A CLINICAL AND IMMUNOLOGICAL STUDY OF $CD8^+$ T CELLS IN MULTIPLE SCLEROSIS

A thesis submitted in requirement of Cardiff University

For the degree of Doctor of Philosophy

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For Dad

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Abbreviations

AG	Arachnoid granulations
AID	Autoimmune disease
APC	Antigen presenting cell
ARR	Annualised relapse rate
BBB	Blood brain barrier
BM-APC	Bone marrow derived antigen presenting cell
BV	Brain volume
CARE-MS	Comparison of Alemtuzumab and Rebif® Efficacy in Multiple Sclerosis
CCL2	C-C chemokine ligand 2
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDR3	Complementarity determining region 3
CDR3-LD	Complementarity determining region 3 length distribution
CIS	Clinically isolated syndrome
CLN	Cervical lymph node
CNS	Central Nervous System
CPL	Combinatorial peptide library
CSF	Cerebrospinal fluid
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DHODH	Dihydroorotate-dehydrogenase
DMF	Dimethylfumarate
DMT	Disease modifying therapy
EAE	Experimental autoimmune encephalomyelitis

EBNA	Epstein-Barr Virus nuclear antigen-1
EBV	Epstein-Barr Virus
EDSS	Expanded disability status scale
FACS	Fluorescence-activated cell sorting
FMO	Fluorescence minus one
Gd	Gadolinium
GFAP	Glial fibrillary acidic protein
GLA	Glatiramer acetate
GWAS	Genome wide association studies
HA	Haemagglutinin
HD	Highly differentiated
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSCs	Haematopoietic stem cells
HV	Healthy volunteers
ICAM	Intercellular adhesion molecule
IFN	Interferon
IIH	Idiopathic intracranial hypertension
IL	Interleukin
ISF	Interstitial fluid
IMGT	International ImMunoGeneTics
ITP	Idiopathic thrombocytopaenic purpura
JAML	Junctional adhesion molecule-like
JC	John Cunningham

LAP	Latency-associated peptide
LFA	Lymphocyte function-associated antigen
LLN	Lower limit of normal
MAG	Myelin-associated glycoprotein
MAIT	Mucosal-associated invariant T cells
MBP	Myelin basic protein
MCAM	Melanoma cell adhesion molecule
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MR1	Major histocompatibility complex class I related protein I
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NAWM	Normal appearing white matter
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NIND	Non-inflammatory neurological disease
OIND	Other inflammatory neurological disease
OND	Other neurological disorders
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PLP	Proteolipid protein
pMHC	Peptide/MHC

PML	Progressive multifocal leucoencephalopathy
PPMS	Primary progressive Multiple sclerosis
RRMS	Relapsing remitting Multiple sclerosis
RT-PCR	Reverse transcription polymerase chain reaction
SAD	Sustained accumulation of disability
SRD	Sustained reduction of disability
TAL	Transaldolase
TCR	T cell receptor
TCRVα	Alpha chain of T cell receptor
TCRVβ	Beta chain of T cell receptor
TGF	Transforming growth factor
Th1	T helper 1 cells
Th7	T helper 7 cells
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TREK1	TWIK-related potassium channel-1
T_N	Naïve T cells
T_{RM}	Tissue resident memory T cells
T_{SCM}	Stem cell memory T cells
T_{CM}	Central memory T cells
T_{TM}	Transitional memory T cells
T_{EM}	Effector memory T cells
T_{EMRA}	Effector memory RA T cells
UHW	University Hospital of Wales

TREK-1	TWIK-related potassium channel-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
WML	White matter lesions

Summary

Prior focus has largely been on the role of $CD4^+$ T cells in Multiple sclerosis (MS) disease pathogenesis, but there is mounting evidence for the role of $CD8^+$ T cells. This thesis aimed to explore the role of $CD8^+$ T cells by; (i) analysing clinical outcomes in MS patients treated with alemtuzumab, (ii) performing an in-depth phenotypic analysis of cerebrospinal fluid (CSF)-resident T cells in MS patients, (iii) examining the CSF-resident T cell receptor (TCR) repertoire in MS patients, and; (iv) identifying the pathogenic triggers/antigenic targets of dominant CSF-resident TCRs. Alemtuzumab was shown to be an effective treatment for relapsing MS. Immunophenotyping demonstrated an increased number of CSF-resident $CD4^+$ and $CD8^+$ T cells in MS patients compared with controls although the majority of CSF-resident T cells were of an effector memory phenotype across all groups. This suggests that effector memory T cells enter the CSF as part of normal central nervous system (CNS) immunosurveillance, and is consistent with the fact that I was able to detect Epstein-Barr virus-specific TCRs in all groups at similar frequencies. Clonal expansions were observed in the $CD4^+$ and $CD8^+$ T cell repertoire of all patient groups and so are not a unique feature of MS. However, I did observe a significant increase in TCR diversity in the $CD4^+$ and $CD8^+$ TCR repertoire in MS patients compared to controls. Overall, the results from the Alemtuzumab study strongly support a central role for T cells in MS pathogenesis. Immunophenotyping and clonotyping analysis suggest that $CD4^+$ and $CD8^+$ T cells with an effector memory phenotype preferentially accumulate in the CSF as part of normal immune surveillance. In MS, increased TCR diversity warrants further investigation as it suggests that a more diverse response to CNS antigens may play a role in disease pathogenesis.

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Chapter 1

Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that causes demyelination and destroys oligodendrocytes, neurons and axons.[1] MS is the most common chronic neurological disease affecting young adults in the Western word, with a lifetime risk of 1 in 400. Clinical disease is heterogeneous but most commonly is characterised by a relapsing/remitting course with episodes of CNS inflammation and demyelination manifesting as subacute episodes of clinical neurological dysfunction, which may include sensory, motor, visual, and cognitive deficits.[2] Clinical presentation is determined by the spatiotemporal dissemination of characteristic MS lesions within the CNS.[3] These CNS lesions are pathognomonic of MS and are caused by parenchymal immune cell infiltrates that promote inflammation, demyelination, gliosis and neuroaxonal degeneration, leading to disruption of neuronal signalling.[3] Autoreactive T cells are considered to initiate the disease process, mounting aberrant immune responses against CNS autoantigens, the exact nature of which remains unknown.[3] Later in the course of the disease, neuroinflammation is less prominent with neurodegeneration becoming the main feature in the secondary progressive phase. A smaller proportion (10-20%) of patients present without discreet episodes of neuroinflammation with a progressive disease course from onset; primary progressive MS (Figure 1.1).[2]

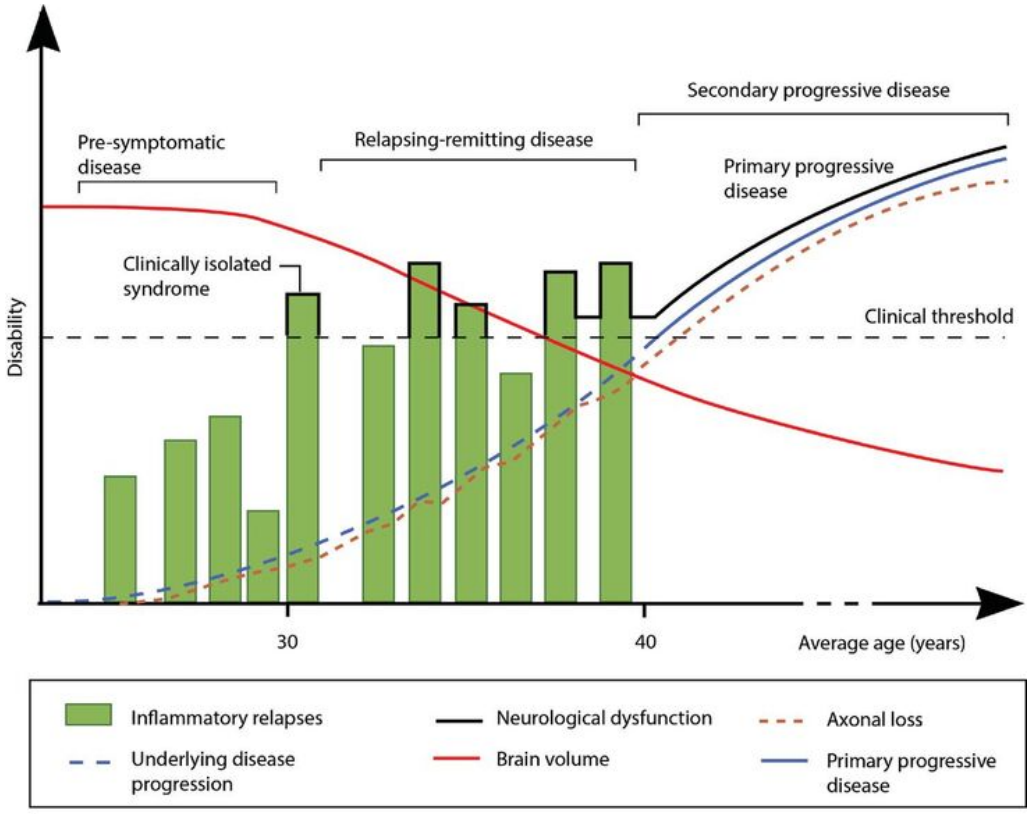


Figure 1.1. Association between inflammation, clinical relapses and progression in Multiple sclerosis. Relapsing-remitting disease is the most common form of disease presentation, characterised by inflammation and demyelination occurring in eloquent areas of the CNS and reaching a threshold that produces clinical symptoms. Recovery occurs as inflammation improves, but with residual disability. Following this relapsing phase, patients become secondary progressive - the disease is no longer characterised by inflammation and relapses but a gradual accrual of disability related to axonal and brain volume loss. Figure adapted from Dendrou et al.[3]

Disease pathogenesis is likely to be multifactorial with a contribution from environmental factors in addition to a genetic predisposition.[2] Genome Wide Association Studies (GWAS) indicate that there is an association with the Human Leukocyte Antigen (HLA) regions with genetic variation thought to account for approximately 30% of the overall disease risk. In total, more than 100 genetic regions have been associated with MS.[3] This, along with evidence from the composition of MS plaques and animal models supports the central role of T cells in MS disease pathogenesis.[2] As such, treatment options are currently focused on the relapsing stage of the disease and are aimed at interfering with cell trafficking,[4, 5] lymphocyte function[6-8] and lymphodepletion.[8, 9]

Although there is a large body of research focusing on the role of $CD4^+$ T cells in MS pathogenesis,[10] interest in the role of $CD8^+$ T cells is increasing with a growing body of evidence suggesting they have a central role to play in disease development. Throughout this introduction the evidence for the role of $CD8^+$ T cells in MS will be explored as a background for setting out the objectives of this thesis. Understanding the possible pathogenic and regulatory role of $CD8^+$ T cells has clear implications for unravelling disease pathogenesis and for the development of novel therapeutics.

1.2 The immune system

The innate immune system is the first line of defence against invading pathogens, incorporating macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and NK cells.[11] The adaptive immune system is divided into humoral and cell-mediated immunity involving B- and T-cells, respectively. B cells secrete antibodies, which bind to epitopes on invading pathogens, identifying them for removal by other components of the immune system, such as macrophages. The cell-mediated immune system, which will be the focus of this thesis, involves the activation of T cells. The majority of T cells express only one T cell receptor (TCR), which after recognition of a specific antigen proliferate by clonal expansion.[12, 13]

1.3 T cells

T lymphocytes or T cells, ubiquitously characterised by the presence of the co-receptor molecules CD4 or CD8, orchestrate human cellular immunity. Central to the interaction between T cells and antigen (presented in combination with major histocompatibility complex (MHC), or HLA in humans) is the T cell receptor (TCR). The majority of TCRs are heterodimers comprised of two subunit chains (α- and β-), which both contain constant and variable domains (Figure 1.2). Because of the need for a large number of unique TCRs given the number of potential TCR/antigen interactions, the immune system has a unique way of introducing diversity. TCR diversity is generated during the early stages of T cell development in the thymus. During cell division, extensive gene recombination occurs between the V(variable)- and J(junctional)- segments, and the V-, D(diversity)- and J- segments, in the TCR α and TCR β genes respectively - a process referred to as V(D)J recombination. The region of TCR β that spans the V-D and D-J junctions is known as the complementarity determining region 3 (CDR3) and is unique

to each TCR β variant. Following this process, T cells that lack sufficient affinity for MHC molecules and those that recognise self-antigens are eliminated (positive and negative selection respectively).[14]

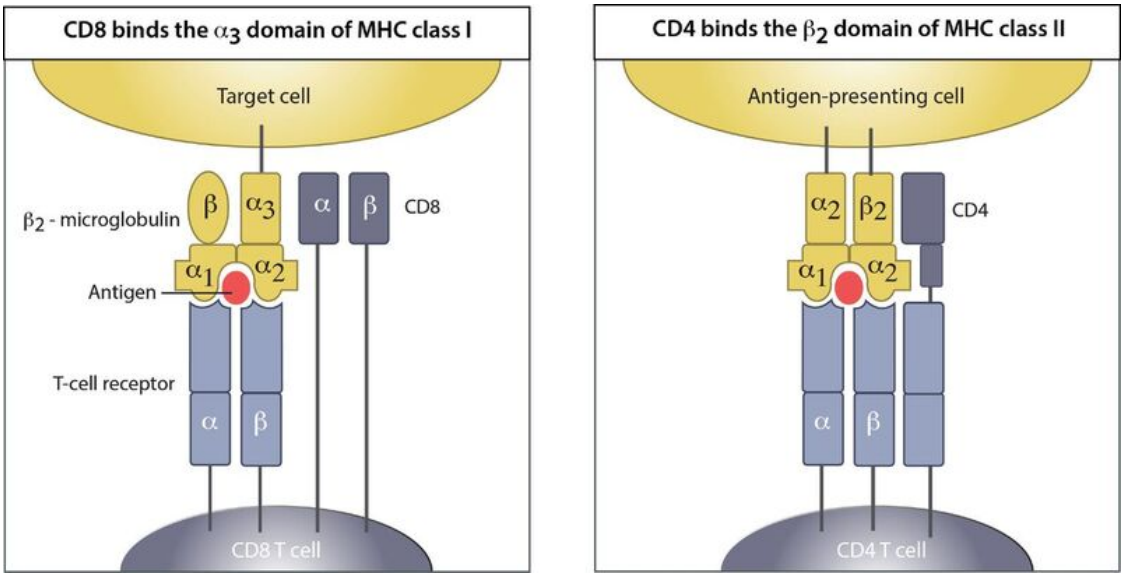


Figure 1.2 Interaction of T cells with antigen presenting cells. $CD8^+$ T cells interact via T cell receptors and peptide/MHC class I on antigen presenting cells. $CD4^+$ T cells interact via peptide/MHC class II binding. Figure adapted from The Immune System, Parham P, Garland Science, 2009.[15]

1.4 T cell immune surveillance of the central nervous system

The central nervous system has long been considered an immune-privileged site for several reasons;[16] (i) the expression of MHC molecules is limited within the CNS parenchyma,[17] (ii) the entry of immune cells into the CNS via the blood-cerebrospinal fluid (CSF) barrier, the CSF-brain barrier and the blood brain barrier (BBB) is restricted,[18] (iii) the antigenic representation in peripheral lymph nodes may not be an accurate representation of the CNS due to the special features of CNS lymphatic drainage (Figure 1.3).[16, 19]

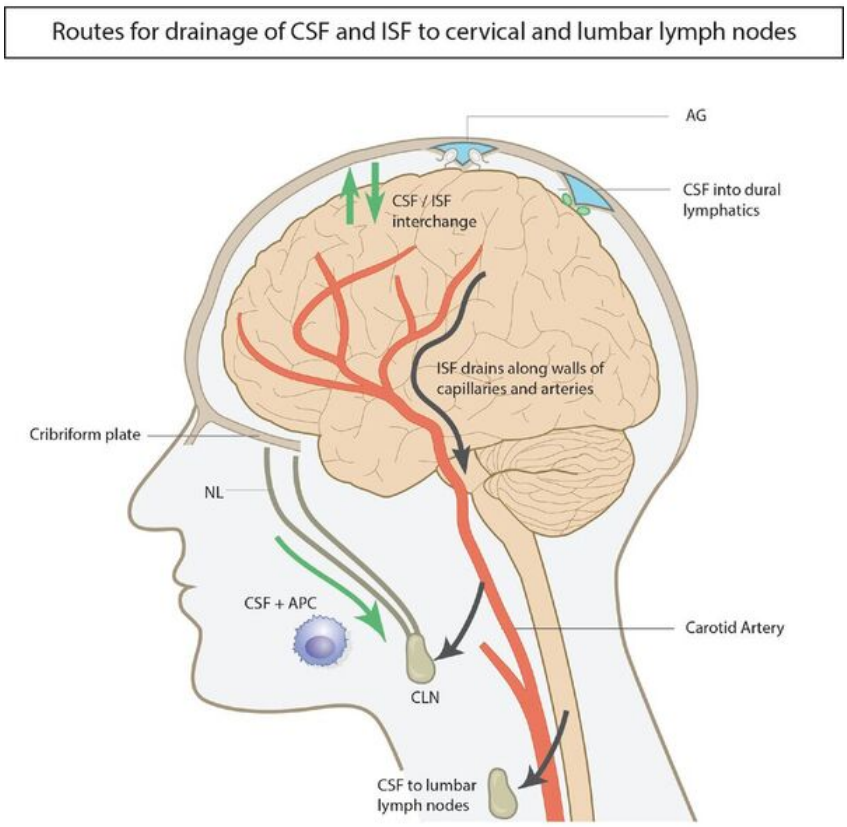


Figure 1.3. Lymphatic drainage of the central nervous system. CSF and interstitial fluid (ISF) drain to lymph nodes by different pathways. (i) CSF drains into venous sinuses through arachnoid villi and granulations (AG). Lymphatic drainage of CSF occurs via nasal and dural lymphatics and along cranial and spinal nerve roots. Channels that pass from the subarachnoid space through the cribriform plate allow passage of CSF T cells and antigen-presenting cells (APC) into nasal lymphatics and cervical lymph nodes (CLN). CSF from the lumbar subarachnoid space drains to lumbar lymph nodes. (ii) ISF from the brain parenchyma drains along basement membranes in the walls of cerebral capillaries and arteries to cervical lymph nodes. There is interchange between CSF and ISF (glymphatic system), as CSF enters the surface of the brain alongside penetrating arteries.[20] Figure adapted from Engelhardt et al.[20]

Despite this, T cells are central for CNS immune surveillance and maintaining homeostasis, with a fine balance to be struck between control of infectious agents and immune-mediated damage. As the CNS is a common target of viral infections and autoimmune disorders, then T cells must be able to access the CNS despite its supposed immune privileged status. As such, the brain and spinal cord are under continual immune surveillance to detect and eliminate potential mediators of infection and damage. Two possible scenarios have been proposed as to how this may occur (Figure 1.4).[16]

