerosis therapies such as the interferons or glatiramer acetate in the 6 weeks prior to the screening visit. If you have taken certain medications such as Natalizumab (Tysabri) in the past 12 months you will not be able to participate in the study. If you have taken other medications such as Fingolimod (Gilenya) in the past 6 months you will be unable to participate in the study. You will be unable to join the study if you have been treated with steroids for a relapse or other reason within 30 days of dosing with another investigational product. You will be unable to do the study if you are intolerant to paracetamol or other anti-inflammatory medicines.
Vaccinations	The eligibility for the study requires that you are up-to-date with your vaccinations of tetanus, diphtheria, pertussis, measles, mumps and rubella. If you are not up-to-date and are willing to be vaccinated, you will be asked to go to your GP surgery to ask to be vaccinated. If you are vaccinated then, you will be asked to provide us with the date of your vaccination, and the vaccination should be 8 weeks or more before the planned date for the 1st dose of GSK2618960. For subjects with expected study during flu (influenza) season (October – April), the eligibility for the study also requires that you are up-to-date with your flu vaccination (within 1 year) or you will

	be invited to have flu vaccine at the CUC if possible. Dosing cannot start before 8 weeks after a vaccination. Please tell us if you have had any recent vaccinations. You should also not receive live vaccines such as yellow fever; measles, mumps and rubella (MMR); and BCG from 1 month before screening until the last visit of the study (the follow-up study).
Food and Drink	You are required not to eat poppy seed-containing food (e.g. poppy cake or bread covered with poppy seeds) during the 4 days before the screening visit and for 4 days before the day of admission to the CUC. You must not consume any caffeine- or xanthine-containing products (e.g. coffee, tea, cola drinks or chocolate) for 24 hours before the start of dosing and while you are in the CUC or CRF. During your in-clinic stay you will be required to eat the standard meals provided by the CUC; no other food will be allowed. On Day 1 of each treatment period, the first meal will be served 4 hours after start of dosing and the dinner approximately 8 hours after dosing. You will be allowed to drink small quantities of water at room temperature from 1 hour after start of dosing.
Activity and Travel	You will be required to refrain for strenuous exercise for 48 hours before your screening visits, during the in-clinic stay and the remaining visit days. You must refrain from traveling abroad to countries with a high prevalence of infectious diseases. Please check with the study team before you book any trips abroad to check whether your destination will be acceptable and also to confirm the timelines as these might change during the study.
Contraception / Reproductive Risks	Men in this study should not father a baby while they are in this study. You will be asked whether you can father a child. If you can father a child, you must agree to one of the following methods of contraception from the day of dosing until the final study visit (the follow-up visit). Acceptable methods of contraception: • Complete abstinence from intercourse. This must be consistent with your preferred and usual lifestyle. Periodic abstinence and withdrawal are not acceptable methods of contraception. • Condom (during non-vaginal intercourse with any partner – male or female). The female partner must also use: oral

	contraception, OR injectable contraception, OR implants contraception, OR hormonal vaginal ring, OR contraception patch, OR intrauterine device, OR occlusive cap (diaphragm or cervical/vault caps), OR vaginal spermicidal agent (foam/gel/film/cream/suppository). If the above is not entirely clear, please consult one of the study physicians. If your partner gets pregnant during this study, call the study doctor right away. You may be asked questions later about the pregnancy and the baby. Women must be abstinent from sex or must use one of the following contraceptive methods: • Oral contraceptive, either combined or progestogen alone • Injectable progestogen • Implants of etonogestrel or levonorgestrel • Estrogenic vaginal ring • Percutaneous contraceptive patches • Intrauterine device (IUD) or intrauterine system (IUS) that meets the <1% failure rate as stated in the product label • Male partner sterilization (successful vasectomy) prior to the female subject's entry into the study, and this male is the sole partner for that subject. • Male condom combined with a female diaphragm, either with or without a vaginal spermicide (foam, gel, cream or suppository). • Male condom combined with a vaginal spermicide (foam, gel, cream or suppository).

Screening visit:

At the screening visit, you will need to have the following examinations, tests or procedures to find out if you can be enrolled into the study. The information and samples collected as part of these screening activities will be kept and used like the rest of the study results. These tests are sometimes part of regular medical care. They may be done even if you do not join the study. If you have had some of them recently, they may not need to be repeated. This is up to the study doctor.

- The screening visit may take up to 4 hours, and some parts will take place in the hospital's CRF and some others in the GSK CUC.

- Consent talk: the doctor will explain the study. If you are happy to join the study, you will continue with the rest of screening visit.
- Medical history: You will be asked about your health and any illnesses you may have or had in the past. You will be asked about medicines you are taking (including over-the-counter medicine, vitamins or herbal treatments), and your possible use of tobacco, alcohol and recreational drugs. You will also be asked whether you are up-to-date with your vaccinations.
- Physical examination: You will receive a complete physical examination, which will include body weight and height, with calculation of Body Mass Index.
- During the study you will also have to undergo the expanded disability status scale (EDSS) which is a neurological examination which helps clinical trials to assess the level of disability you have from your multiple sclerosis. This will be done at the screening visit, once before you receive the study medication and once at week 14 (to count from the first dose of study medication in case you get several doses, and at the follow-up visit).
- Electrocardiograms (ECG): a test that records the electrical activity of your heart (there will be leads connected to some stickers on your chest, wrists and ankles).
- 24 hour monitoring of your heart with a Holter machine (a small box with leads connected to some stickers on your chest, you will have to carry the small box with you at home and the collection of the Holter machine will be arranged by the CUC staff).
- Vital signs: Your weight, height, blood pressure, heart rate and body temperature.
- Blood tests will be taken from a vein in your arm for laboratory tests to check for general health. This will include blood samples to check if you have Hepatitis B or Hepatitis C or HIV.
- You will be asked to provide a urine sample in the CUC.
- An alcohol breath test.
- Questionnaire: You will be asked about your personality and any suicidal thoughts and feelings and any past experiences (see the reason below).

The study physician will review all screening results and you will be invited to participate in the study only if you fulfil all the inclusion criteria for the study. You will not be eligible for the study based on certain medical conditions or blood results that the doctor will be able to explain, or if you are not able or not willing to do the MRI scans, or if you do not consent for the flu vaccination if it is required for the study in your case, or if you meet, or are unable to commit to, some of the restrictions that are listed in the table above. If you cannot be in the study, we will destroy all your blood and urine samples. Should

the doctor at the clinic have any concerns about your test results they will discuss with you and ask for your permission to write to you GP so they can assess you for treatment and/or further investigations.

If you qualify for the study and wish to join, you will be asked to come to the 1st and 2nd baseline MRI visits. And approximately 4 weeks after the 2nd baseline MRI, you will be asked to start Treatment Period 1.