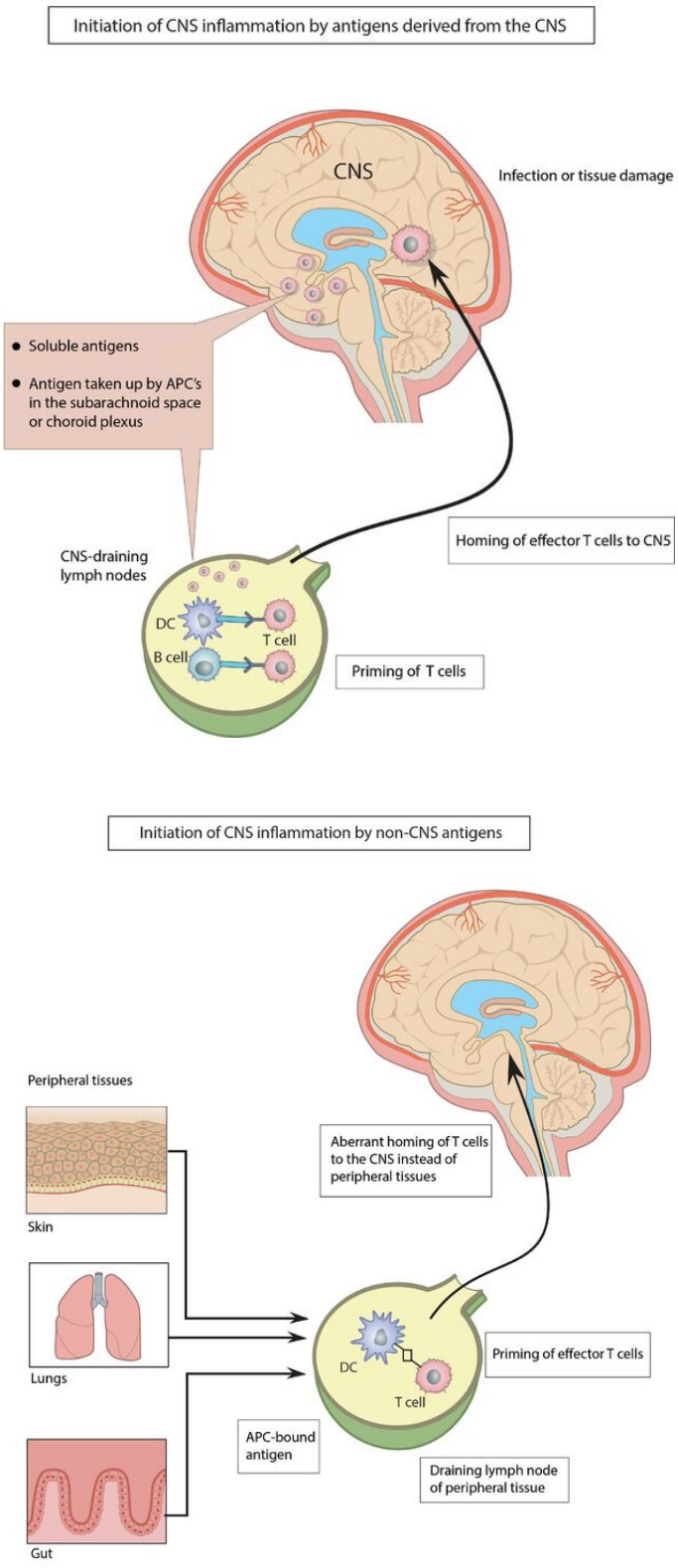


Figure 1.4 Two proposed scenarios of T cell CNS immune surveillance despite supposed immune privileged status. (a) CNS inflammation initiated in the CNS-draining lymph nodes (upper panel). After primary virus infection of the CNS, T cell priming due to aberrant leakage of CNS antigens occurs in the CNS-draining lymph nodes. T cells then home back to the CNS in order to eliminate foreign antigens or to cause autoimmune inflammation. (b) T cells that recognise target antigens in the CNS are primed by non-CNS, peripheral antigens due to cross-reactivity or molecular mimicry (lower panel).[16] Figure adapted from Korn et al.[16]

In the first scenario, CNS inflammation is initiated in the CNS-draining lymph nodes. Up until recently, the lack of lymphatic drainage of the CSF was thought to be a major contributor to its immune-privileged status. However, the recent discovery of a CNS lymphatic drainage system[21] and the newly named 'glymphatic system', related to drainage from the parenchymal interstitial fluid to the CSF,[22] has helped to explain how T cell immunity may occur. Antigen – either virus infected cells or self-antigens - might be exported from the CNS and presented in CNS-draining lymph nodes. After priming of antigen-specific T cells in the CNS-draining lymph nodes, these T cells may then home back to the CNS in order to eliminate foreign antigens or to cause autoimmune inflammation.[16] In an alternative scenario, non-CNS peripheral antigens prime T cells recognising target antigens in the CNS. These antigens may be molecular mimics of CNS antigens or antigens that are produced by viruses that cause a systemic infection before infecting the CNS.[16]

Once homed back to the CNS, T cells may be activated and regain entry via several different proposed routes (Figures 1.5).[23] T cell reactivation may occur in the choroid plexus,[24] the meninges,[25] and the perivascular space within the CNS parenchyma (Figure 1.6).[26] Interestingly, in a mouse model, it has recently been
shown that T cells gain the capacity to enter the CNS after residing transiently in lung tissue.[27]

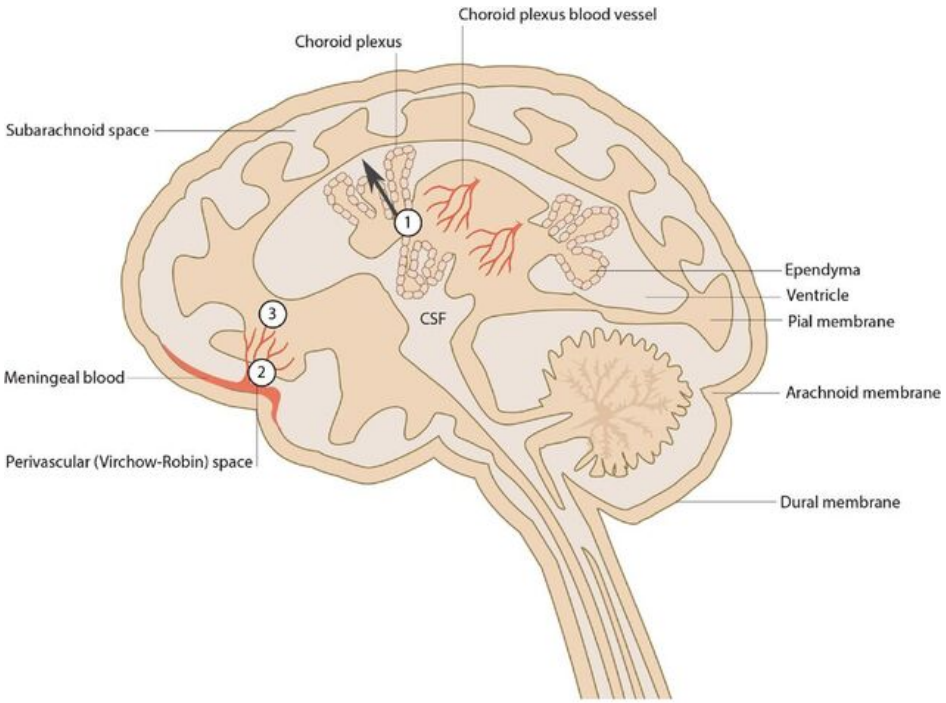


Figure 1.5. Suggested sites of T cell entry into the CNS. (1) Through the fenestrated blood vessels of the choroid plexus, across the ependymal layer and into the CSF, (2) through the perivascular or Virchow-Robin space, (3) directly into the CNS parenchyma through postcapillary venules.[23] Figure adapted from Ousman et al.[23]

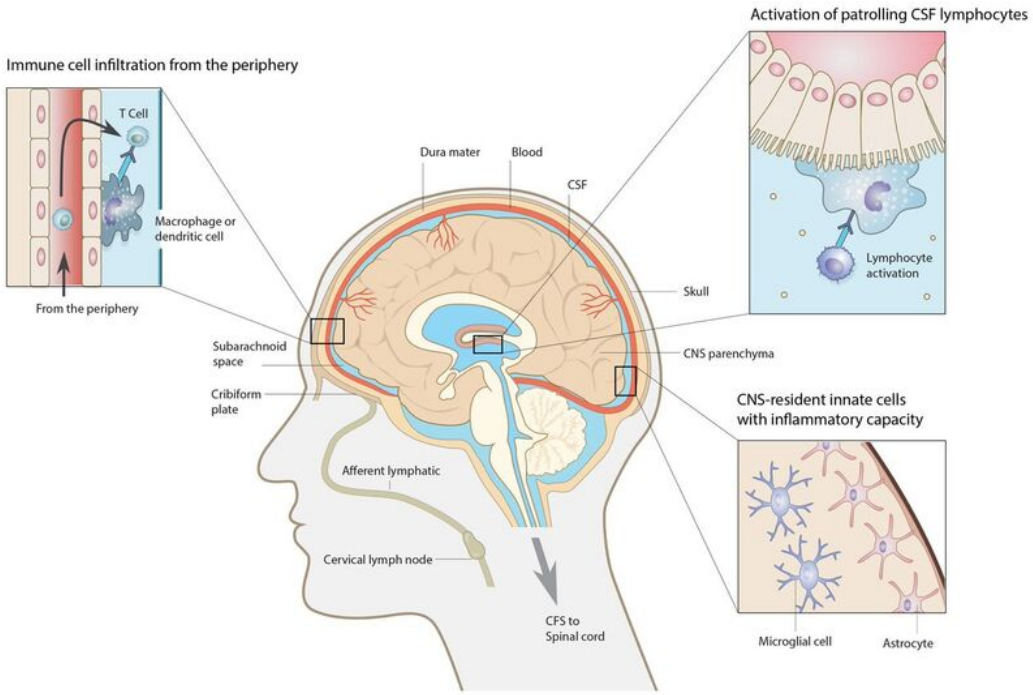


Figure 1.6 Suggested sites of T cell-CNS interactions in MS. (a) peripheral activation of T cells against CNS antigens with subsequent CNS infiltration. (b) CNS-intrinsic T cell activation as a result of normal immune surveillance. (c) Intra-parenchymal inflammation.[3] Figure adapted from Dendrou et al.[3]

In addition to the above scenarios, and with particular regard to $CD8^+$ T cells, protection of the CNS from reinfection was recently found to depend on tissue resident memory (T_{RM}) cells.[26] T_{RM} cells are non-circulating T cells that are located in non-lymphoid tissues to provide immediate protection from re-infection.[28] Interestingly, the systemic reactivation of T cells, which then home to the CNS to clear the pathogen, is usually associated with pathology that ultimately may be more harmful than the potential pathogen-induced damage.[29] Conversely, T_{RM} cells have been shown to clear the CNS of pathogen during reinfection efficiently and with less collateral pathology.[16]

A disruption of the constant immune surveillance that occurs in immunosuppressive disease (e.g. human immunodeficiency virus, HIV) or with immune-modulating drugs highlights its importance. A striking example of this is the occurrence of progressive multifocal leucoencephalopathy (PML) that occurs secondary to John Cunningham (JC) virus.[30] The majority of humans harbour this virus, which is normally controlled by the immune system. However, upon depletion of usual CNS immune surveillance, due to immunosuppressive disease or therapeutics (in particular, natalizumab, a monoclonal antibody against the adhesion molecule very late antigen-4 (VLA-4), preventing lymphocyte egress into the CNS), the virus enters the brain resulting in an untreatable, and often fatal infection.[30]

In summary, potential pathogens are likely to be commonly frequenting the CNS and as such humans require a constant, active, immune surveillance to keep these potential infections under control. Immunosuppressive disease and drugs that alter this fine balance highlight the importance of this. In addition, aberrant immune stimulation and activation has the potential to lead to autoimmune disease such as MS.

1.5 Overview of T cell involvement in the pathogenesis of Multiple sclerosis

The exact cause of MS remains unclear and in particular whether there is a common provoking factor or pathway across all affected individuals. There is a clear genetic

predisposition with contribution from environmental factors but as yet the pathogenic trigger and antigenic target remain elusive.

The question of whether MS originates in the periphery or in the CNS is still to be comprehensively determined although it is clear that there is an ever-evolving immune response. Early in the disease, immune cells infiltrate the CNS parenchyma.[3] These cells, in association with activated microglia and astrocytes, promote demyelination and oligodendrocyte and axonal injury. Later in the disease process, immune cell infiltration is less prominent, with more CNS-intrinsic inflammation and neurodegeneration.[3] Consistent with general CNS immune surveillance, both peripheral and central models of disease pathogenesis have been suggested.

As discussed in section 1.4, the peripheral model of MS pathogenesis suggests autoreactive T cells are activated at peripheral sites through molecular mimicry,[31] bystander activation or the co-expression of TCRs with different specificities.[32] T cells are then thought to traffic to the CNS along with activated B cells and monocytes (Figure 1.7).[3]

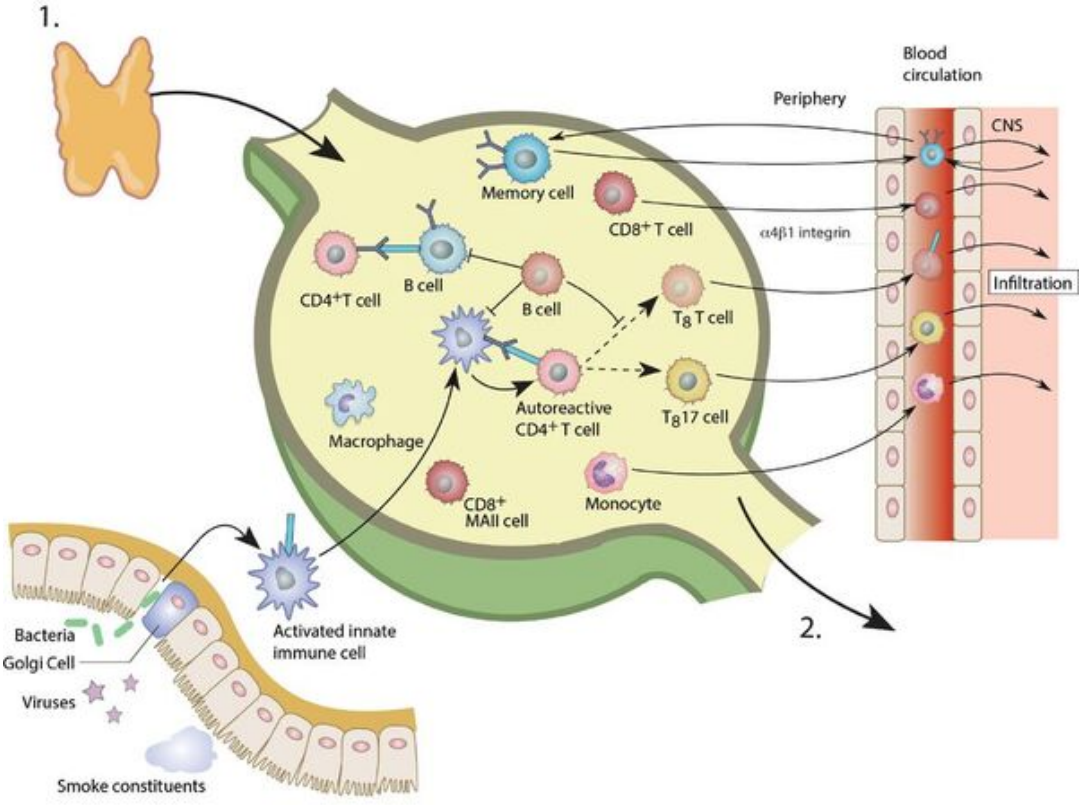


Figure 1.7. Immune system dysregulation outside the CNS. CNS-directed autoreactive T cells that escape both central and peripheral tolerance (1.) may be activated in the periphery and subsequently be pathogenic to CNS antigens (2.). This process may occur through molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen released into the periphery or bystander activation. Genetic and environmental factors, including infectious agents and smoke constituents, contribute to these events. Figure adapted from Dendrou et al.[3]

With the emerging understanding of CNS immune surveillance, an alternative hypothesis suggests central disease development, with a CNS-intrinsic inflammatory response to an as yet unknown CNS viral infection. This may then result in secondary infiltration of autoreactive T cells (Figure 1.4).[16]

In addition to genetic risk discussed later in this chapter, environmental factors also influence disease pathogenesis.[3] Firstly, viruses or microbes may be directly involved in the triggering of autoreactive T cells.[31] Secondly, CNS infectious agents may promote the release of sequestered CNS antigens into the periphery.[33] Thirdly, environmental influences may also alter the activation threshold of T cells.[16] Lastly, cytokines secreted at sites of peripheral inflammation secondary to infection, may also have an influence

on CNS immune responses, leading to local inflammation and disease. Interestingly, this last point raises the possibility that MS could be triggered by infection at a distant site rather than a CNS-directed autoreactive immune response.[3]

In addition to a pathogenic role for T cells, defective regulatory T cells may also have a contributory role in MS pathogenesis.[3] These cells are discussed in more detail later in this chapter. Further to this general introduction to CNS immune surveillance and MS pathogenesis, the remainder of this chapter will focus on the evidence for the role of $CD8^+$ T cells in MS.

1.6 $CD8^+$ T cells

$CD8^+$ T cells are primary effector cells of the adaptive immune system[34] and are the main determinants of immunity to intracellular pathogens and cancer cells.[35] They recognise protein antigens presented in association with MHC class I (MHCI; HLA-A, -B and -C in humans) molecules on the surface of target cells ($CD4^+$ T cells interact with MHC class II molecules (MHCII; HLA-DR, -DQ, -DP in humans)).[35] MHCI is expressed on almost all nucleated cells, enabling the immune system to scan the cell surface to detect internal anomalies.[34] Following activation, $CD8^+$ T cells expand and deliver a range of effector functions. After clearance of the initial infection, only a small proportion of these expanded cells survive and exist as the memory population.[34] Production of the cytokines interferon(IFN)-γ and tumour necrosis factor(TNF)-α occurs after $CD8^+$ T cells differentiate into effector cells, with perforin and granzyme B also offering some direct cytotoxic action.[36] After infection, memory T cell clones persist to fight against recurrent infection.[36] An issue that may be of particular relevance in MS is that if the stimulating antigen cannot be cleared, then $CD8^+$ T cells can become chronically stimulated and cause autoimmune disease.[34, 37]

In addition to their cytotoxic role, $CD8^+$ T cells can also perform regulatory functions, with evidence to suggest relevance to clinical disease.[38] Regulatory $CD8^+$ T cells ($CD8^+$ Tregs) have been shown to exert their regulatory effects by a variety of mechanisms. This can either be via direct cell death, the induction of negative cell signalling molecules through cell-cell interactions and by the secretion of immunosuppressive cytokines such as interleukin(IL)-10.[38] Interestingly, it has been demonstrated that IL-

10 is secreted at the peak of the inflammatory response by $CD8^+$ T cells in a proposed autocrine regulation to prevent unwanted tissue damage.[39]

1.7 Genome wide association studies (GWAS) implicate $CD8^+$ T cells in MS disease pathogenesis

MS has historically been considered to be a $CD4^+$ T cell mediated disease due to strong associations with HLA class II regions in genome wide association studies.[40] In addition to the evidence for the role of $CD4^+$ T cells, there is also some indication from these studies that $CD8^+$ T cells play a role. The first genome wide association studies found disease association with several immunological, neurological and non-neurological genes. Of the immunological genes, risk was shown to be conferred by alleles of the HLA genes. In particular, the class II allele, DRB1*1501 was shown to confer risk along with the class I molecule HLA*0301, although HLA*0201 was shown to be protective.[41] These results suggested a key role for $CD4^+$ (HLA-DR) and $CD8^+$ (HLA-A) T cells in the pathogenesis of MS. However, the associated risk for developing MS with HLA-A*0301 was not found in a subsequent large GWAS, although the protective role for HLA-A*0201 was replicated.[42]

A further meta-analysis has confirmed the association of HLA-DRB1*1501 as well as other class II alleles DRB*0301, *1303, *0404, *0401 and *1401, with HLA-A*0201 again found to be protective, with its association attributed to an amino acid polymorphism in the peptide-binding groove of the HLA-A molecule.[43] Investigation of the mechanism of the potential pathogenic and protective role of the earlier identified HLA-A3 and HLA-A2 alleles was performed in a novel transgenic mouse model. Friese et al developed a humanised transgenic mouse expressing the potential risk variant HLA-A3 either alone or with a myelin proteolipid protein (PLP) TCR. Double transgenic (2D1-TCR and HLA-A3) mice developed spontaneous experimental autoimmune encephalomyelitis (EAE) at a low frequency, which increased following immunisation with the PLP peptide. Interestingly, 2D1-TCR^+CD8^+ T cells were found in typical MS anatomical sites such as the cerebellum and spinal cord, and in contact with HLA-A3 expressing oligodendrocytes with demyelination and axonal damage observed. These findings confirmed that an HLA-A3 restricted myelin specific TCR in a humanised HLA-

A3 mouse model can induce an MS-like disease. Further experiments confirmed that 2D1-TCR$^+$CD8$^+$ T cells mediated earlier disease but later manifestations were due to epitope spreading, with CD4$^+$ T cells more implicated. Intriguingly, the addition of an HLA-A2 transgene to the double transgenic mouse prevented an MS-like disease occurring. This was shown to be secondary to a reduction in numbers of the splenic 2D1-TCR$^+$CD8$^+$ T cells due to altered thymic selection and reduced responsiveness of these cells.[44]

In addition to the associations seen with HLA alleles, 110 genetic variants have now been identified that are associated with susceptibility to MS.[45] Of those identified, genes coding for cytokine pathways, co-stimulatory molecules and signal transduction have been identified[42] in addition to differences in central tolerance mechanisms, peripheral T cell function and activation, cytokine production and homeostatic proliferation in disease pathogenesis.[3] These findings strongly implicate the cellular immune system in disease pathogenesis.

1.8 CD8$^+$ T cells in Multiple sclerosis plaques

In addition to GWAS studies, there is convincing neuropathological evidence for the role of CD8$^+$ T cells in the pathogenesis of MS. CD8$^+$ T cells are not usually present in significant numbers in normal central nervous system tissue.[46] Although the MS plaque comprises several different cell types including T cells, activated macrophages and microglia,[47] CD8$^+$ T cells are the most predominant immune cell present (Figure 1.8). This was first demonstrated in the 1980s by Booss et al with CD8$^+$ T cells outnumbering CD4$^+$ T cells in the CNS from MS patients, with numbers not affected by disease duration, speed of evolution or immunosuppressive therapy.[48] Furthermore, a few years later, perivascular cuffs around MS lesions were shown to contain up to fifty times more CD8$^+$ than CD4$^+$ T cells, as well as CD8$^+$ T cells predominating in normal appearing white matter.[49] This cellular discrepancy has further been demonstrated in active MS lesions where the CD4$^+$:CD8$^+$ ratio is approximately 1:3.[47] This reverses the usual CD4$^+$:CD8$^+$ ratio of 2:1 in normal blood, and 3:1 to 6:1 ratio in CSF.[50] More recent studies have confirmed these findings using modern techniques including single cell analysis.[51-54] In addition to white matter, CD8$^+$ T cells are also found in early cortical lesions.[55]

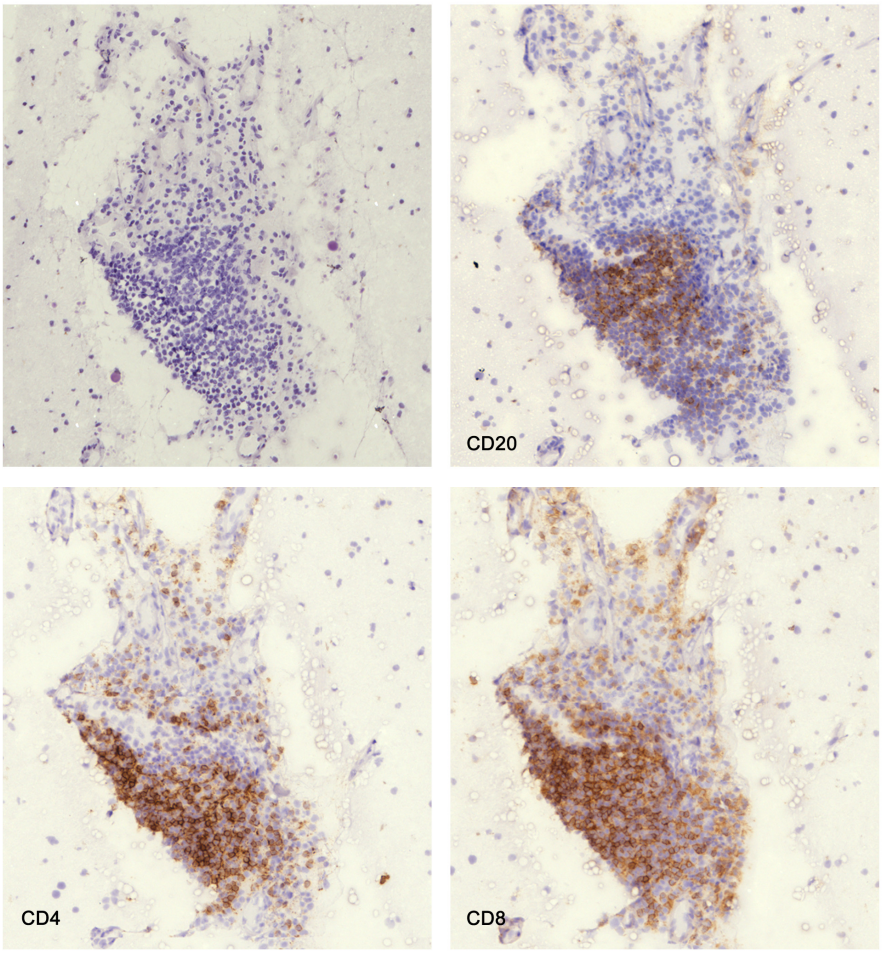


Figure 1.8. Lymphocytic perivascular infiltrate in the human forebrain meninges. Staining of CD20 (top right), CD4 (bottom left) and CD8 (bottom right). Image courtesy of Dr Owain Howell and Prof Richard Reynolds (unpublished).

1.9 Interaction of CD8$^+$ T cells with MHC class I

Although there is evidence that CD8$^+$ T cells are present in MS lesions, in order for CD8$^+$ T cells to interact with host antigen, MHC class I molecules need to be present to allow antigen presentation. Normally neurons and oligodendrocytes only express low levels of MHC class I constitutively, while astrocytes, microglia, blood vessel endothelial cells, and bone marrow derived-APC (BM-APC) do express MHC class I constitutively but do not synthesise myelin antigens.[46, 47, 56-58] However, BM-APC and blood vessel endothelial cells can cross-present exogenously synthesised proteins on MHC class I molecules,[59-61] making them the most likely candidates to be presenting myelin peptides.[1] In addition, although not constitutively expressing MHC class I, oligodendrocytes can express MHC in response to IFN-gamma[56, 58, 62, 63] and therefore CD8$^+$ T cells may be able to target oligodendrocytes once inflammation begins.[1]

In support of this, upregulated MHC class I antigens on neurons, axons, astrocytes and oligodendrocytes in acute, chronic, active MS and inactive MS lesions have been shown to correlate with disease severity and lesion activity making these cells potential targets for pathogenic $CD8^+$ T cells.[56] In addition, $CD8^+$ T cells interact with antigen-presenting cells (APCs) at the margin of chronic and active lesions[64] and acute axonal injury has been correlated with the number of $CD8^+$ T cells and macrophages present.[65, 66] $CD8^+$ T cells have also been shown to directly damage CNS target cells,[67-72] including axonal transection,[69] which has been observed in MS lesions.[73] Damage may also occur through collateral bystander damage.[74] $CD8^+$ T cells are able to exert their cytotoxic effects against neuronal cells, with levels of granzyme A and B increased in the CSF during acute relapse[75] and granzyme B expressing $CD8^+$ T cells found in close proximity or attached to oligodendrocytes or demyelinated axons.[1] In contrast to MHCI expression, only microglia when stimulated *in vitro* express MHC class II molecules perhaps making them unlikely to be the sole driver of disease.[76]

1.10 T-cell trafficking into the central nervous system

In order for $CD8^+$ T cells to exert their effect in the CNS, they need to be able to traffic from the blood into the CNS. As previously discussed, this may occur through three possible routes. These include trafficking from blood to the CSF across the choroid plexus, from blood to the subarachnoid space through meningeal vessels, and from blood to parenchymal perivascular spaces.[25, 77]

It has recently been shown that T cell trafficking may be influenced by the levels of TWIK-related potassium channel-1 (TREK1), with downregulation of TREK1 causing increased migration of immune cells and upregulation blocking it.[78] The α-4 integrin of VLA-4 has also been shown to be involved in the recruitment and passage of $CD8^+$ T cells across the BBB,[79] with its relevance demonstrated by the effectiveness of natalizumab, a monoclonal antibody used in the treatment of MS. Natalizumab targets the α-4 integrin and thus prevents cell migration.[80] A recent study has also highlighted a role for P-glycoprotein, a transporter that influences $CD8^+$ T cell migration across the BBB by regulating endothelial C-C chemokine ligand 2 (CCL2), a chemokine involved in cell migration.[81] Mice lacking P-glycoprotein or CCL2 showed significantly reduced $CD8^+$ migration into the brain.[81, 82] Melanoma cell adhesion molecule (MCAM) has

been shown to be expressed by effector $CD8^+$ T cells and is upregulated during MS relapses. Crucially, blockade of MCAM restricts the transmigration of $CD8^+$ T cells across the BBB *in vitro* and reduces neurological deficits *in vivo* in different experimental autoimmune encephalomyelitis (EAE) models.[83] Junctional adhesion molecule-like (JAML) has also recently been demonstrated to be upregulated in MS patients at the BBB with monocytes and $CD8^+$ T cells with migratory capacity compromised when JAML was blocked.[84] In addition, under inflammatory conditions, $CD8^+$ T cells have been shown to express vascular endothelial growth factor (VEGF), which promotes BBB permeability.[85] Recently, α4β1-integrin has been shown to be involved in $CD8^+$ T cell interaction with BBB endothelium[86], and MHCI expression on BBB endothelial cells has also been shown to be important for $CD8^+$ T cell trafficking in the CNS.[59] Once migrated across the BBB into the CNS[87] $CD8^+$ T cells follow a reticular system of fibres, which is induced by inflammation to guide trafficking.[88] $CD8^+$ T cells have also been shown to be able to induce disruption of the BBB.[89]

1.11 CSF-resident T cells

Whole CSF from healthy individuals contains between 175,000 and 500,000 leukocytes - approximately 1,000 to 3,000 per ml.[50, 90] Although lymphocytes predominate, erythrocytes, monocytes and granulocytes are also present to a lesser extent.[90] Similarly, T cells are the most predominant cell type found in the CSF of patients with MS, other inflammatory and non-inflammatory neurological diseases (NIND) and in healthy controls.[91] In particular, $CD4^+$ T cells outnumber $CD8^+$ T cells[91, 92] with an increased CSF CD4/CD8 ratio seen in MS and inflammatory neurological diseases compared with non-inflammatory disease[93, 94] and a significant increase of $CD4^+$ T cells in CSF compared with controls.[95] The immunophenotype of these CSF-resident cells in MS has been further characterised in several studies although results are inconsistent and the difficulty in obtaining CSF from healthy volunteers in some cases makes interpretation difficult.

Expression of CCR7, a chemokine receptor that helps recruit T cells to the lymphoid system, alongside the naïve cell marker CD45RA helps to identify naïve ($CD45RA^+CCR7^+$), central memory (CD45RA-$CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and effector memory-RA ($CD45RA^+CCR7^-$) T cells.[93] CD45RO can also be used instead of

CD45RA.[96] In previous studies, effector memory $CD4^+$ and $CD8^+$ T cells have been demonstrated to be the predominant CSF-resident population in MS in some studies[79, 93, 97] although memory, and specifically central memory T cells were the dominant population in others.[50, 91, 98-102] Central memory T cell populations have also been shown to be the predominant T cell population in control populations.[50, 98, 99, 103]

Interestingly, although effector memory or central memory T cells have been shown to be enriched in MS CSF, this seems not to be exclusive to this group of patients. For example, Mullen et al demonstrated that both patients with MS and patients with other inflammatory CNS disorders had a higher percentage of effector memory T cells in the CSF compared with non-inflammatory controls.[93]

Giunti et al demonstrated a similar observation albeit with central memory cells being increased in the CSF of patients with MS and other inflammatory neurological diseases.[98] Perhaps more strikingly, Kivisakk et al showed an enrichment of central memory $CD4^+$ and $CD8^+$ T cells in the CSF of patients with non-inflammatory neurological disorders with no difference in MS $CD4^+$ T cells when compared with NIND.[50, 99] Similarly in a relatively large study by de Graaf et al on patients attending for routine surgery, a predominance of central memory T cells was observed in CSF-resident $CD4^+$ and $CD8^+$ T cells.[103] Svenningsson et al[91] did not show any differences between MS patients, other neurological disorders (OND) or healthy volunteers, where the majority of CSF-resident T cells were shown to be of memory phenotype ($CD45RO^+$). Other studies have shown differences between MS CSF and controls[97, 101, 102] or been hampered by the lack of control CSF.[79, 100] In another recent study investigating the phenotype, function and reactivity between peripheral blood, CSF, normal appearing white matter (NAWM) and white matter lesions (WML) in 27 patients with MS,[104] central memory T cells predominated in the CSF and effector memory T cells were enriched in the NAWM and WML. Where no differences have observed between MS patients and controls, it has been suggested that this is consistent with general CNS immune surveillance.[91]

Other T cell subtypes have also been studied in MS, with $CD4^+$ and $CD8^+$ Tregs increased[105, 106] and decreased[107] respectively when compared with peripheral blood,

with $CD8^+$ Tregs decreased during relapse.[108] In addition, regulatory $CD4^+CD25^+$ CSF-resident T cells have been shown to be increased in MS patients compared with controls[109] and can increase during relapse.[110]

Interestingly, a recent study looking at 14 different immune cell subtypes in blood and CSF in a variety of different inflammatory and non-inflammatory neurological disorders concluded that there is a poor correlation between blood and CSF immune cells and therefore inferences about disease pathogenesis cannot simply be made by studying the peripheral compartment.[111]

Markers of T cell migration have also been studied in MS. Expression of CCR2 on $CD4^+$ and $CD8^+$ T cells has been shown to be significantly increased in CSF from MS patients compared with inflammatory neurological disease controls.[112] In addition, CCR2 and CCR5 have been shown to enriched in the CSF compared with blood in patients with MS.[113] CSF $CD4^+$ and $CD8^+$ T cells have been shown to express higher levels of CCR5 and CXCR3 as compared with blood in patients with MS[114, 115] although the percentage of CSF $CD8^+$ CXCR3 cells has been demonstrated to be decreased during relapse.[116] Magnetic resonance imaging (MRI) disease activity has also been shown to be associated with an increase in CXCR3 positive CSF-resident T cells.[117] Intercellular adhesion molecule (ICAM)-1 and 3 have also been shown to be increased and decreased respectively in CSF-resident T cells in MS patients during remission compared with relapses.[118, 119] Lymphocyte function-associated antigen 1 (LFA-1) has also been shown to be enriched in CSF-resident T cells compared with blood in patients with MS.[91] The lack of control CSF in many of these studies however, limits the significance of these findings.

1.12 Clonal expansion of $CD8^+$ T cells

There is a growing body of evidence that $CD8^+$ T cells demonstrate clonal expansion in different tissue compartments in patients with MS. In an early study, Oksenberg et al demonstrated restricted expression of T cell TCRVα gene expression in brain lesions from 3 patients with MS, not observed in control brains.[120] Further to this, oligoclonal T cell clones were observed in the CSF and blood of patients with MS, which in some

cases were identical.[121] Other early studies demonstrated a bias for particular TCRVβ gene rearrangements in MS patients.[122, 123]

Babbe et al subsequently demonstrated oligoclonal repertoires of $CD8^+$ T cells (not observed in the $CD4^+$ compartment) from single cell analysis of brain lesions in 2 patients with MS. Interestingly, the same clonal expansions were seen in the blood at two separate time points in one patient.[51] In a follow-up study, the same clonal expansions seen in the brain were also observed in the CSF and blood, with one sample taken 7 years after the original brain biopsy.[54] Gestri et al also found oligoclonal T cell expansions in MS and other neurological disorders.[124] Muraro et al analysed TCRVβ usage in blood and found that expansions of TCRVβ genes in MS patients were significantly more frequent than in controls, were predominantly oligoclonal and were significantly correlated with inflammatory disease activity detected by MRI.[125] Matsumoto et al demonstrated TCRVβ expansions in blood compared with controls and also in CSF, although no control CSF was available.[126]

Complementarity determining region 3 length distribution (CDR3-LD) alteration has also been shown to be significantly higher in MS patients compared with controls and correlates with lesion activity on MRI.[127, 128] A change in the expression of TCRVβ has also been shown to be different when taken during relapse or remission.[129] Jacobsen et al, studying TCRVβ usage in blood and CSF from 36 MS patients also demonstrated a skewing of the $CD8^+$ CSF repertoire in MS patients although no difference was seen in the peripheral blood repertoire between patients and controls. Of note, no control CSF was available in this study for comparison.[130] A further study of 4 MS brains has demonstrated identical T cell clones detected in separate brain regions including normal appearing white matter and were unique for each patient.[52] CDR3 regions also contained silent mutations suggesting that these clones had responded in response to a particular antigen. In a more recent study of TCRVβ clonality in blood, CSF and brain from 3 patients with MS, $CD8^+$ T cells clones were shown to exhibit strong sharing between the 3 compartments, especially between the CSF and brain lesions.[53] Again, control samples of blood only were available for comparison.

Although these studies make a convincing case for $CD8^+$ T cell clonal expansion being pathogenic, other studies perhaps suggest caution in over interpreting these data. Early studies demonstrated no oligoclonal expansions in CSF-resident T cells in 2 MS patients,[131] and a polyclonal repertoire seen in active MS plaques.[132] In addition, although TCRVβ usage was shown to be skewed in blood, this was not observed in CSF or brain in another study.[133] Gran et al also demonstrated TCRVβ skewing that was present in MS patients and controls, with MS TCRVβ expansion returning to normal when analysed at a second time point.[134] In a separate study, the TCR Vβ5-JB and TCR Vβ17-JB repertoire showed a less diverse pattern in the CSF samples compared with blood not just in MS but also in patients with other neurological diseases.[135] In an interesting twin study of blood, a Gaussian distribution was observed in $CD4^+$ T cells with widely skewed TCR spectratypes in the $CD8^+$ T cell population. However, no correlation was found between oligoclonality and disease, with sequencing revealing shared TCRs between intra- and inter-pair twin members. The authors suggest that this may be a 'MS predisposing trait'.[136] Clonal dominance has also been shown within PLP-specific $CD8^+$ T cells in MS, although clonal dominance in MBP-specific $CD8^+$ T cells was only demonstrated in healthy controls, not seen in MS.[137] Another recent study using deep sequencing technology demonstrated a significantly higher frequency of clonal expansions in MS blood and CSF compared with controls, although cells were not sorted into $CD4^+$ and $CD8^+$ populations.[138]

Although these studies suggest that $CD8^+$ T cell clonal expansion is pathogenic, further evidence is required before this is definitive. In particular, the main limitations of all the studies performed on $CD8^+$ T cell oligoclonality is either the lack of controls entirely or if present, the lack of access to CSF and brain samples to compare the $CD8^+$ T cell repertoire.[51, 53, 54, 127, 130] It is also of note that $CD8^+$ T cell clonal expansions are thought to be a common feature of the normal human T cell repertoire,[139] and may be important for CNS immune surveillance.[52] $CD8^+$ T cell clonal expansion also occurs with increasing age[140], in particular in response to chronic cytomegalovirus infection.[141] Clearly this area needs further exploration to understand the relevance of clonal T cell expansions in MS and if pathogenic then it will be important to identify the antigenic target of these cells in order to develop targeted therapeutics.

1.13 Antigenic targets of CD8$^+$ T cells *in vitro*

Despite candidate proteins being identified *in vitro*, the antigenic trigger and pathogenic target of CNS infiltrating CD8$^+$ T cells remains unknown. Several candidate target proteins have been identified with autoreactive CD8$^+$ T cells being shown to be induced by myelin derived peptides; myelin-basic protein (MBP),[142] proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG),[63, 143] glial fibrillary acidic protein (GFAP)[144] and transaldolase (TAL) a peptide expressed in oligodendrocytes.[145] Furthermore, autoreactive CD8$^+$ T cells induced by PLP have also been shown to cross-react with an environmental organism[146] and a viral infection has been shown to activate CD8$^+$ T cells, whose TCRs are also capable of recognising MBP.[32] Ji et al demonstrated that the expression of dual TCRs allowed activation by a viral antigen and subsequent recognition of MBP.[32] Autoreactive CD8$^+$ T cells specific for apoptotic epitopes (apoptotic T cells) have also been shown to be present at significantly higher frequencies in the blood and CSF of patients with MS, with a strong potential to produce IFN-γ or IL-17.[147] Perhaps mitigating against a myelin antigen being the cellular target is that the frequency of myelin-reactive T cells has been shown to be the same in MS patients and controls,[143] although other studies have shown an increased number of these cells which are of an activated/memory phenotype.[148] A recent study, albeit with small patient numbers, did not demonstrate any substantial CD8$^+$ T cell activity to candidate MS antigens.[149]

In addition to the antigenic target of CD8$^+$ T cells in MS, there is debate about whether a pathogenic trigger exists and if so, what it is. Epstein-Barr virus[150, 151] and human herpes virus-6 (HHV-6)[152] have been suggested as possible candidates, but no definitive link has as yet been proven.