During the Study Days

Treatment Period 1:

You will be expected to do the following things during the inpatient stay:

- Arrive at the CUC, in the morning to get ready for the MRI scan for brain imaging.
- The MRI scan takes place in the MRIS unit in Addenbrookes Hospital, Cambridge. At several times during the study, you will have a MRI scan, which will be similar to the MRI scan used in your diagnosis. For each occasion, the scan will last approximately 30 to 60 minutes. The technician will take you into the MRI scan room where you will lie down on the patient table. The technician positions your head in the middle of the MRI-scanner. The scanner does not touch you, nor do you feel anything. The scanner does make a loud knocking noise as it takes the images. The technician will offer you headphones to listen to music or ear plugs to lessen the sound. The technician leaves the room, while you are in the scanner, but is in full view and communication with you through the observation window in the adjoining room. There is also voice communication at all times through an intercom. It is important for you to lie very still; you may be asked to briefly hold your breath while the scan is taken. You will be given an injection of dye, called gadolinium, into your veins during the scan, which is necessary to detect areas in the brain where your multiple sclerosis may be currently active.
- In the CUC or CRF, you will have a brief consultation, physical exam, ECG, vital signs, alcohol breath test, urine test and blood tests.
- Meal on the CUC then fast from midnight, however water will be permitted until 1 hour pre-dosing.
- On Day 1, you will woken early and have the following procedures to be done to see how the study medicine is affecting your body.
    - You will have 2 Cannula – one in your arm for the frequent blood samples of the day, and one in the other arm for the study medication to be given intravenously.

- ECGs
- Vital signs (blood pressure, heart rate and body temperature)
- Blood tests to check study medication levels and effects on the blood cells
- Heart monitoring: this will be done 1) as continuous heart monitoring (with leads and stickers on your chest) from 1 hour before dosing starts for 6 hours after the dosing starts (you will see your heart activity on the screen next to your bed), and 2) by monitoring of your heart with a Holter machine starting before dosing for 24 hours after dosing started (a small box with leads connected to some stickers on your chest, you will have to carry the small box with you)

- The study medicine will be given intravenously (direct into the blood stream via a vein) as explained on Day 1, and you will have to stay in bed for 6 hours after dosing.
- The tests described above will be done at intervals during the day and night after dosing.
- On the next days you will have a combination of the tests described above at different times.
- It is anticipated that you will be resident in the CUC for 6 days/5 nights, until discharge in the morning of Day 5 before noon after review by the study doctor (you may be discharged earlier at the discretion of the study doctors, but not before noon on Day 2).
- On the morning when you go home, there will be further tests and the suicidality questionnaire, and you will most likely be discharged during the morning.

In the out-patient visits, tests similar to those described will be done (but not the heart monitoring, and not all tests done at all visits), including at the last study visit (See Table above).

What side effects and risks can I expect from this study?

You may have side effects while on this study. Ask the study doctor if you have any questions about the side effects described here.

Side effects may be mild or severe. The study doctor may give you medicine(s) to help lessen any side effects. Some side effects may go away as soon as you stop taking the study medicine. In some cases, side effects can be serious, lasting or may never go away.

Possible side effects from the medicine GSK2618960

GSK2618960 is very specific and does not block IL7R in non-primates so it has not been given to animals other than monkeys. This study will be the first time that GSK2618960 will be administered into humans. In study Part A and Part B, up to 48 healthy volunteers have

received GSK2618960, and the study doctor will let you know what side effects were observed in this small number of subjects when they discuss the study with you. Therefore, there are no known side effects in humans so far. You will be closely monitored for the side effects.

You will receive the study drug in liquid form as intravenous doses (directly into the blood stream). The dose used in Part C of the study will be determined from the information received from the earlier parts of the trial. The study doctor will inform you of the side effects that were observed in the earlier part of the trial, in healthy volunteers.

When GSK2618960 was given to monkeys in very high doses, a very small drop in body temperature of less than a degree was observed in monkeys which were given moderately high doses, but these were not regarded as clinically concerning. Some monkeys developed (in their blood results) some antibodies to GSK2618960 but with no side effects. The body makes such antibodies when it recognises that GSK2618960 is a “foreign drug” so it tries to block it.

During the study, you will be closely monitored for safety and this will include your body temperature and looking for antibodies in your blood tests. The risk of the antibodies is that the medicine will not work if given again in the future as the body will “remember” the medicine.

GSK2618960 blocks a protein present in the body called Interleukin-7 receptor (IL7-R in short) which is involved in a pathway that is crucial to the lymphocytes (which are white blood cells and cells of the immune system). It is anticipated that the number of lymphocytes in the blood (called lymphocyte count) will drop. The drop is not expected to be too large and it is not expected to cause health problems. However, with lower lymphocyte count, there is a theoretical risk that the body's immune system may be less effective at fighting infections, which means that you may have a slightly increased risk of infection during the study.

The following reasons may mean you will not be allowed to take part in the study:

- if you have a low lymphocyte at screening, or

- if you have a history of tuberculosis or fungal infections, or

- if you are not up-to-date with vaccinations (including flu (influenza) vaccine as discussed earlier, or

- if you have a positive screening test result for hepatitis B or C, or HIV

In addition, you will be watched closely during the study for any signs of infection and will need to receive treatment if appropriate if you

develop an infection, whether it is due to participation in the study or not. This is also why there are travel restrictions during the study.

We will also watch the results of other blood tests which show how GSK2618960 works in the body.

There is a risk of an allergic reaction with any drug, particularly those that act on the immune system. No allergic reactions were seen in monkeys given GSK2618960. Allergic reactions can be dangerous if not treated quickly. You will be watched closely for allergic reactions. Symptoms of an allergic reaction may include an itchy rash, having difficulty breathing, wheezing, chest tightness or swelling around the mouth, throat or eyes.

In the unlikely event that you develop signs of allergy or anaphylaxis (a severe allergic reaction) during the observation period, you will be treated within the CUC where all clinical staff are trained to deal with the recognition and treatment of anaphylaxis. You may need to undergo further tests/investigations and you may be transferred to the hospital if required.

Should you develop a generalised rash, itching, severe nausea, swelling of the lips or tongue or difficulty in breathing at any time during your stay in the CUC or after leaving the CUC, you should seek immediate medical attention.

With the understanding of how GSK2618960 works and together with the information from animal studies, we consider the risk of this study to be minimal and we will monitor you closely for side effects.

If there are any changes to the organisation of the study or any dose regimen changes, we will let you know in advance.

Side effects from the study procedures

When you give blood you may feel faint, or experience mild pain, bruising, irritation or redness at the site. In rare cases, you may get an infection. The total amount of blood to be taken during the study is no more than a pint (500 mL), a little bit more than would be donated to the Blood Transfusion Service by a donor in a single session. This should not cause ill effects.

MRI Scan: Risks Associated with gadolinium contrast agent for MRI

Side effects of the gadolinium contrast agent injection may include mild headache, nausea and local pain. Rarely (less than 1% of the time) low blood pressure and light-headedness occurs. This can be treated immediately with intravenous fluids. Very rarely (less than one in one thousand), patients are allergic to the contrast agent. These effects are most commonly a nettle rash and itchy eyes, but more severe reactions have been seen which result in shortness of

breath.