With regard to EBV, in a recent review of systematic reviews, a biomarker of EBV infection (anti-EBV nuclear antigen-1 (EBNA) IgG seropositivity) and a history of infectious mononucleosis (in addition to smoking) showed the strongest consistent evidence of association of developing MS.[153] Interestingly, although EBV infection is common in healthy controls (85-95%), EBV seronegativity in MS is rare.[154] MS risk has also been shown to increase with elevated serum anti-EBNA titres.[155] With regard to CSF, studies have mostly shown higher levels of CSF antibodies that react to EBV

antigens compared with controls,[156-159] although these findings are not universal.[160] The presence of EBV DNA itself in CSF has only been demonstrated in one MS patient.[161, 162]

An increased frequency of EBV-reactive $CD8^+$ T cells has been observed in MS patients[163, 164], and in clinically isolated syndrome (CIS)[165-167]. In contrast, other studies have demonstrated no increase in the frequency of EBV-reactive $CD8^+$ T cells in either CIS or MS.[168, 169] The $CD8^+$ T cell response to EBV has been shown to be dysregulated, with a lower response seen in patients with MS.[164, 170, 171] The presence of EBV in MS brains is controversial with its presence[172], and absence both being reported.[162, 173, 174] A recent study has also demonstrated brisk white matter lesion-derived T cell reactivity (mainly $CD8^+$) towards autologous EBV infected B cells.[104]

1.14 $CD8^+$ T cell driven animal models of MS

Multiple animal models for MS exist but no single model demonstrates the inflammatory mechanisms and neurodegeneration seen in MS in their entirety.[175] Experimental autoimmune encephalomyelitis mediated by class II-restricted, MBP-specific $CD4^+$ T cells is the most widely used, although the phenotype is less diverse than that seen in humans.[176] It has only been in the last 15 years that $CD8^+$ T cell driven mouse models for MS have been developed.

Rivera-Quinones et al were the first to demonstrate the importance of $CD8^+$ T cells in causing demyelination, with MHC class I deficient mice retaining neurological function in a Theiler's murine encephalomyelitis virus (TMEV) model of MS.[177] An anti-CD8 monoclonal antibody in this mouse model also resulted in less meningeal inflammation and fewer demyelinating lesions in the spinal cord.[178] In 2001, Sun et al induced EAE with MOG-specific $CD8^+$ T cells, which was more severe and permanent than with MOG injection alone. Interestingly, these $CD8^+$ T cells were not pathogenic in the absence of beta-2 microglobulin, a component of MHC class I molecules.[179] These findings were later confirmed with $CD8^+$ T cells shown to produce IFN- γ.[180] In the same year as Sun et al, a different group similarly induced EAE but with MBP-specific $CD8^+$ T cells with clinical features more similar to MS than that seen with $CD4^+$ T cell induced EAE. In this study, co-administration with anti-IFN γ abrogated disease severity.[181]

Since these initial investigations, subsequent studies have focused on $CD8^+$ T cell responses to epitopes expressed by oligodendrocytes. Saxena et al produced a transgenic mouse model with haemagglutinin (HA) expressed exclusively by oligodendrocytes. Transfer of pre-activated HA-specific $CD8^+$ T cells led to inflammatory lesions in the optic nerve, spinal cord and brain with focal loss of oligodendrocytes, demyelination and microglia activation similar to that observed in MS pathology. Again in this study activated $CD8^+$ T cells produced IFN- γ in addition to granzyme B.[182] In a similar model with ovalbumin (OVA) expressed by oligodendrocytes, OVA-specific $CD8^+$ T cells developed spontaneous EAE with demyelination and infiltrated lesions, a response exacerbated by the addition of IFN-γ.[183] Blockade of the OVA-peptide/MHCI complex prevented disease development.[184] Humanised mouse models, including that by Friese et al discussed in section 1.7 have also been developed, further implicating a role for $CD8^+$ T cells in disease pathogenesis.[44, 185] Experiments in TMEV have also demonstrated that virus specific $CD8^+$ T cells secreting perforin can induce CNS vascular permeability.[186] $CD8^+$ T cells can also cause demyelination and axonal damage in a living brain tissue system.[74] $CD4^+$ T cell mediated CNS autoimmunity has also been shown to lead to determinant spreading to myelin-specific $CD8^+$ T cells.[187]

1.15 IL-17 producing $CD8^+$ T cells in MS

Several studies have suggested a role for IL-17 producing $CD8^+$ T cells in MS pathogenesis. In a study by Tzartos et al on brain tissue, both $CD4^+$ and $CD8^+$ T cells were shown to express IL-17 in active and chronic active lesions, with expression being lower in chronic inactive lesions and in NAWM.[188] IL-17 producing $CD8^+$ T cells have also been shown to be present at higher percentages in the blood of MS patients during relapse than controls[189] and are required for Th17 induction of EAE in mice.[190]

A distinct subset of $CD8^+$ T cells in humans that produce IL-17 has been discovered to express CD161.[191] In MS patients compared with controls, an enrichment of $CD161^+CD8^+$ T cells has been shown and these cells are also detectable in brain immune infiltrates, capable of producing IFN-γ.[192] $CD161^+CD8^+$ T cells are dominated in peripheral blood by mucosal-associated invariant T (MAIT) cells,[193] which are responsible for elimination of microbes through the MHC-class I related protein I

(MR1)[193] and have been shown to be associated with some human autoimmune diseases.[194] The role of MAIT cells in MS is still unclear[195, 196] but they have been observed in MS brain lesions.[197, 198] Other IL-17 producing $CD8^+$ T cell subsets have also been identified such as those expressing MCAM.[199] Although the significance to clinical disease of these $CD8^+$ T cell subsets is still to be determined, targeting IL-17 producing $CD8^+$ T cells may be a candidate for future therapeutics.[200]

1.16 Regulatory $CD8^+$ T cells ($CD8^+$ Tregs)

The main role of regulatory T cells ($CD8^+$ Tregs) is to maintain immunological tolerance against self-antigens.[201] $CD8^+$ Tregs have stimulated considerable interest in a wide range of autoimmune disorders, and evidence in MS suggests that these cell populations may be important in regulating disease pathogenesis.[202, 203]

The first animal models seemed to show a regulatory function for $CD8^+$ T cells,[204-206] with the mouse protein Qa-1 (HLA-E in humans)[207, 208] and $CD8^+CD28^-$ T cells playing a role in disease suppression.[209] Interaction of Qa-1 with CD94/NKG2A on $CD8^+$ T cells downregulates their suppressive effects with specific disruption of this interaction enhancing suppression and preventing EAE induction.[210] In support of this, a HLA-E restricted $CD8^+$ T cell population expressing an increased level of CD94/NKG2A has been isolated from MS patients during exacerbations[211] and HLA-E expression is upregulated in T and B cells from MS patients. In addition, significantly increased HLA-E expression has also been observed in white matter MS lesions.[212] Interestingly, in Qa-1 deficient mice, $CD4^+$ T cells were resistant to the effects of $CD8^+$ T cell suppressor activity and increased susceptibility to EAE.[207] Further evidence for $CD8^+$ Tregs has been demonstrated by MOG-specific $CD8^+$ T cells transferred from C57BL/6 mice, regulating disease via suppression/killing of $CD4^+$ T cells.[213] Several new regulatory subsets of $CD8^+$ T cells have also been discovered in recent years, including $CD8^+/LAP^+$ cells[214] and $CD8^+/CD122^+$ cells[215] from MS animal studies and $CD8^+/CD161^-/CD56^+$ T cells in vitro.[216] The importance of $CD8^+$ Tregs has also been demonstrated in clinical studies.

$CD8^+$ Tregs have been found in patients with MS, in whom HLA-E-restricted $CD8^+$ T cells display a less regulatory phenotype than those in healthy individuals.[217] A

deficiency of $CD8^+$ Tregs has also been observed during clinical relapse with an increase in numbers during recovery.[218, 219] Similarly, neuroantigen-specific $CD8^+$ T cells may have less suppressive capacity during relapses.[218] $CD8^+$ Tregs expressing HLA-G have also been recognised, with reduced levels associated with post-partum relapses.[220, 221] MRI supports the evidence for a role for $CD8^+$ Tregs with increased radiological lesion load negatively correlated with the number of $CD8^+$ T cells in peripheral blood in MS patients.[222] $CD8^+$ Treg regulatory function is mediated by direct killing of activated $CD4^+$ T cells or by secretion of immunosuppressive cytokines such as IL-10 and transforming growth factor-β.[34] Interestingly, it has been suggested that Tregs may not be important for CNS protection during homeostasis but once tolerance is broken are required to re-establish homeostasis in the CNS.[16]

1.17 The role of $CD4^+$ T cells in MS pathogenesis

$CD4^+$ helper T cells interact with MHC class II molecules on antigen presenting cells and exert their effect by the release of cytokines, which act on target cells.[12] Although this thesis will focus primarily on $CD8^+$ T cells, it is worth noting the evidence for the role of $CD4^+$ T cells in MS disease pathogenesis. As discussed in section 1.7, the genetic association with DRB1*1501 and other immune system genes supports the role of $CD4^+$ T cells in MS pathogenesis.[42]

The differentiation of naïve $CD4^+$ T cells is determined by the exposure to different cytokines secreted by dendritic cells, which are APCs as well as cells of the innate immune system.[223] In the presence of IL-12, naïve $CD4^+$ T cells differentiate into IFN-γ secreting Th1 helper cells. If IL-23 predominates then IL-17 secreting Th17 cells predominate. When these cell types are activated in autoimmune disease, the production of pro-inflammatory effector cytokines is thought to be deleterious.[223]

The current understanding of the role of Th1 and Th17 cells relates to the pathological mechanisms discussed in section 1.5. Activation may occur in the periphery[223] with subsequent migration across the BBB into the CNS. In response to CNS antigens they are reactivated, which in turn induces an inflammatory response.[223] Pro-inflammatory cytokines induce macrophage and microglial activation, which leads to the production of other pro-inflammatory mediators. Then the production of oxygen and nitric oxide

radicals lead to the pathological hallmarks of MS – demyelination and axonal loss.[224, 225]

Both Th1 and Th17 cells have been shown to be present in the brain and plaques in MS patients[226, 227] and knocking out these genes in mice[228, 229] or administering anti IL-17 or IL-23 antibodies suppresses disease activity.[230, 231] In clinical studies, the number of Th17 cells have been found to be increased in the blood of MS patients[232] and during relapse compared to remission.[233] Similarly, the number of Th17 cells in the CSF has also been shown to be increased during relapse.[234] Clearly therefore, there is evidence that $CD4^+$ T cells have a central role in MS pathogenesis but further discussion of this is outside the remit of this thesis.

1.18 Evidence from therapeutics for a central role for $CD8^+$ T cells in MS

The majority of patients with MS present at onset with relapsing disease, with recurrent episodes of subacute clinical disability due to areas of inflammation and demyelination in clinically eloquent areas.[235] The main drug treatments or disease modifying therapies (DMTs) are therefore aimed at reducing inflammation and in turn the relapse rate in these patients. There are currently no neuroprotective therapies available. Understanding the mechanisms of action of disease modifying therapy demonstrates the importance of $CD8^+$ T cells to disease pathogenesis.

Interestingly, therapies aimed purely at $CD4^+$ T cells have not shown benefit in MS patients,[236-238] despite showing promise in animal models.[239] In contrast, those targeting both $CD4^+$ and $CD8^+$ T cells are effective.[36] For example, alemtuzumab, an anti-CD52 monoclonal antibody, which depletes both $CD4^+$ and $CD8^+$ T cells has been shown in clinical trials to be an effective treatment for MS[9, 240, 241] as has fingolimod, which prevents T cell migration[242] and natalizumab, a monoclonal antibody against VLA-4.[80] Although the mechanism of action of fingolimod, natalizumab and alemtuzumab are more direct to $CD8^+$ T cell function, the first-line therapies of interferon-β and glatiramer acetate affect $CD8^+$ T cells in more subtle ways and offer further insights into disease pathogenesis. The effect of different licensed therapeutics on $CD8^+$ T cells will be discussed in more detail below.

1.18.1 Interferon-β

Interferon-β (IFN-β) is an anti-viral treatment,[243] which has a modest effect on relapse prevention but a relatively small side-effect profile.[244] It is thought to modulate cytokine levels, affect the expression of MHCII molecules, and stabilize the BBB, thereby inhibiting transmigration of autoreactive T cells into the CNS. IFN-β is also thought to inhibit T cell activation and proliferation directly.[7] Zafranskaya et al demonstrated that treatment with IFN-β can restore normal levels of $CD45RO^+$ memory T-cells (both $CD4^+$ and $CD8^+$) in the peripheral blood of MS patients and reduce $CD45RO^+$ T cell reactivity towards MOG. IFN-β can also suppress the proliferation of $CD4^+$ and $CD8^+$ T-cells; and attenuate the production of IFN-γ.[7] This finding was confirmed in another study with IFN-β also being shown to expand numbers of $CD4^+$ and $CD8^+$ Tregs during the first year of IFN-β treatment.[245] In addition, the frequency of $CD8^+CD161^+$ T cells in patients treated with IFN-β have recently been shown to be reduced.[246] Therefore, this treatment has the ability to target both $CD4^+$ and $CD8^+$ T-cell reactivity, possibly by exerting a regulatory effect.

1.18.2 Glatiramer acetate

Glatiramer acetate (GLA) is a random copolymer of alanine, lysine, glutamic acid and tyrosine,[247] which along with IFN-β is used as a first line treatment in relapsing-remitting MS.[248] Similar to interferon-β, studies looking at the mechanism of action of this drug offer unique insights into MS pathogenesis and in particular a role for $CD8^+$ T cells. At a cellular level, the beneficial effect of GA on the course of MS is currently hypothesised to be in part due to a Th2 shift induced in GA-reactive $CD4^+$ T cells.[249] As well as this shift of $CD4^+$ T cell phenotype, $CD8^+$ T cell responses were found to be lower in untreated MS patients compared to those treated with GA but upregulation of $CD8^+$ T cell responses was seen post-treatment. $CD4^+$ T cell responses however, were downregulated suggesting that $CD8^+$ T cells may play a role in regulating the role of $CD4^+$ in disease pathogenesis, perhaps by direct cytotoxic killing.[250-252]

There is differing evidence as to whether GA effects the TCR population in MS patients with one study not demonstrating influence on the TCR repertoire in $CD8^+$ populations,[253] but with another demonstrating that an oligoclonal expansion does occur with upregulation of IL-4, IL-5, IL-10 and Transforming growth factor(TGF)-β in

the $CD8^+$ subset indicating regulatory potential. However, restoration of the pro-inflammatory cytokine, TNF-α was also observed.[254] Conversely, in a separate study, GA treatment suppressed the expression of TNF-α, IL-10 and IL-4.[247] As well as affecting T cell populations, GA has also been shown to attenuate the profile of cell-bound adhesion molecules in patients with MS. [255]

1.18.3 Other therapies

Alemtuzumab, an anti-CD52 humanised monoclonal antibody, has recently been licensed for the treatment of relapsing Multiple sclerosis in Europe.[256] The anti-CD52 effect of alemtuzumab results in rapid and profound depletion of circulating lymphocytes after initial infusion as a result of antibody-dependent cell-mediated cytotoxicity.[257] Although the exact mechanism of action remains unclear, it is thought to be related to remodelling of the immune system,[258] rather than immunodeficiency, a hypothesis supported first by the lack of disease activity in most patients despite normalizing levels of lymphocytes and by the relative lack of opportunistic infections seen in treated patients.[9, 240, 241] Although exhibiting a degree of individual variability, the rate of immune reconstitution is not thought to predict disease activity.[259] Clinical trials have demonstrated superior efficacy against an active comparator, with reduction in annualised relapse rates and sustained accumulation of disability at 3 years and sustained efficacy at 5 years.[9, 240, 241, 260]

Natalizumab is reserved for patients with highly active disease, experiencing frequent relapses and active inflammation on MRI. It is a monoclonal antibody targeted against VLA-4, preventing egress across the BBB.[80] Studies of CSF from patients treated with natalizumab show a reduction in both $CD4^+$ and $CD8^+$ T cells[261] with an increase in activated $CD4^+$ and $CD8^+$ T cells in peripheral blood.[262]

Methylprednisolone, either in oral or intravenous form is used to treat clinical relapses in patients with MS.[248] Aristimuño et al demonstrated that it causes a reduction in activated and effector memory T cells and an increase in naïve and regulatory T cells.[263]

Fingolimod, the first licensed oral disease modifying treatment in the United Kingdom for MS, available as a second line treatment,[264] causes internalisation of sphingosine-1 phosphate receptors, trapping T cells in lymph nodes and thus affecting T cell migration.[242] In addition to this mechanism, another separate effect of fingolimod on $CD8^+$ T cell function has been demonstrated, with inhibition of IFN-γ and granzyme B production in $CD8^+$ T cells.[265, 266] In patients taking fingolimod, both $CD4^+$ and $CD8^+$ T cell counts are reduced in the peripheral blood, with naïve and central memory T cells particularly affected.[267] The proportion of $CD8^+CCR7^-$ cells expressing the CCL2 receptor, CCR2 are also significantly reduced with more $CD27^-CD28^-$ (late effector) memory cells, which have less expression of CCR2 compared with early ($CD27^-CD28^+$) effector memory cells. Therefore, fingolimod treatment results in a subset of $CD8^+$ T cells with distinct functional migratory properties.[268] In addition to its anti-migratory effect fingolimod has also been shown to reduce the number of effector T cells producing IFN-γ either alone or in combination with IL-17 and to increase the number of TReg cells.[269] In a separate study by Kowarik et al, fingolimod increased the percentage of $CD8^+$ T cells compared to treatment naive patients in the CSF of MS patients, although more detailed phenotypes were not examined.[270]

Teriflunomide, a new oral treatment for relapsing MS has recently been licensed in the UK.[271] It acts as an inhibitor of dihydroorotate-dehydrogenase (DHODH), a mitochondrial enzyme involved in the de novo synthesis of pyrimidines, and which is particularly active in proliferating cells.[272] It appears therefore to selectively reduce the activity of proliferating T and B cells and has been shown to reduce relapse rates by approximately one third.[273-275] The development of teriflunomide again demonstrates that inhibition of both $CD4^+$ and $CD8^+$ T cells produces a clinical benefit in patients with MS and provides evidence for their role in disease pathogenesis.

Dimethylfumarate (DMF) is another newly licensed oral medication,[276] which acts by activating the nuclear factor (erythroid-derived 2)–like 2 (Nrf2) and subsequent upregulation of anti-oxidant target genes. This effect then leads to cytoprotection for neurons and astrocytes against oxidative stress. It may also be beneficial through increasing mitochondrial function.[277] Other effects of the drug include an observed reduction in circulating $CD4^+$ and $CD8^+$ T cells, although being more marked in the $CD8^+$

compartment.[278] In addition, DMF reduces the central and effector memory T cell populations[279, 280] in peripheral blood with a relative expansion of naïve cells.[280] Interestingly, similar to the effect of natalizumab, DMF has also been demonstrated to inhibit expression of α4 integrin on circulating lymphocytes.[281]

In addition to the established treatments in MS, vitamin D deficiency has been postulated to be involved in disease pathogenesis. Interestingly, addition of vitamin D to $CD8^+$ T cells of MS patients showed that these cells secreted less IFN-γ and TNF-α and more IL-5 and TGF-β suggesting a direct effect of vitamin D on $CD8^+$ T cells.[282] Autologous haematopoietic stem cell transplantation results in the persistent depletion of $CD8^+$ MAIT T cells, suggesting an important role in MS disease pathogenesis.[195]

1.19 Discussion and summary

There is mounting evidence that $CD8^+$ T cells play a role in the complex pathogenesis of MS. Initial results from genome wide association studies mainly suggest a protective role for $CD8^+$ T cells but subsequent evidence from neuropathological, *in vitro* and *in vivo* studies have demonstrated a pathological role as well as a regulatory role for $CD8^+$ T cells in MS. Firstly, clonal expansion of $CD8^+$ T cells has been shown in blood, CSF and brain of MS patients which has not been observed in the $CD4^+$ T cell compartment. Secondly, $CD8^+$ T cells outnumber $CD4^+$ T cells in white and grey matter lesions in MS. Thirdly, MHCI is expressed by various cells types in the CNS, which have been shown to interact with $CD8^+$ T cells resulting in axonal damage. In addition, studies investigating the effect of different MS therapies on $CD8^+$ T cell function add further weight to the argument for a role for $CD8^+$ T cells in the pathogenesis of MS.

It is clear that in the inflamed CNS of patients with MS, a complex milieu of cytotoxic and regulatory $CD8^+$ T cells, $CD4^+$ T cells, B cells and macrophages exists in addition to other inflammatory cells and cytokines. Further understanding of the roles and interactions of all these cells will be key to understanding the pathogenesis of MS. Future therapy may be aimed at $CD8^+$ T cells, either by upregulating regulatory T cells,[283] targeting pathogenic $CD8^+$ T cells,[284] targeting MHC class I/peptide complexes,[184] or with individualised therapy to epitope-specific T cells. In addition to

treating MS, removing select populations of T cells would also mitigate against the risk of infections associated with immunosuppressive therapy, such as progressive multifocal leucoencephalopathy (PML) associated with natalizumab.[285] The key aim is clearly to modulate the immune system to an extent that disease activity is controlled but so that adverse immunosuppressive events are also minimised.

Despite mounting evidence for the role of $CD8^+$ T cells, the antigenic trigger and target antigen in humans is unknown. It is hypothesised that $CD8^+$ T cells may be activated by a pathogen and then due to the promiscuous nature of the TCR cause autoimmunity by attacking a self-antigen.[286] This search is made difficult because of molecular mimicry, epitope spreading, bystander activation, and/or dual TCRs.[287] Interestingly, the frequency of autoreactive T cells to myelin antigens is still debated with some studies showing no difference to healthy controls and other studies suggesting a contrary view. It is postulated that differences in activation state may therefore account for MS disease risk.[288]

Further studies are clearly required to fully elucidate the role of $CD8^+$ T cells in MS. It is still unclear what activates $CD8^+$ T cells in the periphery, what their cellular interactions are and how they mediate damage *in situ*. It is clear however that they have a central role in disease pathogenesis and understanding their role may lead to future therapeutic drug developments.

1.20 Specific aims of this thesis

Aim 1: Clinical outcomes of MS patients treated with the anti-CD52, lymphocyte depleting monoclonal antibody, alemtuzumab.

The first aim of this thesis was to undertake a detailed analysis of clinical outcomes in MS patients treated with alemtuzumab. Although clinical trials performed over a 2-3 year period demonstrate positive clinical outcomes, 'real-world' observational studies are required to confirm the duration of this effect and highlight any potential adverse events. Because alemtuzumab is an anti-CD52, lymphocyte-depleting agent, beneficial long-term outcomes will help confirm the central role of $CD8^+$ and $CD4^+$ T cells in MS disease pathogenesis.

Aim 2: Perform an in-depth phenotypic analysis of T cell populations in the CSF of MS patients.

Recent advances have extended the boundaries of flow cytometric analysis through new developments in instrumentation and fluorochrome technology, enabling the simultaneous and independent measurement of up to 18 colour markers.[289, 290] Although previous studies have attempted to analyse the immunophenotype of $CD4^+$ and $CD8^+$ T cells of CSF-resident T cells, the results have been inconsistent. The second aim of this thesis therefore was to perform an in-depth phenotypic analysis of CSF-resident T cells in patients with MS. In addition, we aimed to perform a similar analysis in patients with idiopathic intracranial hypertension (IIH) and other neurological disease (OND) as controls.

Aim 3: Examine the CSF-resident T cell receptor (TCR) repertoire in MS patients and identify dominant TCRs.

Although clonal expansions of $CD8^+$ T cells has been observed in MS, the numbers of patients in these studies are small and control populations are either limited or absent altogether. I therefore aimed to analyse the TCR usage in CSF-resident T cells in MS using a strand-switch anchored RT-PCR approach that enables the quantitative characterization of TCR gene usage without bias.[291] In addition, cells sorted from IIH patients and OND were to be used as control populations.

Aim 4: To identify the pathogenic triggers and antigenic targets of dominant CSF-resident TCRs.

To identify the pathogenic triggers and antigenic targets of dominant CSF-resident TCRs I aimed to use two different strategies. The first strategy was to perform a TNFα capture assay to activate and sort EBV-specific T-cell populations for clonotypic analysis.[292] Any overlap between TCR usage in the CSF and the peripheral EBV-specific repertoire would then be determined. Secondly, I aimed to use combinatorial peptide library (CPL) screening[286, 293] technology to identify the peptide sequences recognized by dominant MHCI-restricted TCRs. Here, the TCR sequences would be built into a lentiviral construct and expressed on the surface of primary $CD8^+$ T-cells.

The main focus of this thesis was $CD8^+$ T cells because of the more limited literature

regarding the role of these cells in MS pathogenesis. However, results from $CD4^+$ T cells were also analysed in parallel.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Buffers and media

PSG medium: Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermofisher, Waltham, MA, USA) with 2mM L-glutamine (Invitrogen, Carlsbad, California, USA), 100 units/ml penicillin (Invitrogen, Carlsbad, California, USA) and 1% 100 µg/ml streptomycin solution (Invitrogen, Carlsbad, California, USA).

R10 medium: PSG medium containing 10% Foetal Bovine Serum (Invitrogen, Carlsbad, California, USA).

R2 medium: PSG medium containing 2% Foetal Bovine Serum (Invitrogen, Carlsbad, California, USA).

DMEM medium: Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermofisher, Waltham, MA, USA).

D10 medium: Dulbecco's Modified Eagle Medium with 2mM L-glutamine (Invitrogen, Carlsbad, California, USA), 100 units/ml penicillin (Invitrogen, Carlsbad, California, USA), 1% 100 µg/ml streptomycin solution (Invitrogen, Carlsbad, California, USA) and 10% Foetal Bovine Serum (Invitrogen, Carlsbad, California, USA).

Freezing medium: 90% foetal bovine serum with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK).

Cytotoxic T lymphocyte (CTL) medium: PSG (22.5 ml), Click's medium (22.5 ml, Sigma-Aldrich, Gillingham, UK) with 10% foetal bovine serum.

LB medium: 25 capsules of LB-medium (10g Tryptone, 5g Yeast Extract, 10g NaCl, MP Biomedicals, Santa Ana, California, USA) was put in 1 litre water and autoclaved at 121^{o}C.

MACS buffer: Dulbecco's Phosphate buffered saline (dPBS, Sigma-Aldrich, Gillingham, UK) with 0.5% Bovine Serum Albumin (Sigma-Aldrich, Gillingham, UK) and 2 mM EDTA (Sigma-Aldrich, Gillingham, UK).

T cell medium: PSG with 10% heat inactivated AB serum (Welsh Blood Service, Pontyclun, UK), IL-15 (25 ng/ml, PeproTech, Rocky Hill, NJ, USA), IL-2 (200 iu/ml, Pharmacy department, University Hospital of Wales, Cardiff, UK).

Agarose gel: 1% agarose gel was made by combining 1X Tris Acetate-EDTA (TAE) buffer (Sigma-Aldrich, Gillingham, UK) with 1g of UltraPure agarose (Thermofisher, Waltham, MA, USA) per 100ml buffer (50ml/gel). The gel was then microwaved at 800W for approximately 1 minute until the agarose had dissolved and then cooled on ice for approximately 2 minutes. The agarose gel was then poured into a gel tank and allowed to cool. Solidified gel was covered in 1X TAE buffer.

2.2 Reagents

2.2.1 Cell lines

C1R-A2 lines were courtesy of Professor Linda Wooldridge (University of Bristol) and T2-B7/T2-A2 from Professor Scott Burrows (University of Queensland). 293T cells were used courtesy of Sian Llewelyn-Lacey (Cardiff University).

2.2.2 Human antibodies used for flow cytometry

Antibodies used are outlined in table 2.1.

Specificity	Flourochrome	Volume used (in 50 µL PBS)	Supplier
CD14	V500	1.5 µL	BD, Oxford, UK
CD19	V500	1.5 µL	BD
CD3	APC-H7	4 µL	BD
CD8	BV711	1 µL	Biolegend, San Diego, USA
CD4	PECy5.5	0.5µL	Thermofisher, Waltham, USA
CD27	Qdot605	0.5µL	Thermofisher
CD45RA	ECD	4 µL	Beckman Coulter, Brea, USA
CD57	FITC	1µL	BD
CD95	PE-Cy5	3µL	Biolegend
CCR7	PE-Cy7	3µL	BD
CD127	BV421	3µL	Biolegend
CD49d	APC	3µL	Biolegend
PD-1/CD279	PE	5µL	BD
Pan γδ	PE	0.5µL	BD
TNFα	PECy7	15µL	BD
CD28	N/A	3µL	BD
CD49d	N/A	3µL	BD
Rat CD2	PE	2µL	Biolegend

Table 2.1 Antibodies used for flow cytometry of CSF.

2.2.3 Primers

Primers used are outlined in table 2.2 below;

Primer name	Sequence
SMARTer oligo	5’- AAGCAGTGGTATCAACGCAGAGTACXXXXX
5’ CDS	5’-$(T)_{25}$VN-3’
Universal Primer Mix Long Universal Primer Short Universal Primer	 5'–CTAATACgACTCACTATAgggCAAgCAgTg- gTATCAACgCAgAgT–3' 5'–CTAATACgACTCACTATAgggC–3'
MBC2	5’-tgcttctgatggctcaaacacagcgacct-3’
MAC2	5’-GGAACTTTCTGGGCTGGGGAAGAAGGTGTCTTCTGG-3’
M13F	TTT TCC CAG TCA CGA C
M13R	CAG GAA ACA GCT ATG AC

Table 2.2 Primers used for clonotyping of sorted CSF-resident T cells.

2.3 Methods

2.3.1 Patient selection and ethics

Patients were recruited from the neurology day unit at the University Hospital of Wales (UHW), Cardiff. Patients attending for diagnostic lumbar puncture for investigation of Multiple sclerosis were consented, in addition to patients being investigated or treated for idiopathic intracranial hypertension and other neurological diseases. Consent was obtained under pre-existing ethics agreements - An epidemiological study of Multiple sclerosis in South East Wales (05/WSE03/111) and Welsh Neuroscience Research Tissue Bank (15/WA/0073). In most cases patients were attending for diagnostic investigations and subsequent clinical case note review was performed to confirm eventual diagnoses.

2.3.2 Sample collection

Lumbar puncture was performed in the left lateral position under aseptic technique. 10 ml cerebrospinal fluid (CSF) was obtained from the L3/4 or L4/5 intervertebral space and collected into either a 30 ml universal container or 15 ml falcon tube. 20 ml peripheral blood was collected immediately following the procedure.

2.3.3 Isolation of peripheral blood mononuclear cells (PBMC) and storage

Ten millilitres of peripheral blood was carefully layered on top of two 50 ml falcon tubes containing 20 ml of histopaque (Sigma-Aldrich, Gillingham, UK) and spun at 2000 rpm for 20 minutes with the break off. The buffy coat was removed using a pastette pipette, washed with 50 ml PSG and spun at 1500 rpm for 10 minutes. The cell pellet was then resuspended in 50 ml PSG and centrifuged at 1500 rpm for 6 minutes. The resulting cell pellet was resuspended and cells counted manually with a haemocytometer.

2.3.4 Freezing of PBMCs and cell lines

If not used immediately (for Epstein Barr Virus (EBV) B95.8 lymphoblastoid cell line generation, see section 2.3.8.1) PBMCs were spun again (1500 rpm for 5 minutes),

resuspended in freezing media and aliqouted at 10 x 10^6 cells/ml. 1ml cryovials were frozen slowly in Mr. Frosty (Thermofisher, Waltham, MA, USA) in a -80°C freezer, then subsequently transferred to liquid nitrogen for long-term storage.

2.3.5 Thawing of PBMCs and cell lines

Frozen PBMCs were removed from liquid nitrogen and placed in a water bath at 37°C until thawed. Cells were then added to 9ml PSG and centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and cells resuspended in 1ml PSG and counted.

2.3.6 Immunophenotyping of CSF-resident T cells

2.3.6.1 CSF preparation

Within 1 hour of collection, CSF was transferred to the laboratory and centrifuged at 2000 rpm for 10 minutes. CSF supernatant was collected and 300 µL aliquots stored at -80°C. Although the cell pellet was never visible, 50µL PBS was added and cell resuspension performed by pipetting. This was subsequently transferred to a FACS tube with a pastette pipette.

2.3.6.2 Antibody staining of CSF

With the light in the CATII safety cabinet turned off, Aqua (Thermofisher, Waltham, MA, USA) was reconstituted with 50µL DMSO to a new vial, and then diluted 1 in 40 with PBS. 8µL of the diluted aqua solution was then added to the cell suspension and incubated for 10 minutes at room temperature. Following this time, the following antibody panel was added and the cell suspension incubated at 4°C for 20 minutes; CD14 V500, CD19 V500, CD3 APC-H7, CD8 BV711, CD4 PECy5.5, CD27 Qdot605, CD45RA ECD, CD57 FITC, CD95 PE-Cy5, CCR7 PE-Cy7, CD127 BV421, CD49d APC and PD-1/CD279 PE. For some samples, PD-1 PE was substituted for pan γδ PE. Of note, the CSF stain for patients LC20552 and LJ20639 used different antibodies for CD127, CD95 and CD27. For LC20552 – PE, APC and PECy5 respectively; for LJ20639 - Pacific blue, PE and PECy5 respectively. In addition, CD49d was not used for these samples. (For a full list of antibodies used for each patient, see Appendix, Section 8.1).

During cell incubation, compensations were made. Firstly, 8 drops of anti-mouse Ig κ/negative control compensation particles (BD, Oxford, UK) were added to 800µL PBS in a FACS tube. 50µL of this solution was then added to each individual compensation FACS tube. The same amount of corresponding antibody used in the cell stain was then added to the relevant tube. Antibodies were left to stain for 10 minutes at room temperature when 150µL PBS was then added. For the CCR7 PE-Cy7 compensation tube, 50µL of anti-rat Ig κ/negative control compensation particles (BD, Oxford, UK) was used instead.

Following 20 minutes incubation at 4^{o}C, 1ml PBS was added to each cell suspension tube and then centrifuged for 2 minutes at 2000 rpm. Supernatant was subsequently discarded with the tube then blotted onto paper. Cells or beads were then resuspended in 100µL PBS and transferred for cell sorting.

2.3.6.3 Flow cytometry and cell sorting

$CD4^+$ and $CD8^+$ T cells were sorted on a BD FACSAria II (BD, Oxford, UK) into RNAlater (Ambion, Thermofisher, Waltham, MA, USA). After sorting, cells were centrifuged at 13,000 rpm for 2 minutes then stored at -80^{o}C until required.

2.3.7 Clonotyping of sorted CSF-resident T cells

2.3.7.1 mRNA extraction of sorted $CD4^+$ and $CD8^+$ T cells from CSF

mRNA isolation was performed using Miltenyi Biotec's µMACS mRNA Isolation kit (Miltenyi Biotec, Bisley, UK) in a dedicated RNA laboratory. The worktop was cleaned with bleach before use and the worktop, pipettes, pipette tip boxes, magnet, sample rack and collection boxes cleaned with RNAase away (Sigma-Aldrich, Gillingham, UK) before use. When ready for use, sorted cell samples were removed from -80^{o}C storage and left to thaw on the bench. When almost fully defrosted, vials were transferred to a centrifuge pre-chilled to 4^{o}C and spun at 15000 g for 7 min. During centrifugation, MACS columns were placed in the magnet and lysateclear columns put in plastic centrifuge tubes. Following centrifugation, RNAlater was removed, 900 µL lysis/binding buffer added and the vial vortexed vigorously for 1 minute. Samples were then spun for 2 minutes at 13000 rpm to remove the foam caused by lysing. Lysate was then

added on top of the lysate clear column and centrifuged for 3 minutes at 13000 rpm. 50 µl Oligo(dT) beads were then added to the cleared lysate and mixed by pipetting. MACS columns were then rinsed with 100 µl lysis/binding buffer and allowed to run through. Lysate (950 µL total) was then applied onto the column and left to run through. The column was then washed with 2x 200 µl lysis/binding buffer followed by 4x 100 µl wash buffer. Elution buffer was heated to 72^{o}C and 27 µl of hot buffer added directly to the column to remove wash buffer from the column. RNA was subsequently eluted with 30 µl hot elution buffer into a 1.5 ml screw-topped microtube.

2.3.7.2 cDNA synthesis

cDNA was synthesised using SMARTer^{TM} RACE cDNA Amplification kit (Takara Clontech, Saint-Germain-en-Laye, France). Reagents were stored at -20^{o}C and defrosted before use. 1 µl 5'CDS (oligo dT primer) was added to a 1.5 ml screw-topped microtube and 6 µl mRNA added. The remaining mRNA was then stored at -20^{o}C and SMARTer oligo thawed out, after storage at -20^{o}C. Samples were then heated to 72^{o}C for 3 minutes, then 42^{o}C for 2 minutes. The following reagents were then added to each sample; SMARTer oligo (1µl), 5xRT Buffer (2µl), DTT (1µl), dNTPs (1µl), RNase inhibitor (1µl), SuperScript II (1µl, Thermofisher, Waltham, MA, USA). RNAase inhibitor and SuperScript II were kept at -20 ^{o}C until needed for use. Samples were then placed at 42^{o}C for 2 hours. After 2 hours, 10µL tricine buffer was added with the sample then placed at 72^{o}C for 8 minutes. Approximately 24µl of cDNA was made and either used immediately for cDNA PCR or stored at –80^{o}C.

2.3.7.3 Amplification of MBC2 (TCR β-chain) or MAC2 (TCR α-chain) gene product

In a 0.2 ml PCR tube, the following reagents were added for cDNA PCR; sigma water (19µl, Sigma-Aldrich, Gillingham, UK), 10x Buffer (5µl), 10x UPM (10µl), MBC2 or MAC2 primer (1µl, Thermofisher, Waltham, MA, USA), dNTPs (1µl), cDNA (13µl), AdvanTaq2 (1µl, Takara Clontech, Saint-Germain-en-Laye, France). 13 µl sigma water was used instead of cDNA as a negative control. Samples were then placed in a Gene Amp PCR thermocycler (Thermofisher, Waltham, MA, USA) and run on the following settings;

Temperature (°C)	Duration (seconds)	Number of cycles
95	30	1
95 72	5 120	5
95 70 72	5 10 120	5
95 68 72	5 10 120	35

2.3.7.4 Isolating PCR product by agarose gel electrophoresis

6µl of 1kb ladder was added to the first lane of each gel and 10µl of 6x TrackIt Cyan/Orange Loading Buffer (Sigma-Aldrich, Gillingham, UK) was added to each sample. Samples were then loaded onto the gel and run at 65V/180mA (per gel) for 50 minutes. After 50 minutes, gels were transferred to a blue tip box top and covered with the TAE buffer from the gel box. 15µl SYBR Gold (Carlsbad, California, USA) was then added to the gel box and placed on a rocker for at least 25 minutes. Gels were then visualised under low intensity UV light to identify a band between 500 and 700 bp (Figure 2.1). If present, the DNA band was cut out on a UV light box with sterile scalpels and placed into 1.5 ml screw-topped microtubes.

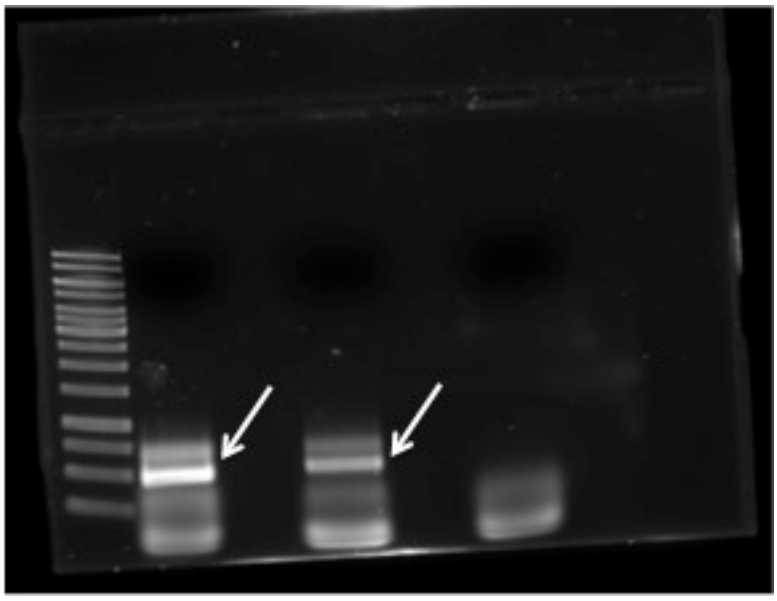


Figure 2.1 Isolation of PCR product by agarose gel electrophoresis. Amplified DNA was observed between 500 and 700 bp (arrows).