Screening for HIV, Hepatitis B and C

At the screening visit you will have a blood test for HIV, Hepatitis B and C viruses. We test for these viruses to ensure that subjects are completely well, to avoid the risk of the GSK medicine of harming them, and to stop the risk of interference of HIV in the results of the study. These infections can be treated with anti-viral medication to prevent a serious illness developing in the future. If you are infected with one of these viruses then it could affect your relationships with friends and family, and you might find it more difficult to get life insurance or a mortgage. It could also limit the type of work that you can do. If your viral test is positive, you will be asked to have a repeat blood test. Please note tests can sometimes show a positive result even though you may not be infected. These are called false positives. If the repeat test confirms that you have been infected with one of these viruses then we will refer you to your GP or to an appropriate hospital specialist for counselling and follow-up treatment with your permission. Please ask us if you have any questions about testing for viruses.

Screening for drugs of abuse

During the screening and before the dose of study medicine is taken on the study days, your urine will be tested for drugs (cocaine, morphine, amphetamines, benzodiazepines, barbiturates, cannabis, tricyclic antidepressants and methadone). For this purpose, you will be required to provide a sample of your urine, which has been produced in the study centre, prior to any other study procedures. If any of these tests are positive you will not be able to continue in the study. The results of the test will be discussed with you privately, and will be kept confidential.

Suicidality questionnaire

GSK2618960 is a potential treatment for immune pathways that interact with the nervous system and therefore has the small potential to change your mood or the way you think, including having thoughts about hurting or killing yourself (committing suicide). Although there is no evidence to show that the study medication is associated with people having these thoughts in people in your age group, GSK considers it important to monitor for such events in clinical studies.

If you have thoughts of hurting or killing yourself or have any other unusual or distressing thoughts or feelings at any time during this clinical study, you should tell the study doctor or go to the nearest hospital immediately.

Other risks

There may be other side effects that may happen that are not known now. For example, all medicines can cause an allergic reaction in some patients. Certain problems can become worse if not treated quickly.

If the study doctor notices changes for example in your liver or kidney function, or heart rhythm, you may be asked to return to the CUC or the CRF for more tests (may include further blood tests). The study doctor will explain these tests to you if required. You may also need to stop taking the study medicine after talking with the study doctor.

If you experience an adverse event which you or your family consider is serious or life threatening (e.g. feel very tired or faint, difficulty breathing, develop itching or a bad skin rash, have yellow eyes or skin, or dark urine, or become confused), dial 999 for an ambulance and do not delay treatment by attempting to contact the doctor in charge of the study.

Multiple sclerosis relapse

It is possible that the study medication may cause worsening of multiple sclerosis. The study team do not think this is at all likely, but one genetic study showed multiple sclerosis patients might have more 'active' IL7R than people who do not have multiple sclerosis. On the other hand, a similar drug to the study medication was successful in treating a disease similar to multiple sclerosis in animals called "EAE". However if you do experience new neurological symptom/s or worsening symptoms during the trial then you must contact the study staff within 48 hours of the start of the new symptom/s for assessment. You may be asked to come for an extra visit to the clinic within 7 days of the start of the new symptoms. At this clinic visit, the EDSS neurological examination will be performed and you may have other tests done. The study doctor may require an extra MRI if it is required for the diagnosis of a relapse. In some circumstances, you may not be allowed to receive the next dose of GSK2618960, and the study doctor will discuss this with you. In that case, you will be asked to continue with the study visits to continue monitoring for the safety of the dose/s of GSK2618960 have already received.

What benefits can I expect from this study?

You may not benefit from taking the study medication. Not enough is known about its effect on multiple sclerosis to be sure, at this stage. Certainly, other people with multiple sclerosis, now and in the future, will benefit from the knowledge that this study will bring, on the effect of GSK2618960 on the body and on multiple sclerosis. It may help doctors better understand the different ways the body handles GSK2618960 (for example blood levels or results of blood tests), or

different ways people tolerate the study medication, and help to further develop anti-IL7R monoclonal antibodies. Improved knowledge of the safety and tolerability of the study medication can potentially help doctors conduct further studies to investigate its effectiveness.

Are there alternatives to taking part in this study?

You may continue to take your current disease-modifying treatment, if any, for multiple sclerosis. You may be eligible for other multiple sclerosis therapies on the NHS; the study team will make this clear at your screening visit.

Will I receive payment to be part of this study?

No.

GSK will reimburse you for the reasonable costs of travelling to and from study visits, including – if appropriate - for occasional overnight accommodation. Please provide receipts for your expenses.

Will I have to pay anything to be part of this study?

As part of the study, you will receive the study medicine and all the study tests and procedures at no cost to you.

Do I have to stay in the study?

No. Your participation in the study is voluntary. You may choose to stop taking part in the study at any time, without giving a reason. Tell the study staff if you want to stop being in the study. Your decision will not affect your medical care now or in the future. It will not affect other benefits you receive outside of the study.

What happens if I leave the study?

In some circumstances if you experience a relapse, the study doctor will ask you to be withdrawn from the study for safety reasons. In this situation, or in the situation where you decide to leave the study, you and the study doctor will discuss the best way to do this. You will be asked to attend the clinic to continue monitoring for the safety of the dose/s of GSK2618960 that you have already received. All the data and samples collected before you left the study will still be used for the study.

What happens to my personal and medical information?

It is very important that your personal and medical information stay confidential and secure. GSK will protect your information in accordance with current law.

When you sign this consent form you agree that GSK can use your personal and medical information as described here:

- Your personal and medical information may be checked by GSK

and others (like agencies that approve and monitor studies). This is to make sure that the study is being run properly.

- Only the researchers at this study site can use information that identifies you (such as name and address) and only for the purpose of the study.
- Your study information will be labeled with a code number (for example, 1234782). It will not include your name or address. The study doctor will have the link between your name and the code number.
- The link between your name and the code number will not be shared. Only the code number and coded information will be sent to GSK.
- GSK will use your coded information for research only.

GSK may:

- Keep your coded information electronically, and analyse it by computer to find out what the study is telling us. This may be done by GSK or a third party, in which case GSK will ensure that the third party is required to keep your data secure,
- Share the information with regulatory agencies that approve new medicines,
- Share the information with people who check that the study is done properly (like the ethics committee or review boards),
- Combine the information with results from other studies to improve disease understanding. This may help us to assess the risks and benefits of the study medication.
- Publish study results for medical journals, meetings and on the internet for other researchers to use; your name will not appear in any publication,
- Share coded information with other companies, organisations or universities to carry out research. This may include research looking at improving the quality and efficiency in conducting clinical research trials in general.

Personal and medical data collected during the study may be moved, stored and used in the country where you live or another country where GSK or those working with GSK work.

Use of this information may take place in countries with lower data protection rules than the country where you live. GSK will make sure that if your data are moved to another country, it will still be treated as stated in this Participant Information Sheet and Consent Form.

A description of this clinical study will be available on the GSK

Clinical Study Register: http://www.gsk-clinicalstudyregister.com/ and may also appear in clinical trial/study registries in the UK.

GSK will be the owner of the study results. GSK plans to use the results, and may get patents, or sell the study medicine in the future, or make profits other ways. You will not be paid any part of this.

If you withdraw your consent for use of your personal information, you will no longer be able to continue in the study. However all the information and samples collected before you left the study, or at any follow up visit, will still be used as set out in this consent form.

At any time, you may ask the study doctor to see your personal information and correct it, if necessary. In some circumstances, you may not be able to access your study information while the study is ongoing. However, the study doctor will share any important medical information if it is relevant to your health during the course of the study.

What happens to my blood/tissue samples?

If you take part in this study, you will be asked to give blood and urine samples for doing laboratory tests to check your well-being, laboratory tests to check the way the body breaks down GSK2618960 and to check the effect of GSK2618960 on various aspects of the activity of lymphocytes, and urine tests of drugs of abuse, and alcohol breath test. Similar to information collected in the study, your samples may also be used by GSK or shared by GSK with other companies or universities to better understand the effect of GSK2618960 on the body, the different ways the body handles GSK2618960 (for example blood levels or results of blood tests), or different ways people tolerate GSK2618960, and help to further develop anti-IL7R monoclonal antibodies. In addition, improved knowledge of the safety and tolerability of GSK2618960 can potentially help doctors conduct further studies to investigate its effectiveness.

Your blood and urine samples will be given the same code as your other study information and kept in locked storage. Anyone who works with your samples will hold the information and results in confidence.

GSK may store your tissue samples for up to 15 years after the end of the study after which time your samples will be destroyed.

Whom should I call if I have questions?

You will receive a yellow emergency contact card recording the study reference number, treatment and emergency telephone numbers should you wish to contact a doctor outside normal working hours. You should keep this card with you for the duration of the study.

Who has reviewed this study?

This study has been reviewed by GSK internal review committees to ensure that the relevant scientific and safety issues are addressed.

This study has also been reviewed and approved by an independent research ethics committee (14/LO/1670 National Research Ethics Service Committee, London, UK). An ethics committee consists of an independent group of people who review research studies to protect the rights and well being of the people taking part in the study.

What is the "pharmacogenetics" part of this study and why is it being done?

Scientists intend to look at whether variations in people's genes (DNA) for IL7R might be associated with different ways the body handles GSK2618960 (for example blood levels or results of blood tests), or different ways people tolerated GSK2618960, or different levels of efficacy of GSK2618960.

A blood sample of about 2 teaspoons will be required to do the pharmacogenetics research. If there is a problem looking at your blood sample, we will ask to take the sample again. The risks associated with giving a pharmacogenetics blood sample are the same as the risks for giving any blood sample in this study.

Your blood sample will be given the same code as your other study information and kept in locked storage. Anyone who works with your sample will hold your sample and results in confidence, and the rules are as follows:

- Patients who consent for the pharmacogenetics study will not be able to deduce any individual genotype investigated, as studies conducted using this information will always be blinded to the patient, to the study staff in the CUC and CRF, and the scientists who analyse the gene results.
- Genetic data will be anonymised and pooled for statistical testing for research, so results of individual gene tests will not be provided to any study participant or to any party.
- All genetic analyses will be conducted at the end of the study.
- Genetic results from research will not be disclosed to insurers according to the Department of Health (UK) and Association of British Insurers (ABI) Concordat and Moratorium on Genetics (2011).

Your sample and information derived will be used by GSK or shared by GSK with other companies or universities to better understand the different ways the body handles GSK2618960 (for example blood levels or results of blood tests), or different ways people tolerated GSK2618960, or the treatment of multiple sclerosis, and to further

develop anti-IL7R monoclonal antibodies.

GSK may store and use your sample for up to 15 years after the end of the study. After 15 years, your sample will be destroyed.

In this study, participation in the pharmacogenetics research is part of the protocol and if you do not wish to participate in the pharmacogenetics study, you will not be eligible to participate in the rest of the study. If you withdraw from the study during the study and you already have given your DNA sample, the sample will be tested as described and will only be destroyed after all results have been analysed and the study results published.

What benefits can I expect from the pharmacogenetics part of the study?

You will not receive any direct benefit from taking part in the pharmacogenetics part of the study. The analysis may help scientists understand whether variations in people's genes (DNA) for IL7R might be associated with different ways the body handles GSK2618960 (for example blood levels or results of results of blood tests), or different ways people tolerated GSK2618960, or different levels of efficacy of GSK2618960. This may help identify better ways to treat multiple sclerosis and who is more likely to benefit from GSK2618960 and who may have side effects.

Further information and contact details

If you have any questions about this study please phone the dedicated answer phone on:
01223 216187. We will respond within one working day.

We can also be contacted by email:

Alasdair Coles (Chief Investigator)
ajc1020@medschl.cam.ac.uk
Onajite Kousin-Ezewu (Co-Investigator)
ok256@medschl.cam.ac.uk
Karen May (Research Nurse)
km480@medschl.cam.ac.uk

In the event of an emergency please call:

The Addenbrooke's contact centre on **01223 245151** and ask to be put through to a member of the "Campath team" (Drugs Trial Rota).

Thank you for taking the time to read this document, and for considering taking part in the study.

9.2 THE CAMTHY TRIAL

Keratinocyte growth factor and thymic recovery from lymphopenia in humans (CAMTHY): a single centre, double-blind randomised, placebo control phase 2 trial.

Authors Alasdair J Coles, Laura Azzopardi, Onajite Kousin-Ezewu, Harpreet Kaur Mullay, Sara J Thompson, Lorna Jarvis, Jessica Davies, Sarah Howlett, Judith Babar, Timothy J Sadler, William Brown, Edward Needham, Sarah Dawson, Ruth Seggewiss, Daniel C Douek, John Isaacs and Joanne L Jones.

Abstract

Background: The lymphocyte-depleting antibody alemtuzumab is a highly effective treatment of relapsing-remitting multiple sclerosis (RRMS); however 50% of patients develop novel autoimmunity post-treatment. Most at risk are individuals who reconstitute their T-cell pool by proliferating residual cells, rather than producing new T-cells via the thymus; raising the possibility that autoimmunity might be prevented by increasing thymopoiesis. Keratinocyte growth factor (KGF) promotes thymopoiesis in non-human primates. So in this study we tested its ability to: (i) increase thymopoiesis and (ii) reduce autoimmunity post-alemtuzumab. Here we report results from the pre-planned interim analysis.

Methods: In this randomised, double-blind, placebo-controlled trial we recruited individuals with RRMS (disease duration ≤10 years; expanded disability status scale ≤5.0; with ≥2 relapses in the previous 2 years). All patients received 12mg/day alemtuzumab for 5 days at baseline and 3 days at M12. Following a dose-tolerability sub-study, patients were assigned (1:1) to receive placebo or 180mcg/kg/day palifermin, given for 3 days immediately prior to and after each cycle of alemtuzumab, with repeat doses at M1 and M3. The interim primary endpoint was naïve (CCR7+CD45RA+) CD4+ count at M6. Exploratory endpoints included: number of recent thymic-emigrants (RTEs: CD31+CCR7+CD45RA+CD4+) and signal-joint T-cell receptor excision circles (SjTRECs)/mL of blood.

Findings: Individuals receiving palifermin had fewer naïve CD4+T-cells at M6 compared to placebo ($2.229x10^7$/L vs. $7.733x10^7$/L; p=0.007). Those treated with palifermin also had fewer RTEs (M6: 16.05% vs. 33.95%) and lower SjTRECs/mL (M6: 1100 vs. 3396). At M30, no difference was observed in the rate of autoimmunity between the two groups: 4/14 palifermin vs. 5/13 placebo (one patient was lost to follow-up).