2.3.7.5 Gel extraction of PCR product

PCR product was extracted and cloned using the TOPO® TA Cloning® Kit for Sequencing with One Shot® MAX Efficiency® DH5α-T1R *E. coli* as per the manufacturers instructions (Thermofisher, Waltham, MA, USA). Screw-topped tubes containing gel DNA-amplicons were weighed and 2µL NT buffer added for each 1mg of gel. Tubes were then placed in a heating block at 50^{o}C for 10 minutes and vortexed every 2-3 minutes to dissolve the gel. If not dissolved after 10 minutes, the sample was heated for longer until no gel was visible. 700µL of the NT solution was then pipetted onto the binding column and centrifuged at 11,000 xg for 30 secs. Flow-through was then removed from the collection tube by pipetting. This step was repeated if more than 700µL solution was present.

700µL of NT3 buffer was then pipetted onto the column (after addition of ethanol to the NT3 buffer). The column was then centrifuged at 11,000 xg for 30 secs and flow-through removed by pipetting. The column was then centrifuged again at 11,000 xg for 1 minute to remove excess ethanol. To finally elute DNA, the column was removed from the collection tube and placed in a 1.5 mL screw-topped tube and 30µL of NE buffer added directly onto the column and left for 1 minute. The tube was then centrifuged at 11,000 xg for 1 minute with the flow-through containing the amplified DNA. Extracted DNA was then either stored at -80^{o}C or used immediately for the ligation step.

2.3.7.6 Product ligation into plasmid vector

If frozen, DNA product was thawed to room temperature. 4µL of DNA product was then added to a fresh 1.5 mL screw-topped tube. 1µL salt solution was added to the DNA product, followed by 1µL linear TOPO vector (stored at -20^{o}C, transferred to ice when ready to use and used immediately). Tubes were then mixed gently and incubated at room temperature for a maximum of 30 minutes. Following 30 minutes incubation, samples were put on ice to stop the reaction then proceeded to bacterial transformation.

2.3.7.7 Bacterial transformation

Max Efficiency DH5a Competent *E. coli* cells were allowed to thaw on ice (1 tube/ligation sample). Once thawed, 50µL of bacterial cells were added to each ligation tube without mixing. Samples were subsequently left on ice for a maximum of 30 minutes. Samples were then heat shocked at 42°C for exactly 30 seconds and then returned to ice for 2 minutes. Next, using sterile technique, 950µL SOC media (Thermofisher, Waltham, MA, USA) was added and samples incubated on a thermomixer at 37°C, 750rpm for 1.5 hours.

2.3.7.8 Preparation of LB-AIX plates for bacterial growth

32 capsules of LB agar medium (Sigma-Aldrich, Gillingham, UK) were added to 800ml water and placed in an autoclave at 121^{o}C and allowed to cool to ~60^{o}C. 800µl ampicillin (100 mg/ml, Sigma-Aldrich, Gillingham, UK) was then added to a final concentration of 100 µg/ml. 50mg X-GAL (Invitrogen, Thermofisher, Waltham, MA, USA) was then dissolved in 2.5ml of dimethyl formamide (DMF, Sigma-Aldrich, Gillingham, UK), with 2ml X-Gal then added. Bacterial medium was then plated out in petri dishes at 20ml/dish and allowed to set. Plates were kept at 4^{o}C for long-term storage.

2.3.7.9 Plating bacteria

100-200µL transformed bacteria were transferred to an LB-AIX plate and spread evenly with a glass L-shaped spreader after flaming in ethanol. 2 plates were spread for each sample. Once spread, plates were put in an incubator overnight at 37^{o}C with subsequent white-coloured colonies containing inserts.

2.3.7.10 Colony PCR of inserted CDR3 amplicon

Polymerase chain reaction (PCR) mix was made with the following reagent volumes per plate to be analysed; 10x PCR buffer, 250 µL (Takara Clontech, Saint-Germain-en-Laye, France); dNTP, 50 µL (Thermofisher, Waltham, MA, USA); M13F, 100 µL (Thermofisher, Waltham, MA, USA); M13R, 100 µL (Thermofisher, Waltham, MA, USA); Advantage 2 Taq, 5 µL (Takara Clontech, Saint-Germain-en-Laye, France); Sigma water, 1995 µL (Sigma-Aldrich, Gillingham, UK). Mastermix was poured into a plastic reservoir and 25µL/well pipetted into a 96 well plate. Using sterile toothpicks, white colonies

were selected and transferred to the 96 well plate (one colony/well). Plates were then centrifuged briefly to 600 rpm and PCR performed with the following program; 95°C for 30 secs, 57°C for 30 secs and 68° C for 3 mins for 35 cycles.

2.3.7.11 Checking for amplification

In order to check for amplification following colony PCR, 12 of the 96 PCR products were run on a 1% agarose gel (Figure 2.2). 5µl SYBR Safe (Invitrogen, Thermofisher, Waltham, MA, USA) was added to a 1% agarose gel (one gel/96 well plate) and covered with 1X TAE buffer. Gels were protected from the light whilst being allowed to set. 1µL of TrackIt Cyan/Orange Buffer (Sigma-Aldrich, Gillingham, UK) was subsequently mixed with 5µL of PCR product from the following wells: A1, B2, C3, D4, E5, F6 G7, H8, G9, F10, E11, D12 and added to the gel. The gel was subsequently run at 65V and 180mA for 30 minutes. After this time, the gel was visualised under UV light for bands at approximately 750bp. Following confirmation of amplification, plates were prepared for sequencing by diluting the PCR product with 25µL Sigma water (Sigma-Aldrich, Gillingham, UK) and 25µL transferred to a new, skirted 96-well plate. Plates were covered with an aluminium plate cover, wrapped in clingfilm and stored at -80^{o}C until sent for sequencing.

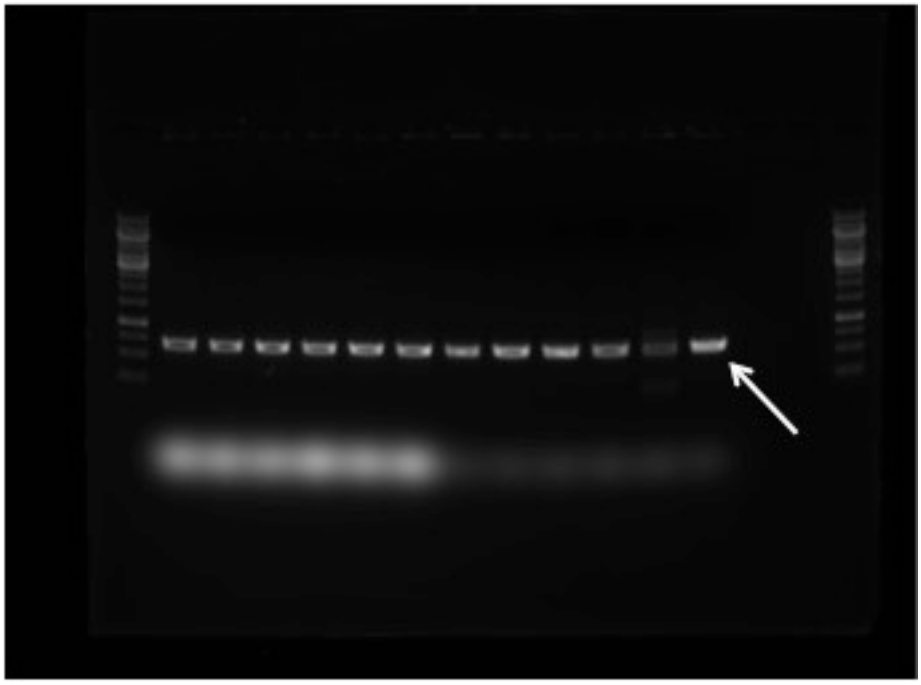


Figure 2.2 Confirming amplification of colony PCR. DNA bands were observed at approximately 750bp (arrows).

2.3.7.12 Sequencing of plates

96 well plates were sent to genewiz (South Plainfield, USA) for sequencing.

2.3.7.13 Analysis of sequencing data

Analysis of sequencing data was performed using sequencher software (Gene codes corporation, Ann Arbor, USA) and the IMGT (international ImMunoGeneTics information system, Montpellier, France) website.

2.3.7.14. Data processing and analysis

Following sequencing, data was imported into sequencher and sequence ends trimmed. If quality scores for each individual sequence were <50%, data was excluded from the analysis. Included data was then converted to TCR sequences using the IMGT website and exported into Microsoft Excel. TCRs were then filtered and only 'in-frame' sequences chosen for further analysis. Once determined, TCR frequencies were sorted according to the following hierarchy; (i) frequency count (ii) CDR3 amino acid length (iii) Highest TRBV (iv) highest TRBJ. Data analysis was performed in Microsoft Excel and Graphpad prism (La Jolla, USA). TCRs were checked against the IMGT CDR3 expected sequences, (Table 2.3)[294] and excluded if amino acid sequences were not consistent.

IMGT TRBV gene	FR3-IMGT (positions 66-104)	CDR3-IMGT (positions 105-115)
TRBV2	IRSTKLEDSAMYFC	ASSE.....
TRBV3-1	INSLELGDSAVYFC	ASSQ.....
TRBV4-1	LHALQPEDSALYLC	ASSQ.....
TRBV4-2	TENNSVP.SRFSPECP.NSSHLFLHLHTLQPEDSALYLC	ASSQ.....
TRBV4-3	VENNSVP.SRFSPECP.NSSHLFLHLHTLQPEDSALYLC	ASSQ.....
TRBV5-1	VSTLELGDSALYLC	ASSL.....
TRBV5-3	VSALELGDSALYLC	ARSL.....
TRBV5-4	VNALELDDSALYLC	ASSL.....
TRBV5-5	RQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
TRBV5-6	RFSGHQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
TRBV5-7	QFSGHQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
TRBV5-8	VNALELEDSALYLC	ASSL.....
TRBV6-1	SLRLESAAPSQTSVYFC	ASSE.....
TRBV6-2*	GLESAAPSQTSVYFC	ASSY.....
TRBV6-3*	GLESAAPSQTSVYFC	ASSY.....
TRBV6-4	LASAVPSQTSVYFC	ASSD.....
TRBV6-5	LLSAAPSQTSVYFC	ASSY.....
TRBV6-6	LELAAPSQTSVYFC	ASSY.....
TRBV6-7	KLESAAPSQTSVYFC	ASSY.....
TRBV6-8	LVSAAPSQTSVYLC	ASSY.....
TRBV6-9	PLRLESAAPSQTSVYFC	ASSY.....
TRBV7-1	FQRTQQGDLAVYLC	ASSS.....
TRBV7-2	TIQRTQQEDSAVYLC	ASSL.....
TRBV7-3	IQRTERGDSAVYLC	ASSL.....
TRBV7-4	IQRTEQGDSAVYLC	ASSL.....
TRBV7-6	QDKSGLPNDRFSAERP.EGSISTLTIQRTEQRDSAMYRC	ASSL.....
TRBV7-7	PDKSGLPSDRFSAERP.EGSISTLTIQRTEQRDSAMYRC	ASSL.....
TRBV7-8	KIQRTQQEDSAVYLC	ASSL.....
TRBV7-9	IQRTEQGDSAMYLC	ASSL.....
TRBV9	LSSLELGDSALYFC	ASSV.....
TRBV10-1	LESAASSQTSVYFC	ASSE.....
TRBV10-2	LESATRSQTSVYFC	ASSE.....
TRBV10-3	LESATSSQTSVYFC	AISE.....
TRBV11-1	IQPAELGDSAMYLC	ASSL.....
TRBV11-2	IQPAKLEDSAVYLC	ASSL.....
TRBV11-3	IQPAELGDSAVYLC	ASSL.....
TRBV12-3*	STLKIQPSEPRDSAVYFC	ASSL.....
TRBV12-4*	STLKIQPSEPRDSAVYFC	ASSL.....
TRBV12-5	ATLKIQPSEPRDSAVYFC	ASGL.....
TRBV13	MSSLELGDSALYFC	ASSL.....
TRBV14	VQPAELEDSGVYFC	ASSQ.....
TRBV15	IRSPGLGDTAMYLC	ATSR.....
TRBV16	IQATKLEDSAVYFC	ASSQ.....
TRBV17	IHPAEPRDSAVYLY	SSG.....
TRBV18	IQQVVRGDSAAYFC	ASSP.....
TRBV19	VTSAQKNPTAFYLC	ASSI.....
TRBV20-1	VTSAHPEDSSFYIC	SAR.....
TRBV23-1	ILSSEPGDTALYLC	ASSQ.....
TRBV24-1	LESAIPNQTALYFC	ATSDL.....
TRBV25-1	LESARPSHTSQYLC	ASSE.....
TRBV27	LESPSPNQTSLYFC	ASSL.....
TRBV28	LESASTNQTSMYLC	ASSL.....
TRBV29-1	VSNMSPEDSSIYLC	SVE.....
TRBV30	SKKLLLSDSGFYLC	AWS.....

Table 2.3. Expected CDR3 region starting sequences for given TRBV genes.

2.3.7.15 Additional bio-computational analysis

Further to my original analysis, additional analysis was performed by Dr Vanessa Venturi (Infection Analysis Program, The Kirby Insitute, UNSW Austrialia, Sydney, NSW 2052, Australia) and Dr Adel Rahmani (School of Mathematical Sciences Physical Sciences, University of Technology Sydney, 15 Broadway, Ultimo, NSW 2007, Australia). Dr Venturi and Dr Rahmani have expertise in using computational biology approaches in order to understand immunological data.

2.3.8 Tumour necrosis factor alpha (TNFα) capture assay to determine antigen specificity of CSF-resident T cells

2.3.8.1 Generation of Epstein Barr Virus (EBV) B95.8 lymphoblastoid cell lines (EBV-LCLs)

When setting up from frozen samples, donor PBMCs were thawed in a water bath, added to 9ml R10 and centrifuged at 1500 rpm for 5 mins. Supernatant was discarded and cells resuspended in 2ml R10. 1ml was then pipetted into 2 wells of a 24 well plate ($5x10^6$ cells/well). Epstein Barr Virus B95.8 (European Collection of Authenticated Cell Cultures, ECACC, Public Health, Porton Down, UK) was passed through a 0.45 µm filter and 1 ml added to each well containing PBMCs. 20 µl (10 µl/ml) ciclosporin (50 µg/ml, Pharmacy department, University Hospital of Wales, Cardiff, UK) was then added to each well and the plate incubated at 37^{o}C for 1 week. After 1 week, 1 ml of media was removed and fresh R10 added in addition to a further 20 µl ciclosporin. Following this, cells were grown, split and fed as necessary and kept in a T75 flask with R10 media. If lines were synthesised from freshly processed PBMCs, $5x10^6$ cells were put in one well of a 24 well plate and the same protocol followed.

2.3.8.2 Generation of EBV stimulated T cell lines

PBMCs were thawed, counted and $2x10^6$ resuspended in 2ml of CTL media in one well of a 24 well plate. γ-irradiated (40 Gy), autologous EBV-LCLS were then added at a ratio of 40:1 ratio (PBMC:LCLs). After 9-12 days, cells were removed, added to R10 and centrifuged at 1500 rpm for 5 mins. Cells were then resuspended in fresh CTL medium at $0.5x10^6$ cells/ml and restimulated with irradiated autologous EBV-LCLs at a ratio of 4:1 (i.e. $1x10^6$:$2.5x10^5$ – CTL:EBV-LCLs). At days 13-16, cells were fed with fresh

medium containing IL-2 at 50 U/ml. Cells were then stimulated weekly using a 4:1 ratio of CTL:EBV-LCLs with twice weekly addition of IL-2.

2.3.8.3 TNFα capture assay

On the day before the experiment, EBV stimulated T cell lines were restimulated with EBV-LCLs at the following ratios in FACS tubes; CTL:LCL 1:1, 1:0 and 1:1 ratio without anti-TNFα. To each tube, 10µl (10µM) of TAPI-O (Millipore, Watford, UK) (TAPI-O (1mg) reconstituted in 2.19ml of DMSO and 100µl aliquots made) was added. 15µl of anti-TNFα PECy7 (BD, Oxford, UK) was then added to each FACS tube along with 3µl each of CD28 (BD, Oxford, UK) and CD49d (BD, Oxford, UK). Samples were then incubated overnight at 37^{o}C. The following day, 1 ml PBS was added to each tube and centrifuged at 2000 rpm for 2 minutes. Supernatant was discarded and the wash step was repeated. 8µL aqua (diluted in PBS at a 40:1 ratio) was subsequently added to each tube and left to incubate for 10 minutes at room temperature. Compensations were made as described above in section **2.3.6.2**. Samples were stained with the following antibodies and incubated for 20 mins at 4^{o}C; CD14, CD19, CD3, CD8 and CD4. After 20 minutes, 1ml PBS was added and samples centrifuged for 2 minutes at 2000 rpm. Supernatant was then discarded and 100µL PBS added. Flow cytometry and cell sorting was performed of TNF^{+}CD4^{+} and CD8^{+} populations. Following cell sorting, clonotyping of these cell populations was performed as previously described.

2.3.9 Cloning of donor TCR and lentivirus synthesis

2.3.9.1 TCR design

Patient TCR constructs were codon optimised and synthesised by Genewiz Inc (USA). Figure 2.3 demonstrates the vector map with TCR and rat CD2 inserts.

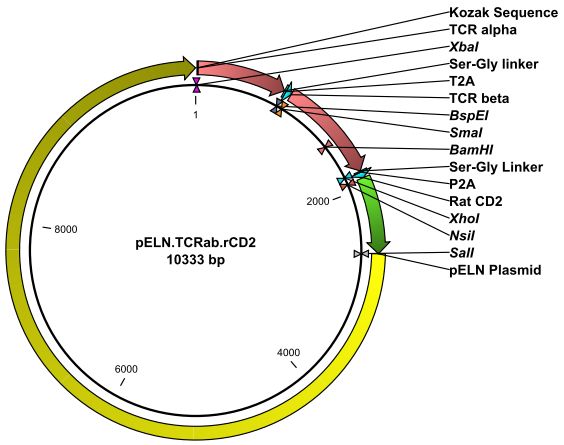


Figure 2.3. Vector map demonstrating TCR and rat CD2 inserts. Figure courtesy of Dr John Bridgeman.

2.3.9.2 Digest and ligation of donor TCR into pELN

Donor TCR was used at 0.1 µg/µl. Donor TCR and pELN.003 (James L Riley, UPenn, USA) were digested with XbaI (Thermofisher, Waltham, MA, USA) and BamHI enzymes (Thermofisher, Waltham, MA, USA) on ice as follows; XbaI 1µl, BamHI 1µl, FD buffer 2µl, donor TCR 10 µl and 6 µl molecular biology water to make up to 20 µl. For pELN, 1µl of the plasmid was used with 15 µl water. Digests were performed in PCR tubes at 37^{o}C for 1 hour. Digest products were subsequently run on a 1% agarose gel with donor TCR and pELN.003 plasmid cut out under UV light and gel extracted as per section **2.3.7.5** (Figure 2.4). After DNA elution, nanodrop was performed to quantify the amount of DNA present. Following this, samples were set up for ligation reactions at 3 different vector:insert ratios; 1:0.5, 1:1 and 1:2. Ligase buffer (2 µl, Thermofisher, Waltham, MA, USA) and ligase (1 µl, Thermofisher, Waltham, MA, USA) were added along with water to make up to 20 µl. Samples were incubated at 4^{o}C overnight.

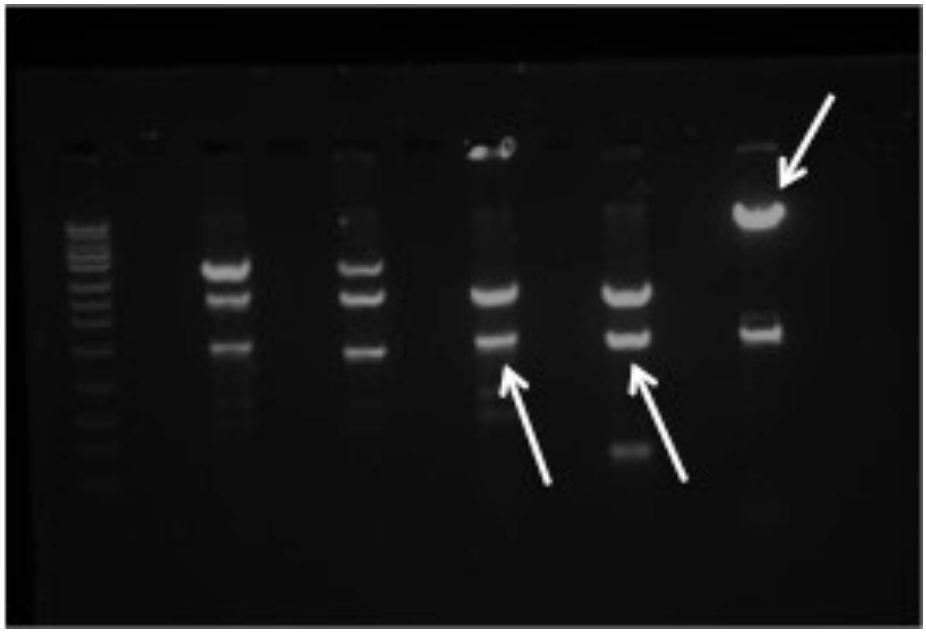


Figure 2.4. Digest of donor TCRs. Lane 3 and 4; donor TCRs (arrows). Lane 5; pELN.003 (arrow).