Interpretation: Unexpectedly, in contrast to animal studies, KGF significantly reduced thymopoiesis after alemtuzumab treatment of RRMS. Following this result, recruitment to the trial was terminated. To date no increase in autoimmunity has been observed.

Funding: MRC and Moulton Charitable Trust

Introduction

T-cell lymphopenia is strongly associated with autoimmunity (Datta and Sarvetnick, 2009, King et al., 2004, Krupica jr et al., 2006, Khoruts and Fraser, 2005, Le Campion et al., 2009). A striking example is autoimmunity following treatment of relapsing remitting multiple sclerosis (RRMS) with the lymphocyte-depleting humanised anti-CD52 monoclonal antibody alemtuzumab (Lemtrada). Two short courses of alemtuzumab given 12 months apart effectively suppress RRMS for many years (Coles et al., 2008a, Coles et al., 2012c, Cohen et al., 2012a, Coles et al., 2012a, Havrdova et al., 2017), however between 6 months and five years after treatment 40% of patients develop thyroid autoimmunity (typically Graves' disease). A further 2% of individuals develop idiopathic thromobocytopenia purpura (ITP), 0.1% Goodpasture's syndrome and rare cases of autoimmune haemolytic anaemia, autoimmune neutropenia and autoimmune pancytopenia have been reported. An additional 20% of patients develop novel asymptomatic autoantibodies (Coles et al., 2008a, Coles et al., 2012c, Cohen et al., 2012a, D. Wynn, 2013, M Habek, 2012, Tuohy et al., 2014).

We have previously shown that while B-cell reconstitution after alemtuzumab is rapid, via the generation of new cells from the bone marrow,(Thompson et al., 2009) CD4 and CD8 cells take 35 and 20 months respectively to reach normal range (Hill-Cawthorne et al., 2011). And that paradoxically, for at least nine months after treatment, thymopoiesis (determined by measuring naïve T-cell production, recent thymic emigrants and T-cell receptor excision circles) is reduced (Jones et al., 2013). Instead, T-cell reconstitution occurs by the proliferation of cells that have escaped depletion. As result the post-treatment T-cell pool is dominated by "memory-like cells" with a restricted T-cell receptor (TCR) repertoire (Jones et al., 2013). In keeping with animal studies demonstrating the pro-autoimmune nature of lymphopenia induced T-cell proliferation (Baccala and Theofilopoulos, 2005, Khoruts and Fraser, 2005, Krupica jr et al., 2006, King et al., 2004), we have shown that individuals with the least thymic function and most restricted TCR repertoire after alemtuzumab are at greatest risk of developing autoimmune complications (Jones et al., 2013). These observations raised the possibility that autoimmunity after alemtuzumab might be reduced if thymic function could be restored.

Keratinocyte growth factor (KGF) promotes thymopoiesis through its trophic effects on thymic epithelial cells (TECs). TECs play a pivotal role in T-cell development providing essential growth factors and presenting self-antigen to developing thymocytes. When administered to mice undergoing bone marrow transplantation (BMT) or experimental graft-versus-host disease (GvHD) KGF enhanced thymopoiesis (Min et al., 2002, Rossi et al., 2002). And in macaques, KGF enhanced thymic naive T cell production and reduced lymphopenia-induced

T-cell proliferation after myeloablation and peripheral blood progenitor cell autologous transplantation (Seggewiss et al., 2007). In this model KGF (given as palifermin at a dose of 250mcg/kg per day for three days before and after transplantation) was well tolerated, and its positive effects on the thymus were maintained for up to 12 months. In 2005 palifermin was licensed (as Kepivance) to prevent mucositis induced by chemotherapy. In its pivotal trial 60mcg/kg of palifermin was given for three days prior to conditioning, then for three days after haematopoietic stem cell transplantation (HSCT) (Spielberger et al., 2004). This regime was well tolerated. Later, a trial of three doses of palifermin (60mcg/Kg) before conditioning and up to nine doses after allogenic HSCT was shown to be safe, but it had no impact the incidence of acute GVHD(Blazar et al., 2006) or absolute lymphocyte count recovery (Rizwan et al., 2011b). Although thymic function was not directly studied in these patients, the result suggested that higher doses of palifermin might be required to see positive immunological effects.

Therefore, we designed a study to explore the tolerability of higher doses of palifermin (90, 120 and 180 mcg/kg/day, given for three days prior to and after alemtuzumab with further doses at months 1 and 3); and then test the efficacy of the highest tolerated dose in a placebo-controlled trial aimed at testing two hypotheses: (i) that palifermin increases thymic T-cell reconstitution after alemtuzumab and (ii) thereby reducing the risk of alemtuzumab induced autoimmunity. Here we report the unexpected results of a pre-planned interim analysis (aimed at testing hypothesis one) which led to protocol-defined termination of recruitment.

Methods:

Study design and participants:

CAMTHY was a single-centre, double-blind, placebo-controlled trial of palifermin in the prevention of autoimmunity following alemtuzumab treatment of multiple sclerosis. It was conducted in accordance with the International Conference on Harmonisation Guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki and was approved by NRES Committee London – Hampstead (Rec: 12/LO/0393). All participants gave written informed consent.

Participants were 18-50 years with: relapsing-remitting multiple sclerosis(Polman et al., 2011); disease duration of 10 years or less; at least two relapses in the previous 2 years with at least one in the previous 12 months (untreated or on beta interferon or glatiramer acetate) and an expanded disability status scale (EDSS) score of 5.0 or less. Exclusion criteria included: progressive forms of multiple sclerosis; previous thymectomy; previous treatment with alemtuzumab, natalizumab, mitoxantrone, cyclophosphamide, cladribine, rituximab or any other immunosuppressant or cytotoxic therapy; a

history of malignancy, or a history of clinically significant autoimmunity other than multiple sclerosis.

A "stop-go" interim analysis, testing the effect of palifermin on naïve T-cell reconstitution (as a read-out of thymic function), was planned when 28 patients reached month 6. An independent trial steering committee adjudicated the results of the interim analysis.

Randomisation and masking

Participants were randomised (1:1) to receive palifermin or placebo using an online randomisation service. Because palifermin's known adverse effects (skin reddening and tongue discolouration) may compromise blinding, samples for immunological assays were recoded with a randomly generated identifier for each participant-visit and were analysed blind in batches. Radiological assessments of thymic size and density were performed by masked assessors outside of the core trial team.

Procedures

Drug treatments: All patients received 12mg/day alemtuzumab for 5 consecutive days at baseline, followed by 12mg/day for 3 consecutive days at month 12, with methylprednisolone pre-treatment on days 1, 2 and 3 of each cycle. As is standard practice, all patients were given 200mg oral acyclovir twice a day for 28 days after each cycle of alemtuzumab to reduce the risk of oral herpes simplex.
For the open label dose escalation tolerability sub-study, 3 individuals were treated at each of the following palifermin doses: 90mcg/kg/day, 120mcg/kg/day and 180mcg/kg/day given as an intravenous bolus injection on days -5, -4 and -3 prior to each cycle of alemtuzumab and on days 8, 9 and 10. Three further doses were given at month 1 (+/- 7 days) and month 3 (+/- 2 weeks) after each cycle of alemtuzumab. Each dose level was separated by a minimum of 10 days (from the day 10 dose) and escalation between doses only occurred if no adverse events greater than a grade 2 occurred. As all doses were equally tolerated (appendix), for the subsequent placebo-controlled study, participants received 180mcg/Kg/day of palifermin, or an equivalent volume of normal saline.

Assessments: In addition to standard alemtuzumab safety monitoring, at each three-monthly visit, for 30 months of follow-up, participants were assessed clinically and their blood assayed for markers of thymic function including: immune-phenotyping, signal joint T cell receptor excision circles (SjTRECs) in whole blood (Lorenzi et al., 2008) and T cell receptor (TCR) sequencing using the

immunoSEQ Assay (Adaptive Biotechnologies), from which Shannon's Entropy and clonality were derived (Carlson et al., 2013, Robins et al., 2009). Shannon's entropy is a measure of sample richness (i.e., the number of unique sequences present) and the uniformity their frequency distribution. Clonality describes the shape of clonal distribution, and ranges from 0 to 1.0. A value of 0 means that all sequences are equally abundant, higher numbers indicate increasing clonal asymmetry in which a few clones are present at high frequencies. To assess thymic size and density, a low dose unenhanced thoracic CT scan was performed on all participants at baseline and month 6.

Outcomes and Statistical analysis

The pre-planned efficacy threshold for the interim analysis, was a statistically significant increase in the number of peripheral naïve (CCR7+CD45RA+) CD4+ cells in the palifermin group, by at least 50%, compared to placebo at month 6 post-alemtuzumab (as an indicator of thymopoiesis). We believed this to be a conservative estimate as palifermin increases naive CD4 numbers threefold in monkeys and twofold in mice (maximal at 3-9 months in macaques and 30-80 days in mice). Power calculations suggested that 28 patients had 80% power to detect this increase.

Multivariate linear regression was used to model naïve CD4 cell count at 6 months with explanatory variables of treatment group, age, baseline naïve CD4 count and total dose of palifermin received. To aid interpretation of the model intercept, the continuous variables were median-centred. An unpaired two-tailed t-test and Mann-Whitney U test were also performed on naïve CD4 cell count at 6 months, comparing palifermin versus placebo. For exploratory end-points, summary statistics were calculated by treatment arm, no formal statistical tests were applied (exploratory end-point p values reported in the text are given for descriptive purposes only). Continuous variables were summarised using n (non-missing sample size), mean, standard deviation, median, maximum and minimum. Categorical variables were reported as frequency and percentages (based on the non-missing sample size) of observed levels. For any laboratory tests where the measurement made was considered to be less than the detectable limit, the value was replaced in the analysis with the lower limit of detection divided by the square root of 2 ($LLD/\sqrt{2}$).

If the interim analysis were successful, 80 patients would have been recruited to the trial which would have given a 78% power to detect a relative risk reduction of 50% of autoimmunity after alemtuzumab, using a 2-sided 5% significance level.

Role of the funding source
The Medical Research Council and the Moulton Charitable trust had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to the data. The corresponding author had final responsibility for the decision to submit for publication.

Results:
Between June 2013 and February 2015 and 28 patients were enrolled, their baseline characteristics are shown in Table 1. The pre-planned interim analysis was conducted by independent statisticians and the unblinded results reported first to the Trial Steering Committee who took the decision to recommend early

termination of the trial, as per protocol; no further patients were recruited and no further palifermin was given. All enrolled patients completed the study, and all analyses were completed with blinding intact, after which one investigator (AC) was unblinded.

As we have previously reported (Jones et al., 2013) thymic function (assessed by measuring naïve T-cells, recent thymic emigrants (RTE) and TRECs/mL) was significantly reduced following treatment with alemtuzumab. However unexpectedly, this was made worse by palifermin. The interim analysis endpoint, mean naïve (CCR7+ CD45RA+) CD4 cell count at month 6, was reduced in the palifermin group: 2.23 $x10^7$/L (SD 2.0) versus 7.73 $x10^7$/L (SD 5.74) in those receiving placebo, p=0.007 (Figure 1, appendix). This difference was also evident at months 1 and 3 post treatment: 0.036 $x10^7$/L (SD 0.025) versus 0.341 $x10^7$/L (SD 0.25), and 0.387 $x10^7$/L (SD 0.68) versus 1.326 $x10^7$/L (SD 1.29) respectively (appendix). The difference in naïve T-cell numbers was greatest at month 1 suggesting that palifermin's negative effect on thymic function occurred early. This was not due to globally reduced T-cell numbers but due to a specific reduction in naïve T-cells. Palifermin also reduced the mean proportion of recent thymic emigrants (RTEs) in the CD4 pool (month 1: 2.94% (SD 2.77) versus 7.93% (SD 8.71); month 3: 4.83% (SD 7.88) versus 13.29% (11.75); month 6: 16.05% (13.21) versus 33.95% (18.68), (Figure 1, appendix). Mean TRECs/mL in the palifermin group was reduced at months 3 and 6: 64.6 (SD 27.9) and 1100.1 (SD 1721.6) versus 846.1 (SD 1980.6) and 3395.5 (SD 3038.8) respectively. In keeping with reduced thymopoiesis, there was a trend towards more restricted CD4 and CD8 TCR repertoires after palifermin; for instance, Shannon's Entropy was 12.7 versus 13.3 in the placebo group and the mean CD4 clonality score was 0.102 versus 0.067. Palifermin reduced the number of unique clones per ug of DNA (75,111 versus 84,017; appendix). As per our previous reports, the CD8 TCR repertoire was more restricted than the CD4 repertoire at baseline, becoming increasingly restricted after treatment, particularly in the palifermin treated group (appendix).

Following alemtuzumab, mean proportions of T effector RA (TEMRA) and in particular effector memory (EM) cells were increased in the CD4 pool, particularly in the palifermin arm. Similar changes were seen in the CD8 T-cell pool (appendix). Palifermin had no effect on the usual rise in the relative number of CD4 T regulatory cells (CD4+CD25hiCD127lo) cells after alemtuzumab (appendix). No difference was seen in thymic size or density between the two arms of the study.

In view of the unexpected negative effects of palifermin on thymopoiesis, we retrospectively assessed thymic function in patients treated on the dose-escalation sub-study. Naïve CD4 T-cells and TRECs/mL at 6 months were lower

in the 90mcg/Kg arm of the dose-escalation (n=3) compared to placebo, and lower still in the three patients on 120mcg/Kg or 180mcg/Kg of palifermin. Given the significant variation in TRECs/mL between individuals prior to treatment, we normalised TRECs/mL at 6 months to baseline levels; this was lowest - at 9.64% of baseline- in the 180mcg/Kg group versus 13.57% after 120mcg/Kg and 28.89% following 90mcg/Kg palifermin.