2.3.9.3 Bacterial transformation of ligation reactions

4 µl of ligation reaction was added to 45 µl XL10 gold bacteria (Agilent Technologies, Santa Clara, USA). Tubes were mixed gently and incubated on ice for 30 mins. Samples were then heat-shocked for 30 secs at 42^{o}C followed by 2 minutes. 900 µl SOC media was then added and incubated for 1 hour at 37^{o}C with shaking at 225-250 rpm. Samples (150 µl) were then spread on plates made as described (**2.3.7.8**) but without XGAL and incubated overnight at 37^{o}C. The following day, 5 separate colonies were picked with a pipette tip and added to a 5ml universal container containing 5ml of LB media. Prior to inserting in the universal containers, colonies were streaked on a separate plate. Universal containers were then placed on a rocker overnight (220 rpm) at 37^{o}C and miniprep performed the following day.

2.3.9.4 Miniprep of amplified donor TCRs

Following overnight incubation, pipettes were removed and universal containers centrifuged at 0^{o}C for 5 minutes at 4000 rpm. Supernatant was discarded in bleach with the pellet resuspended in 600 µl PBS and added to a 1.5 ml centrifuge tube. A miniprep (Zymo Research, Irvine, USA) was performed as per the kit instructions. 100 µl 7x lysis buffer was added to the centrifuge tube and inverted 4-6 times. Within 2 minutes, 350 µl neutralisation buffer was added and inverted until the sample turned yellow. The tube was then spun at 13,000 xg for 2 mins and 900 µl of the supernatant

added to a centrifuge column. The column was then centrifuged at 11,000 xg for 15 secs with the flow through discarded. 200 µl endo-wash buffer was then added and the sample spun at 11,000 xg for 15 secs. 400 µl zyppy wash buffer was added and centrifuged at 11,000 xg for 30 secs. Flow through was transferred to a clean tube and 30 µl zyppy elution buffer added and allowed to stand for 1 min at room temperature. Samples were then centrifuged at 11,000 xg for 15 secs to elute DNA. DNA was quantified on a nanodrop and sent for sequencing to confirm the presence of amplified TCR.

2.3.9.5 Maxiprep of amplified donor TCRs

After confirmation of the amplified donor TCR, maxiprep (Thermofisher, Waltham, MA, USA) was performed as per manufacturers instructions. 5 µl ampicillin was added to 5 ml LB media in a universal container. From the stored streak plate, the colony containing the amplified donor TCR was then picked and added to the container and incubated at 37^{o}C during the day on a rocker. At the end of the day, the culture was added to 500 ml LB media in a 2L conical flask (with 500 µl ampicillin) and incubated overnight on a rocker. Two small and two large plastic pots, lids and rubber seals were autoclaved in preparation for maxiprep. Optical density was measured before proceeding with maxiprep. 30ml equilibrium buffer was added directly into the filtration cartridge inserted in the maxi column. LB media was added to the two large autoclaved pots and weighed to within 0.1 g of each other. Samples were centrifuged at 4000 xg for 10 minutes with pellets then resuspended in 10ml resuspension buffer. 10ml of lysis buffer was then added and incubated at room temperature for 5 minutes. 10ml precipitation buffer was then added and the precipitated lysate transferred to the column. The lysate was allowed to filter through the column by gravity flow. Next, the inner filtration cartridge was discarded and the column washed with 50ml wash buffer. A sterile 50ml centrifuge tube was placed under the filter column and 15ml elution buffer added. The solution was allowed to drain by gravity flow and the column discarded with the elution tube containing the purified DNA.

To precipitate DNA, 10.5 ml propan-2-ol was added to the eluate and mixed well. The tube was then centrifuged at >12,000 xg for 30 minutes at 4^{o}C and the supernatant removed. 5ml 70% ethanol was added to the pellet and the tube centrifuged at

>12,000 xg for 5 minutes at 4°C and the supernatant removed. The pellet was air-dried for 10 minutes and resuspended in 200 µl TE buffer and nanodrop performed.

2.3.9.6 Culture of 293T cells

When culturing 293T cells, D10 media was used. For feeding, D10 media was removed and cells washed with 10ml PBS. 10ml 0.05% Trypsin-EDTA (1X) (Gibco, Thermofisher, Waltham, MA, USA) was then added and left for 2-3 mins for cells to disassociate from the plastic. Trypsin was then removed (2ml left behind) and 25ml D10 added.

2.3.9.7 $CaCl_2$ transfection for lentiviral production

On the day prior to transfection, 15-20 x 10^6 293T cells were put in a T175 flask. In 2x 50ml falcon tubes, 1.25ml 1M Hepes buffer (Sigma Aldrich, Gillingham, UK) was added and topped up to 50ml with serum free DMEM. Sodium hydroxide (Sigma-Aldrich, Gillingham, UK) was added by pastette pipette so that one volume of media was at pH 7.1 and the other pH 7.9. Both volumes of media were filtered through 0.2 µm filters. 1M $CaCl_2$ solution was made by adding 50ml water to 7.35g hydrated $CaCl_2$ (Sigma-Aldrich, Gillingham, UK) and filtered. Aliquots were stored in the freezer (-20°C).

In a 15ml falcon, the following reagents were added; 15 µg pELN lentivirus vector (James L Riley, UPenn, USA) containing donor TCR, 18 µg pRSV.Rev, 7 µg pVSVg, 18 µg pMDLg/pRRE, pH7.1 media (to make up to 2850 µl – added first) per flask, 150 µl of $CaCl_2$ (added last). This solution was vortexed and incubated at 10-30 mins at room temperature.[295]

Old media was removed from 293T cells and 12ml pH 7.9 media gently added. The DNA mix was then vortexed briefly and added dropwise to the surface of the media then incubated at 37°C. The following day, media was replaced with 20ml D10 and returned to the incubator. 48 hours post transfection the media was collected and put through a 0.45 µm filter. Supernatant was replaced with 20 ml fresh D10. 72 hours post transfection, a second collection of media was performed. Supernatants were then centrifuged at 24,000 xg for 2 hours at 4°C. Following centrifugation, the supernatant was removed and the pellet resuspended in 1ml T cell medium. Aliquots of lentivirus were snap frozen on dry ice and stored at -80°C until further use.

2.3.9.8 $CD8^+$ T cell isolation

PBMCs were prepared as described (Section 2.3.3). $CD8^+$ T cells were then isolated from PBMCs by MACS separation as per manufacturers instructions (Miltenyi Biotec, Bisley, UK). MACS buffer (80 µl/10^7 cells) was added to the PBMC cell pellet followed by 20 µl CD8 beads/10^7 cells. Cells were incubated in the fridge for 15 mins. During incubation, MACS column was prepared and 500 µl MACS buffer added. Following incubation, 4 ml MACS buffer was added and centrifuged for 10 mins at 1500 rpm. Supernatant was removed with a pastette pipette and resuspended with 500 µl MACS buffer, then added to the MACS column. Following flow through, 3 x 500 µl MACS buffer was added. The column was then placed over a universal container and 2 ml MACS buffer added. A plunger was then used to push through isolated $CD8^+$ T cells. Cells were then centrifuged at 1500 rpm for 5 mins and resuspended in 1 ml T cell media and counted. 75 µl of $CD3^+CD28^+$ (Thermofisher, Waltham, MA, USA) beads were then added and the cell suspension put in 1 well of a 24 well plate and incubated overnight at 37°C.

2.3.9.9 Lentivirus transfection of isolated $CD8^+$ T cells

Following overnight incubation of $CD8^+$ T cells $CD3^+CD28^+$ beads, one lentivirus aliquot (1ml) was added along with 1 µl/ml (2 µl) of polybreen (Insight biotechnology, Wembley, UK). One week later, lentivirus positive cells were sorted as follows. Cells were centrifuged (1500 rpm, 5 mins) and resuspended in 1 ml PBS and added to a FACS tube. Cells were further centrifuged (2000 rpm, 2 mins) and the supernatant discarded. Cells were stained with the following antibodies; Aqua, CD14, CD19, CD8 and ratCD2 and incubated at 4°C for 20 mins. Following this, 1ml PBS was added and cells centrifuged at 2000 rpm for 2 mins. Cells were then resuspended in 150 µl PBS and transferred for cell sorting. Rat CD2 positive cells were sorted into TCM. The following day, PBMCs from 3 separate donors were prepared to feed cells. PBMCs were separated as described and irradiated for 13 mins at 3000 Rads. Cells were mixed, centrifuged (1500 rpm for 5 mins) and resuspended in 5ml TCM. After counting, 15x10^6 cells were added to a T25 flask and 20ml TCM added. 20 µl PHA (Thermofisher, Waltham, MA, USA) was added to the flask along with the sorted lentivirus positive $CD8^+$ T cells. The flask was then incubated at 37°C (flask tilted). After 7 days, $CD8^+$ T cells were centrifuged (1500 rpm for 5 mins) and resuspended in 2-3 ml. Cells were

counted and resuspended in a 24 well plate at $2x10^6$ cells/well. 14 days after lentivirus transfection, cells were prepared for flow cytometry as described to check for purity. Cells were either frozen or $10x10^6$ cells resuspended in 5x wells of a 24 well plate in TCM. Cells were fed when necessary with fresh TCM.

2.3.10 Sizing scan and combinatorial peptide library (CPL) screen of $CD8^+$ T cells expressing dominant CSF-resident TCRs

2.3.10.1 Sizing scan and combinatorial peptide library (CPL) screen

On day 1, $CD8^+$ T cells were washed in PSG and put in R2 overnight. On day 2, cell cultures were set up for sizing scans or CPL screens. 5 µl of each either sizing scan or CPL peptide mix (at a concentration of 10mM or 1mM, respectively) per well was plated in 96 well round bottom plates (in duplicate).

For the sizing scan, the following mixtures were used to define the MHCI-peptide length preference of the donor TCR: X^8, X^9, X^{10}, X^{11}, X^{12}, and X^{13} (where X is any of the 19 proteogenic L-amino acids excluding cysteine; Pepscan, Lelystad, The Netherlands). Sizing scan parameters are detailed in the table 2.4. The 8mer CPL was synthesized in positional scanning format (Figure 2.5, Pepscan, Lelystad, The Netherlands).[35] CPL parameters are detailed in Table 2.5.

Sizing scan ID	Sequence of sizing scan mixture	Total no. of peptides in scan mixture (19^n)	Concentration of each peptide in scan mixture*
8mer	XXXXXXXX	1.7×10^{10}	5.9×10^{-15} M
9mer	XXXXXXXXX	3.2×10^{11}	3.1×10^{-16} M
10mer	XXXXXXXXXX	6.1×10^{12}	1.6×10^{-17} M
11mer	XXXXXXXXXXX	1.2×10^{14}	8.6×10^{-19} M
12mer	XXXXXXXXXXXX	2.2×10^{15}	4.5×10^{-20} M
13mer	XXXXXXXXXXXXX	4.2×10^{16}	2.4×10^{-21} M

Table 2.4 Sizing scan parameters.[293] n indicates the number of degenerate positions. *When mixtures are used at a concentration of 100µM. Figure adapted from Ekeruche-Makinde et al.[293]

CPL ID	Sequence of position one sublibraries	Total no. of peptides in library (a + 19) × 19n	No. of sublibraries	No. of peptides in each sublibrary (19n)	Concentration of each peptide in sublibrary*
8mer	OXXXXXXX	2.4×10^{10}	160	8.9×10^{8}	1.1×10^{-13} M

Table 2.5. CPL scan parameters.[293] n indicates the number of degenerate positions; O, fixed sequence position (1 of the 20 proteogenic L-amino acids; O is moved systematically through the peptide backbone in a full CPL); X, degenerate position (1 of 19 proteogenic L-amino acids, excluding cysteine); and a, full peptide length. *When mixtures are used at a concentration of 100µM. Figure adapted from Ekeruche-Makinde et al.[293]

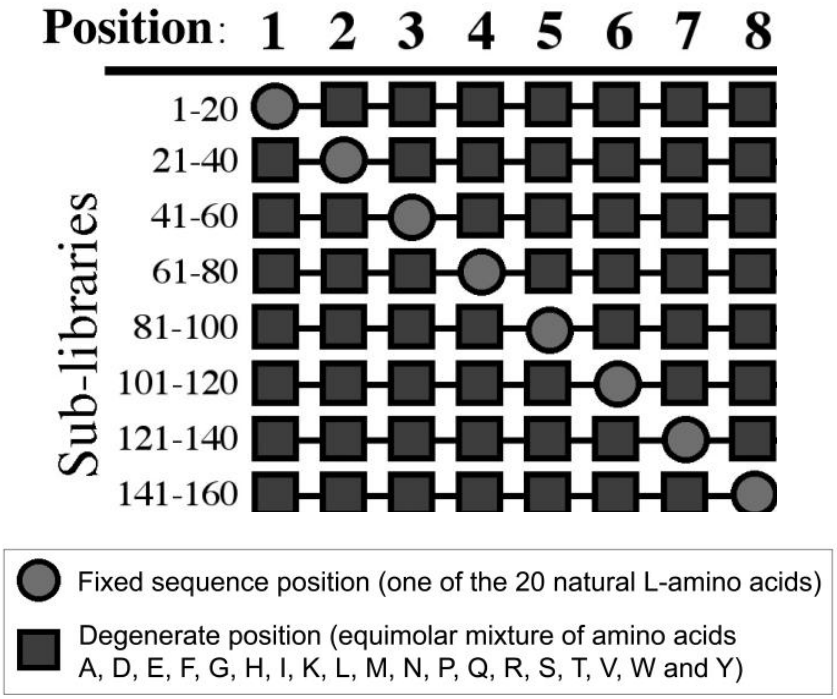


Figure 2.5. Schematic representation of a 8mer combinatorial peptide library (CPL). The 8mer combinatorial peptide library contains a total number of 2.4×10^{10} ($(8+19)\times19^8$) different 8mer peptides and is divided into 160 different peptide mixtures (or sub-libraries) as indicated. In every peptide mixture, one of the 20 natural proteogenic L-amino acids is fixed at one position (circles) but all other positions are degenerate (squares), with the possibility of any one of 19 natural L-amino acids being incorporated in each individual position (cysteine is excluded). Thus, each library mixture consists of 8.9×10^8 (19^8) different 8mer peptides in approximately equimolar concentrations. Figure adapted from Wooldridge et al.[35]

Target cells were centrifuged at 1500 rpm for 5 mins, counted and resuspended in R2 to a concentration of $1.3\text{x}10^6$ cells/ml. 45 µl (approx. 60,000) target cells were added to each well of the peptide plates. Plates were then incubated at 37°C for 1-2 hours for peptide pulsing. $CD8^+$ T cells were similarly prepared to a concentration of $6\text{x}10^5$ cells/ml. Following peptide pulsing, 50 µl (approx. 30,000 cells) of $CD8^+$ T cells were added to each well. Control wells of T cells only, target cells only and both together were also performed, as well as PHA (Thermofisher, Waltham, MA, USA) as a positive control. Plates were incubated overnight. For MIP1-β ELISA (Duoset kit, R&D Systems Inc. Minneapolis, MN, USA), plates were coated with capture antibody at a concentration of 0.9 µg/ml. 50 µl per well was added and the plate incubated overnight at room temperature.

The following day, 50 µl supernatant was harvested from each well into a new plate and diluted in 70 µl R2. The coated plates were next washed 3 times with 200 µl/well wash buffer (PBS tween – PBS made by adding one tablet (Oxoid, Thermofisher, Waltham, MA, USA) per 100ml water with 0.05% tween 20 (Sigma-Aldrich, Gillingham, UK)). Following this, the plate was blotted dry and 150 µl reagent diluent (PBS with 1% BSA, (Sigma-Aldrich, Gillingham, UK)) added per well and incubated for a minimum of 1 hour at room temperature. The plate was washed again and either 50 µl of cell supernatant or standards (1000, 500, 250, 125, 62.5, 31.2, 15.6 and 0 pg/ml) added and incubated for 1 hr 15 mins at room temperature. After further washing, 50 µl detection antibody (diluted 1 in 200 in reagent diluent) was added to each well and incubated for a further 1 hr 15 mins. After this time, plates were washed again and 50 µl streptavidin-HRP (diluted 1 in 200) added and incubated for 20 mins at room temperature being kept away from direct light. After further washing, 50 µl per well of colour reagents A & B were added and incubated until blue colour seen (approximately 20 mins). 25 µl/well of stop solution was added to stop the reaction and plates read at 450nm on a Biorad iMark microplate absorbance reader (Biorad, Hercules, USA). The readouts from the standards were utilised to calculate the concentration of MIP1-β present for each well, with the background readout subtracted. N.B. The above protocol was used whether performing a sizing scan or peptide library screen.

2.3.10.2 Analysis of combinatorial peptide library screening

Results from the CPL screen were inputted into the Warwick Systems Biology Centre webtool (http://wsbc.warwick.ac.uk/wsbcToolsWebpage/resetpass.php, University of Warwick, UK) for peptide identification from CPL screens.[296]

2.3.10.3 Assessing TCR response against chosen peptides identified by combinatorial peptide library screen

Peptides were chosen based on scores from the CPL screens and for disease relevance (Pepscan, Lelystad, The Netherlands). Peptides were first diluted to an 8mM stock and then further diluted in PSG to a concentration of 1mM (10^{-3}M). Peptides were then diluted to concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}. 5µl of each peptide concentration was then moved to an ELISA plate and made up to final concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} following the addition of

45µl of target cells. MIP-1β ELISA was then set up in duplicate as described in section 2.3.10.1.

2.3.10.4 HLA typing

Donor PBMCs were HLA typed at A, B and C loci by Proimmune (Oxford, UK).

2.3.11 TCR V beta staining of peripheral blood

PBMCs were thawed and centrifuged at 1500 rpm for 5 mins. Cells were resuspended in 8ml PBS and divided between 8 FACS tubes. Tubes were then centrifuged at 2000 rpm for 2 mins and supernatant discarded. 8 µl aqua was then added to each sample and incubated for 10 mins at room temperature. After 10 mins, V beta antibodies (A-H) (IOTest® Beta Mark, Beckman Coulter, Brea, USA) were added to each of the 8 tubes along with the following antibodies (CD14, CD19, CD3, CD8, CD4, CD27, CD45, CD95, CCR7, CD127, CD49d) and incubated for 30 mins at 4°C. Following this, 1 ml PBS was added to each tube and centrifuged at 2000 rpm for 2 minutes. Supernatant was then discarded, 100 µl PBS added and samples analysed on the FACSAria II. The IOTest® Beta Mark Kit is a multi-parametric analysis tool designed for quantitative determination of the TCRVβ repertoire of human T lymphocytes by flow cytometry. Eight vials (labeled A-H) each containing 3 different TCRVβ mAb are used. The first mAb within each labelled vial is FITC-conjugated, a second one is PE-conjugated and a third one is a mixture of a PE- and a FITC-conjugated form. The 8 vials containing mixtures of conjugated TCRVβ antibodies correspond to 24 different specificities (about 70% coverage of normal human TCRVβ repertoire). The TCRVβ included in this assay are as follows; 4-1 4-2 4-3, 5-5, 28, 3-1, 19, 14, 5-1, 18, 30, 6-5 6-6 6-9, 6-6, 12-3 12-4, 5-6, 10-3, 20-1, 9, 11-2, 13, 2, 25-1.

2.4 Methods for chapter 3: Treatment of Multiple sclerosis with alemtuzumab; an anti-CD52, lymphocyte depleting monoclonal antibody

2.4.1 Patients and data collection

Patients referred to and assessed in the neurology department at the University Hospital of Wales, Cardiff were identified as candidates for treatment with

alemtuzumab if they had a relapsing disease course and evidence of aggressive disease characterised by a high relapse rate, active disease on cranial MR imaging, rapidly accumulating disability, early motor, cerebellar or cognitive dysfunction or combinations of these factors, and were considered to have poor prognosis. A smaller number of patients were treated locally at regional specialist neuroscience centres in Swansea and Bristol following regional network case-based discussions. Patients receiving alemtuzumab as part of externally sponsored clinical trials were excluded from analysis.