Adverse events were common in both arms of the study (Table 2). In keeping with the chemotherapy experience, palifermin caused an infusion syndrome consisting of: an erythematous rash, oedema of the hands and face, oral symptoms (sensory and/or altered taste) and discolouration of the tongue. Unexpectedly 10/14 patients treated with palifermin developed transient hair thinning (lasting weeks to months) after treatment, in one individual this was marked. Palifermin administration before alemtuzumab did not alter its well reported infusion-associated symptoms, except that chest tightness was reported less commonly. At the interim analysis cut off (M6) no SAEs/SUSARs were reported.

Although the protocol-defined early termination of the trial meant it was underpowered to detect an effect of palifermin on the development of autoimmunity, patients were categorised at month 30 into those who had developed a clinical autoimmune disease during the trial, those who had developed novel asymptomatic autoantibodies (measured on at least two occasions six months apart) and those with no expression of autoimmunity. There were no differences between the groups. 4/14 palifermin patients developed a clinical autoimmune disease, compared to 5/13 on placebo (one patient was lost to follow-up in the placebo group; Fisher's exact test, two-sided, p=0.69). 5/14 patients on palifermin developed either clinical autoimmunity or de novo autoantibodies, compared to 8/13 on placebo (p=0.2).

Discussion

Here we report the unexpected finding that palifermin (keratinocyte growth factor) exacerbates alemtuzumab's negative impact on thymopoiesis. We have demonstrated this by three independent techniques: naïve CD4 count (the primary interim outcome measure), circulating numbers of recent thymic emigrants and T-cell receptor excision circles (TRECs)/mL. Since the overall aim of the trial was predicated on palifermin's ability to boost thymopoiesis to reduce autoimmunity after alemtuzumab, in accordance with the trial protocol, recruitment to the study was halted and further dosing of palifermin suspended.
Our results contradict palifermin's ability to enhance thymopoiesis in murine and non-human primate models (Min et al., 2002, Rossi et al., 2002, Seggewiss et al., 2007). Although a species difference is possible, the fact that the KGF

receptor (FGFR2IIIb) is expressed on human epithelial cells makes this unlikely. We also do not believe that this is a dose effect. Our decision to test the efficacy of the highest tolerated dose of palifermin was based on our interpretation of results from a trial of palifermin in preventing GvHD following allogeneic HSCT. In this study, three 60mcg/Kg daily doses of palifermin before conditioning and up to 9 doses after transplant did not accelerate total lymphocyte recovery, nor reduce the incidence of acute GVHD. Whilst the absence of detailed immune phenotyping data and lack of information on TRECs and TCR repertoire makes it difficult to distinguish palifermin's effect on the thymus versus lymphopenia induced proliferation, the result suggested to us that 60mcg/Kg was unlikely to have a positive effect on thymopoiesis. Our suspicion was confirmed by the results of another trial of palifermin, published during the course of this study, which demonstrated that up to 3 doses of 60mcg/kg of palifermin did not increase CD4 counts, nor improve thymic function (assessed by measuring naïve CD4 cells, RTEs and thymic size on CT scan) in HIV-infected patients with persistent CD4 lymphopenia despite virologically effective anti-retroviral treatment (Jacobson et al., 2014); suboptimal dosing was postulated as a cause for their negative result. Importantly, no previous study has reported a reduction in thymic function with palifermin. In our own study, none of the doses tested in the tolerability sub-study had a positive effect on thymopoiesis. With the caveat that only 3 individuals were treated at each dose level, all doses (from 90 to 180mcg/Kg/day) impaired thymic function after alemtuzumab, with an apparent dose effect.

We have recently learnt that murine TECs express CD52 (the target of alemtuzumab) at least at the mRNA level (personal communication from Prof George Hollander, Oxford). Although we are yet to confirm whether this is the case in humans, it raises the possibility that alemtuzumab impairs thymic function by damaging CD52 expressing TECs. In this study, palifermin's negative effect on thymic function was most marked at the earliest time points, at the point of co-administration with alemtuzumab. For example, the biggest difference in the number of naïve CD4 cells between the two arms of the study was at month 1, where there was a 9.5 fold difference compared to a 3.4 fold difference at month 3, and a 2.6 fold difference at month 6. A similar effect was seen in the TREC/mL data where the biggest difference between the two arms of the study was at month 3 (the earliest point measured; a 13 fold difference vs. a 3 fold difference at month 6). These data suggest that whilst the initial doses of palifermin exaggerate alemtuzumab-induced thymic damage, later doses may be protective. Our working hypothesis is that palifermin worsens alemtuzumab's impact on thymic function by causing TECs to upregulate CD52 expression, so making them more susceptible to damage.

Although palifermin significantly reduced thymopoiesis in our patients, there was no evidence that it increased the risk of developing autoimmunity at 30 months of follow up. However autoimmunity can occur for up to 5 years after alemtuzumab so we will continue to monitor these patients clinically and immunologically.

In conclusion we have shown that palifermin (180mcg/kg/day given over 12 days) worsens thymic function following alemtuzumab treatment of RRMS. Our study acts as a reminder to be cautious in extrapolating efficacy data and dosing regimens from animal studies, and when co-administering drugs that may interact. It remains to be seen if alemtuzumab induced autoimmunity can be reduced by preserving thymic function.

Acknowledgements

This research was supported by the Cambridge NIHR BRC Cell Phenotyping Hub. All patients were treated on the NIHR/Wellcome Trust Clinical Research Facility at Addenbrooke's Hospital in Cambridge. JJ is funded by the Wellcome Trust.

A Case of Anaphylaxis to Alemtuzumab

C. J. S. Nye[1], A. Wagner[2], O. Kousin-Ezewu[3], J. L. Jones[3], A. J. Coles[3]

1 – University of Cambridge, 2 – Department of allergy and immunology, Cambridge University Hospitals NHS foundation trust, 3 – Department of clinical neurosciences, Cambridge University Hospitals NHS foundation trust

Corresponding author: Charles Nye – charles.nye@nhs.net, 07943875671

Acknowledgements: The patient was treated on the NIHR Cambridge Clinical Research Facility

Funding: The Medical Research Council funded the CAMTHY trial

Conflicts of interest: Both AC and JJ have received honoraria and travel costs for attending scientific advisory boards.

Keywords: Anaphylaxis, Alemtuzumab, relapse-remitting Multiple sclerosis, Allergy, Drug, Antibody

Abstract

A 22-year-old female with relapsing-remitting multiple sclerosis developed anaphylaxis to the first dose of the second cycle of alemtuzumab. This is the first reported case of confirmed anaphylaxis to the drug.

Introduction

Alemtuzumab (Lemtrada) is a humanised monoclonal antibody targeting CD52 found on lymphocytes and monocytes, and is a highly effective treatment of relapsing-remitting multiple sclerosis [1–3]. It is given as five consecutive daily doses of 12mg IV at baseline, with no further treatment until 12 months later, when patients receive three consecutive doses of 12mg IV.

Over 90% of patients receiving alemtuzumab experience infusion associated reactions. Work in the 1990s showed that these could be reduced or ameliorated by pretreatment with corticosteroids [4] and that the underlying mechanism was a programmed release of cytokines from natural killer cells, triggered by Fc cross-linking [5]. When severe, these reactions may include a rash, fever, hypotension and bronchospasm and so mimic anaphylaxis; they are therefore termed “anaphylactoid”. This phenomenon has led to confusion in the current literature as to whether patients may develop genuine anaphylaxis to alemtuzumab.

Case report

A 22-year old female, with relapsing remitting multiple sclerosis, and no history of atopy, had previously received her first cycle of alemtuzumab without complication. This had occurred in the context of a clinical trial where, one week before alemtuzumab, she had received either placebo or palifermin to promote thymic reconstitution (CAMTHY; EudraCT Number: 2011-005606-30). No further investigational drug was given to the patient. As is common, she had developed Grave's disease 6 months later and was being treated with carbimazole (40mg OD) and thyroxine (75ug OD).

One year later, she received the first dose of the second cycle of alemtuzumab. Beforehand, as usual, she had received 1g of methylprednisolone and anti-histamines. 40 minutes into the infusion (1/6th of the dose) she had generalized urticaria, facial swelling, tongue swelling, stridor, hypotension and wheeze. The infusion was stopped and her symptoms quickly resolved, without further medications.

Her serum IgE was elevated at 314, rising to 374 the following day (upper limit of normal 170ku/l) and her serum tryptase, taken during her symptoms, was elevated at 22 (upper limit of normal 14). Her baseline tryptase, recorded 10 days after the event and one year later was 3 ruling out mastocytosis and helps to confirm this is true anaphylaxis. Eighteen months later, she had allergy testing. A skin prick test of alemtuzumab 10mg/ml was positive at 4mm and an

intradermal test (1:100) of alemtuzumab was positive with a 12mm wheal and a flare greater than 30mm (see Figure 1).

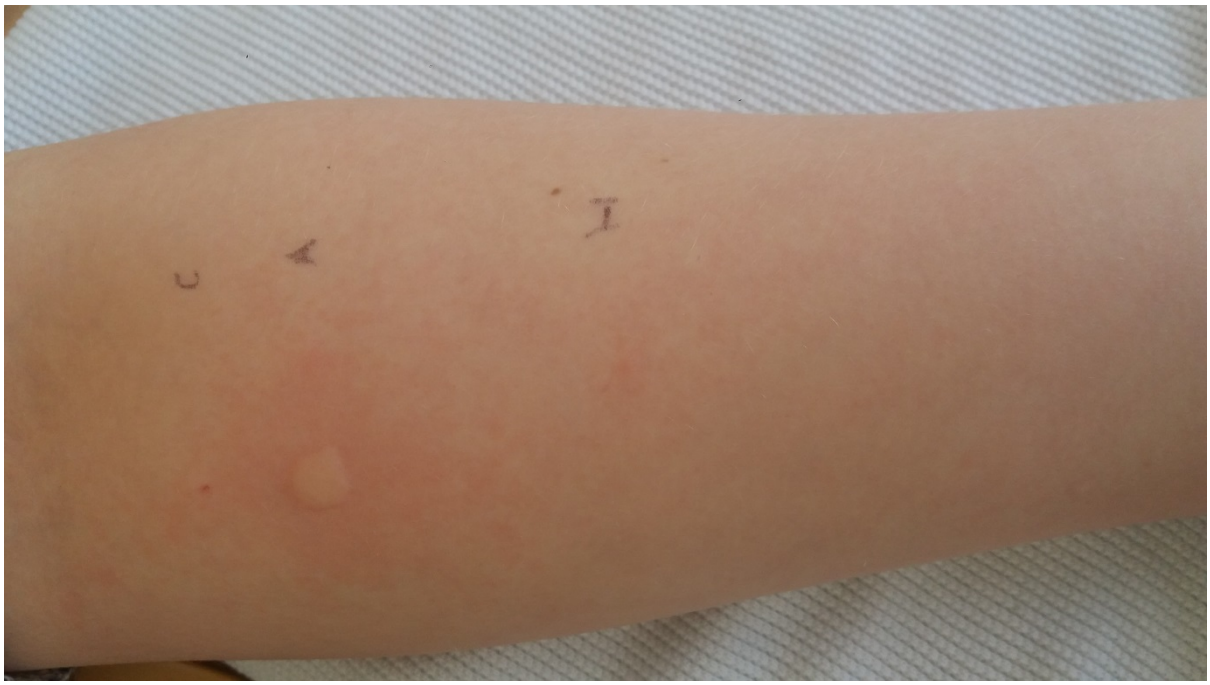

Fig. 1 Result of the skin prick testing showing the positive reaction to alemtuzumab and negative control

The patient switched to fingolimod and has done well.

Discussion

This is the first case of true anaphylaxis to alemtuzumab, confirmed by skin prick, intradermal testing and the confirmed rise in serum tryptase. Another case in the literature is likely to represent anaphylaxis (Caon et al. 2015) [6], but did not have formal allergy testing. However, other reported cases of anaphylaxis more likely represent severe anaphylactoid cytokine-induced infusion reactions.

A typical example of the confusion is the case of " anaphylaxis" with the first administration of alemtuzumab, as a treatment for B-cell CLL [7] clearly, this is very unlikely to be true anaphylaxis. Features of anaphylaxis that would not be expected in a cytokine-release syndrome are stridor and facial and tongue swelling, as seen in this case. This report also shows the benefit of formal allergy testing to confirm true IgE mediated anaphylaxis versus the unavoidable adverse effects due to the physiological action of the drug.

In this case, her symptoms resolved on stopping the infusion of alemtuzumab. Presumably, the premedication with corticosteroids prevented worse manifestations of her allergy. Nonetheless, further exposure to normal concentrations of the drug would be dangerous, so we elected to switch her to an alternative treatment. Another strategy might have been to induce desensitisation to alemtuzumab.

References

1. Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung H-P, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. The Lancet. 2012 Nov 24;380(9856):1819–28.

2. Coles AJ, CAMMS223 Trial Investigators, Compston DAS, Selmaj KW, Lake SL, Moran S, et al. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. N Engl J Med. 2008 Oct 23;359(17):1786–801.

3. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. The Lancet. 2012 Nov 24;380(9856):1829–39.

4. Coles AJ, Wing MG, Molyneux P, Paolillo A, Davie CM, Hale G, et al. Monoclonal antibody treatment exposes three mechanisms underlying the clinical course of multiple sclerosis. Ann Neurol. 1999 Sep 1;46(3):296–304.

5. Wing MG, Moreau T, Greenwood J, Smith RM, Hale G, Isaacs J, et al. Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. J Clin Invest. 1996 Dec 15;98(12):2819–26.

6. Caon C, Namey M, Meyer C, Mayer L, Oyuela P, Margolin DH, et al. Prevention and Management of Infusion-Associated Reactions in the Comparison of Alemtuzumab and Rebif® Efficacy in Multiple Sclerosis (CARE-MS) Program. Int J MS Care. 2015;17(4):191–8.

7. Moreton P, Kennedy B, Lucas G, Leach M, Rassam SMB, Haynes A, et al. Eradication of Minimal Residual Disease in B-Cell Chronic Lymphocytic Leukemia After Alemtuzumab Therapy Is Associated With Prolonged Survival. J Clin Oncol. 2005 May;23(13):2971–9.