Prior to treatment, all patients had a normal blood count, thyroid function tests, routine blood indices and white cell immunophenotyping. At the time of treatment no patient had evidence of active infection and treatment during relapse was avoided whenever possible. Consent for treatment was obtained and explanation of potential risks and benefits provided.

2.4.2 Treatment regimen and adverse event monitoring

Prior to 2006, patients received an initiation dose of 24–30 mg alemtuzumab intravenously per day for 5 days, with 1 g intravenous methylprednisolone given as pre-treatment on the first 3 days only in order to ameliorate the expected infusion reaction side-effects related to cytokine release.[297] After 2006, the daily dose of alemtuzumab was reduced to 12 mg. Routine top-up treatment was administered after 12 months, consisting of 3 daily doses of alemtuzumab with concurrent steroid pre-treatment. Additional courses were given as indicated after intervals of not less than 12 months, as a result of one or more of the following factors; (1) disabling clinical relapse, (2) evolving disability with or without objective change in EDSS, (3) the development of new or enhancing lesions on MRI performed 12 months or more after a prior treatment cycle.

A monitoring program for adverse autoimmune events included monthly full blood count and urea and electrolytes in addition to thyroid function tests with anti-thyroperoxidase antibodies at least 6 monthly intervals. Urinalysis was performed when indicated or during concomitant illness. Additional tests for relevant

autoimmune immune disease(AID)-related antibodies were performed when appropriate.

2.4.3 Data analysis

Patients were identified from a regional clinical database[298] and a systematic review of notes was performed to validate the dataset. Data was collected on demographics, EDSS scores, relapses, adverse events and prior medication use in order for further analysis to be performed. Final data capture was performed on 23rd April 2015. Retreatment rates, annualised relapse rates (ARR) pre- and post- treatment, disability outcomes, adverse events including rates of AID and outcomes of pregnancies were investigated. 6-month sustained accumulation (SAD) and reduction of disability (SRD) was calculated according to established definitions[299]

Chapter 3

Treatment of Multiple sclerosis with alemtuzumab; an anti-CD52, lymphocyte depleting monoclonal antibody

Willis MD *et al.* Mult Scler. 2016;22(9):1215-23

3.1 Introduction

3.1.1 The use of alemtuzumab in Multiple sclerosis

The range of effective treatments for relapsing MS is rapidly expanding, leading to an ever-greater choice for both patients and clinicians. Although the new disease-modifying therapies have undergone rigorous clinical trials before reaching the clinic, post-marketing surveillance and reporting are essential in order to fully understand safety and efficacy, and in some cases have been key in modifying use in clinical practice. In particular, given the proposed role of $CD8^+$ T cells in MS, studies on the effectiveness of therapeutics that affect the function of these cells can offer further insights into their role in disease pathogenesis.

Alemtuzumab was first proposed as a treatment for MS in the 1990s.[300, 301] Following clinical trials demonstrating a dramatic effect on relapse rates, in addition to a positive effect on longer-term disability outcomes,[9, 240, 241] it has now been approved for use in 49 countries worldwide.[302] Its primary indication is for active relapsing disease, either as first or second line treatment, although in a small number of countries has been restricted to patients who have had an inadequate response to two or more established disease-modifying therapies. As well as having an impressive clinical effect across a number of end points including relapse rate, brain atrophy and measures of disability, treatment with alemtuzumab has also offered some fascinating insights[297, 303] into clinical aspects of MS and allowed a greater understanding of disease pathogenesis. In addition, one of the recognised adverse events of alemtuzumab is the side effect of disease specific autoimmunity. This has provided an unintended, but

intriguing window into the origins of human autoimmune disease the discussion of which is outside the remit of this thesis. By studying the clinical outcomes of alemtuzumab, the effects of lymphocyte depletion *in vivo* can be observed and indicate a pathogenic role for T cells.

3.1.2 Mechanism of action of alemtuzumab

Alemtuzumab is a humanised monoclonal antibody, which targets CD52[304], a 12 amino acid glycosylated glycosylphosphatidylinositol-linked protein expressed on the cell surface of lymphocytes, monocytes, macrophages, eosinophils and NK cells.[305-307] The function of this molecule is largely unknown, although is thought to contribute to T cell activation,[308] migration[309] and the induction of regulatory T cells.[309] The anti-CD52 effect of alemtuzumab results in rapid and profound depletion of circulating lymphocytes following intravenous infusion, as a result of antibody-dependent cell-mediated cytotoxicity,[257] complement-dependent cytolysis and induction of apoptosis.[310] However, CD52 is not expressed on haematopoietic precursors, so allowing beneficial immune reconstitution and return of immune competency.[258, 311]

Reconstitution occurs via two mechanisms; proliferation of mature lymphocytes that escape deletion ('homeostatic proliferation') and via bone marrow/thymic repopulation.[312] Following treatment, rates of lymphocyte recovery vary by cell type, with B cells first to recover, followed by $CD8^+$ and $CD4^+$ T cells.[259, 313, 314] Although controversial, the rate and pattern of lymphocyte reconstitution is not currently thought to correlate with subsequent re-emergence of disease activity.[259, 315, 316] As immune reconstitution becomes more established, regulatory $CD4^+$ T cells (Tregs) dominate the T cell population, and is considered to be one of the factors contributing to long-term efficacy rather than this being solely a result of lymphodepletion.[317-319] In particular, a recent study reporting results from the phase III trials has demonstrated a significant increase in Treg cell percentage at 24 months after treatment.[320] An increased representation of memory T cells is also observed,[321] although the impact of this phenomenon is less clear. Furthermore, mRNA levels of pro-inflammatory cytokines and anti-inflammatory cytokines are down- and up-regulated respectively following treatment, which may also contribute to the drug's unique durability in MS.[320]

3.1.3 Early experience

Prior to its use as a therapy for MS, alemtuzumab was licensed for fludarabine resistant chronic lymphocytic leukaemia in addition to its application in organ transplantation and other autoimmune disorders.[322] Early in the clinical development program for MS, alemtuzumab was used in patients with advanced progressive disease. Although radiological outcomes were encouraging, disability accumulation continued with increased cerebral atrophy 7 years after treatment.[300, 301, 303, 322] In contrast, patients with relapsing disease experienced a reduction in annualised relapse rates (ARR) and an improvement in disability. This dichotomy of clinical outcomes between patients treated at an earlier stage of disease and those with progressive disease offered important insights into disease pathogenesis and timing of interventions. Early disease was concluded to be the result of a more active inflammatory demyelinating phase and followed by a later phase of axonal degeneration and accumulation of disability. Subsequent investigation therefore focused on the inflammatory disease subtype characterized clinically by a relapse dominant disease course, with two open label trials in treatment naïve and treatment refractory patients showing encouraging clinical outcomes.[323, 324]

3.1.4 Clinical trials (CAMMS223, CARE-MSI & CARE-MSII)

Early open label studies demonstrated a marked reduction in relapse rates and slowing of disability accumulation when given early in the course of disease.[300, 301, 303, 322-324] The phase II (CAMMS223)[9] and two phase III (Comparison of Alemtuzumab and Rebif® Efficacy in Multiple Sclerosis (CARE-MS) I & II)[240, 241] clinical trials were undertaken following positive early experiences. CAMMS223 compared low- and high-dose alemtuzumab against a high dose active comparator (subcutaneous interferon beta 1-a, Rebif®, 44µg three times weekly) in patients with early, active, relapsing-remitting MS.[9] CARE-MSI[240] and CARE-MSII[241] investigated the use of alemtuzumab in treatment naïve patients and in patients previously on disease modifying therapy who had experienced an inadequate response (≥1 relapse) respectively. As with the phase II study, interferon beta 1-a was used as an active comparator. Inclusion criteria and clinical outcomes for these trials are summarised in Table 3.1.

	CAMMS223	**CARE-MSI** (treatment naïve)	**CARE-MSII** (previous treatment)
	All patients		12-mg group only
Number of alemtuzumab treated patients	222	376	426
Follow-up (years)	3	2	2
Relapse rate reduction (alemtuzumab vs interferon beta-1a)	74% ($p < 0.001$)	55% ($p < 0.0001$)	49% ($p < 0.0001$)
Annualised relapse rate (alemtuzumab vs. interferon beta-1a)	0.10 vs. 0.36	0.18 vs. 0.39	0.26 vs. 0.52
% patients with 6-month SAD	9% vs. 26% ($p < 0.01$)	8% vs. 11% (not significant)	13% vs. 21% ($p < 0.01$)
Change in mean EDSS from baseline	Improvement of 0.39 compared with deterioration of 0.38 on Interferon beta-1a ($p < 0.01$)	No significant change	Improvement of 0.17 compared with deterioration of 0.24 on interferon beta-1a ($p < 0.0001$)
Deaths	1 (ITP), 1 (myocardial infarction)	1 (RTA)	1 (RTA), 1 (aspiration pneumonia)
Autoimmunity			
Thyroid	26%	18%	17%
ITP	0.90%	0.80%	1%
Goodpasture's syndrome	0	1	0
Neoplasia (alemtuzumab vs. interferon beta-1a)	2.8% vs. 0.9%	0.5% vs. 0	0.6% vs. 1.5%

Table 3.1. Clinical outcomes and adverse events of alemtuzumab treated patients in phase II (CAMMS223) and phase III (CARE-MSI and II) clinical trials. Table adapted from Coles AJ.[325] Abbreviations: SAD; sustained accumulation of disability. RTA; road traffic accident. EDSS; expanded disability status score.

3.1.5 CAMMS223

334 treatment naïve patients with a diagnosis of relapsing-remitting MS were randomised to alemtuzumab 12mg/day, alemtuzumab 24mg/day or high dose subcutaneous interferon beta 1-a three times weekly. Results from this study were impressive both for clinical and radiological outcomes. The pooled (12mg and 24mg) alemtuzumab groups demonstrated a reduction in annualised relapse rate (ARR) of 74%, reduction in sustained accumulation of disability (SAD; a ≥1-point increase in Expanded Disability Status Score (EDSS)[326] from baseline if baseline EDSS >0, or ≥1.5 point increase if baseline EDSS=0, persistent over a 6-month period) of 71% and improvement in mean EDSS score of 0.39 points at 36 months. In contrast, patients treated with interferon beta 1-a experienced a worsening of EDSS score of 0.38 points over the same time period. Radiologically, reduction in brain volume was significantly less in the pooled alemtuzumab treatment group. Similarly, although reduction in lesion volume on T2-weighted MRI was seen in both alemtuzumab and beta interferon patients, this was more notable in the alemtuzumab groups, with significance seen at 12 and 24 months; however, at 36 months this effect was not significant.[9]

The cohort of patients involved in CAMMS223 continued to demonstrate improvements in EDSS at 5 years of follow-up although the majority of this effect was in the first 36 months.[260] A post-hoc analysis using a new disability outcome, sustained reduction of disability (SRD, a reduction from baseline of at least 1 EDSS point confirmed over 6 months for patients with a baseline EDSS ≥2.0) demonstrated more alemtuzumab treated patients achieved this outcome compared with interferon treated patients.[327]

3.1.6 CARE-MSI & CARE-MSII

In the phase III follow-up to CAMMS223, CARE-MSI and CARE-MSII investigated alemtuzumab therapy in treatment naïve and treatment experienced patients respectively. These studies were conducted over a 2-year period with the primary endpoints of ARR and time to 6-month SAD.[240, 241] In CARE-MSI, patients received alemtuzumab at a dose of 12mg/day.[240] In CARE-MSII alemtuzumab patients were randomised to a dose of either 12mg/day or 24 mg/day although after one year of the study, all patients received 12mg.[241] Discontinuation of randomization to the

24mg/day group was undertaken because of safety concerns following the reported case of ITP but also to aid recruitment to the remaining study groups.

Once again alemtuzumab demonstrated superiority to interferon beta 1-a. Patients experienced a reduction of ARR in CARE-MSI and CARE-MSII by 55% and 49% respectively.[240, 241] EDSS score was also improved in the alemtuzumab groups in both studies. Although this was significant in CARE-MSII (improvement of 0.17 points on alemtuzumab vs. a worsening of 0.24 in the interferon beta 1-a group) both groups experienced an improvement in EDSS in CARE-MSI (improvement of 0.14 points in both groups), which did not achieve significance.[240, 241] Similarly, in CARE-MSII significantly fewer patients had SAD (13% vs. 20%) and more patients had SRD (22% vs. 9%) in the alemtuzumab group. Again, in contrast to CARE-MSII significance was not achieved in SAD in CARE-MSI, although SRD was not measured.[240, 241]

Radiological outcomes were also significantly better in the alemtuzumab treated patients compared with interferon beta 1-a. In particular, change in brain volume (BV), gadolinium enhancing lesions and patients with new or enlarging T2 hyperintense lesions on MRI were significantly better in the alemtuzumab groups in both studies.[240, 241]

Interestingly, it has been suggested that the improvement in disability observed following treatment might be as a result of increased lymphocytic delivery of neurotrophins to the CNS aiding neuroprotection.[328]

3.1.7 Side-effect profile of alemtuzumab

Despite the clear beneficial effects of alemtuzumab on MS disease activity, there have been concerns regarding its side-effect profile, initially cited by the US Food and Drug Administration as a reason not to approve its use in the United States, although later revoked. In particular, secondary autoimmune disease (AID) is said to affect approximately 30% of patients, with the thyroid gland the most common target.[325] Other serious, but less common forms of AID include idiopathic thrombocytopaenic purpura (ITP), haemolytic anaemia, autoimmune neutropaenia and glomerulonephritis (Goodpasture's syndrome).[9, 240, 241, 325] In addition, predictable adverse infusion related

reactions including headaches, rigors, pyrexia and rash affect the majority of patients.[297] Because of these issues, long-term follow-up data from both controlled trials and open label studies will continue to be of value in informing patient selection, retreatment strategies and long-term surveillance protocols.

3.1.8 Aims and objectives

Alemtuzumab has been used in selected centres in the UK since 2000 as a practical, alternative treatment for patients with early, aggressive disease with poor prognostic indicators at a time when access to more effective treatments was limited, and therefore offers a unique opportunity to access long term follow-up data collected in routine clinical practice. Here, I aimed to conduct a study of patients presenting with high relapse rates[329] together with poor prognostic features[330-332] who were considered candidates for treatment with alemtuzumab. In this chapter, data is presented of a 'real-world' experience of the use of alemtuzumab in MS across three UK MS centres, focusing on relapse rates, disability data, re-treatment rates and adverse events.

3.2 Methods

3.2.1 Patients and data collection

Patients referred to and assessed in the neurology department at the University Hospital of Wales, Cardiff were identified as candidates for treatment with alemtuzumab if they had a relapsing disease course and evidence of aggressive disease characterised by a high relapse rate, active disease on cranial MR imaging, rapidly accumulating disability, early motor, cerebellar or cognitive dysfunction or combinations of these factors, and were considered to have poor prognosis. A smaller number of patients were treated locally at regional specialist neuroscience centres in Swansea and Bristol following regional network case-based discussions. Patients receiving alemtuzumab as part of externally sponsored clinical trials were excluded from analysis.

Prior to treatment all patients had a normal blood count, thyroid function tests, routine blood indices and white cell immunophenotyping. At the time of treatment no patient had evidence of active infection and treatment during relapse was avoided whenever possible. Consent for treatment was obtained and explanation of potential risks and benefits provided.

3.2.2 Treatment regimen and adverse event monitoring

Prior to 2006, patients received an initiation dose of 24–30 mg alemtuzumab intravenously per day for 5 days, with 1 g intravenous methylprednisolone given as pre-treatment on the first 3 days only in order to ameliorate the expected infusion reaction side-effects related to cytokine release.[297] After 2006, the daily dose of alemtuzumab was reduced to 12 mg. Routine top-up treatment was administered after 12 months, consisting of 3 daily doses of alemtuzumab with concurrent steroid pre-treatment. Additional courses were given as indicated after intervals of not less than 12 months, as a result of one or more of the following factors; (1) disabling clinical relapse, (2) evolving disability with or without objective change in EDSS, (3) the development of new or enhancing lesions on MRI performed 12 months or more after a prior treatment cycle.

A monitoring program for adverse autoimmune events included monthly full blood count and urea and electrolytes in addition to thyroid function tests with anti-thyroperoxidase antibodies at least 6 monthly intervals. Urinalysis was performed when indicated or during concomitant illness. Additional tests for relevant AID-related antibodies were performed when appropriate.

3.2.3 Data analysis

Patients were identified from a regional clinical database[298] and a systematic review of notes was performed to validate the dataset. Data was collected on demographics, EDSS scores, relapses, adverse events and prior medication use in order for further analysis to be performed. Final data capture was performed on 23rd April 2015. Retreatment rates, annualised relapse rates (ARR) pre- and post- treatment, disability outcomes, adverse events including rates of AID and outcomes of pregnancies were investigated. 6-month sustained accumulation (SAD) and reduction of disability (SRD) was calculated according to established definitions.[299]

3.3 Results

3.3.1 Demographics

One hundred patients treated with alemtuzumab since 2000 were identified (female 67, male 33) with a total follow-up of 607 patient-years. Ninety-seven patients had relapsing onset disease at time of first treatment. Three patients were subsequently re-classified as secondary progressive disease with frequent relapses with the benefit of hindsight. Demographic characteristics of the cohort are summarised in Table 3.2.

Demographics	All patients	Responders	Non-responders
Number of patients	100	96	4
Female	67	63	4
Relapsing disease	97	93	4
Mean age at disease onset (SD)	28.4 years (8.9)	28.5 (8.9)	26.5 (10.5)
Mean baseline EDSS (SD)	4.0 (1.9)	3.8 (1.9)	4.5 (1.1)
Mean time from disease onset to first treatment (SD)	4.4 years (3.7)	4.4 years (3.7)	4.4 years (2.1)
Mean time from diagnosis to first treatment (SD)	2.0 years (2.0)	2.0 (2.1)	1.3 (1.7)
Mean follow-up post first treatment	6.1 years	6	8.2
Median follow-up post first treatment (range)	6.2 years (0.2–12.9)	6.0 (0.2–12.9)	8.5 (6.2–9.7)
Prior DMT use	27	26	1

Table 3.2. Demographics and baseline characteristics of 100 patients treated with alemtuzumab. SD = Standard deviation. DMT = Disease Modifying Therapy. Non-responders were classified as patients with ≥10 post treatment relapses and a combined post treatment ARR of >1.

The majority of patients (79%) have been followed-up for between 2 and 10 years with a small proportion being followed-up for less than 2 (9%) and more than 10 (12%) years respectively. 27% patients had been on at least one prior disease-modifying therapy (DMT). These included Avonex (4), Azathioprine (2), Betaferon (5), Copaxone (5), Extavia (1), Methotrexate (1), Mycophenolate mofetil (2), Natalizumab (3) and Rebif (18). Of the 3 patients treated with Natalizumab, 2 patients developed thyroid autoimmunity; another patient was subsequently diagnosed with haemolytic anaemia and ITP. Three patients commenced alternative DMTs a mean of 3.4 years following first alemtuzumab infusion. Two patients from this cohort have died; one following an ischaemic stroke 6.9 years after initial treatment and another patient 8 years after the first treatment infusion from aspiration pneumonia.

3.3.2 Retreatment rates

The majority of patients (53%) underwent or were planned to complete the standard 2 cycles of treatment. 28% patients received 3 treatments, 11% 4 treatments and 1 patient 5 treatments. Seven patients received 1 treatment cycle only, with the commonest reasons being: concerns related to monitoring adherence (n=2), development of precancerous comorbidity (n=1) or severe infusion reactions (n=3). One of the first patients to be treated also only received one cycle when experience of using alemtuzumab was more limited. Indications for 53 re-treatment cycles in 40 patients are outlined in Table 3.3.

Reason for retreatment	Number of treatment cycles	%
Clinical relapse only	13	25
New radiological lesions (with or without enhancement) only	14	26
Clinical relapse and new lesions (with or without enhancement)	19	36
Worsening disability and new lesions	2	4
Worsening disability only	5	9

Table 3.3. Reasons for retreatment.

Figure 3.1 demonstrates the temporal relationship between retreatment events and duration of follow-up. Between 2 and 5 years, 27% of patients had been retreated increasing to 51% and 58% at 5 to 10 years and greater than 10 years follow-up respectively.

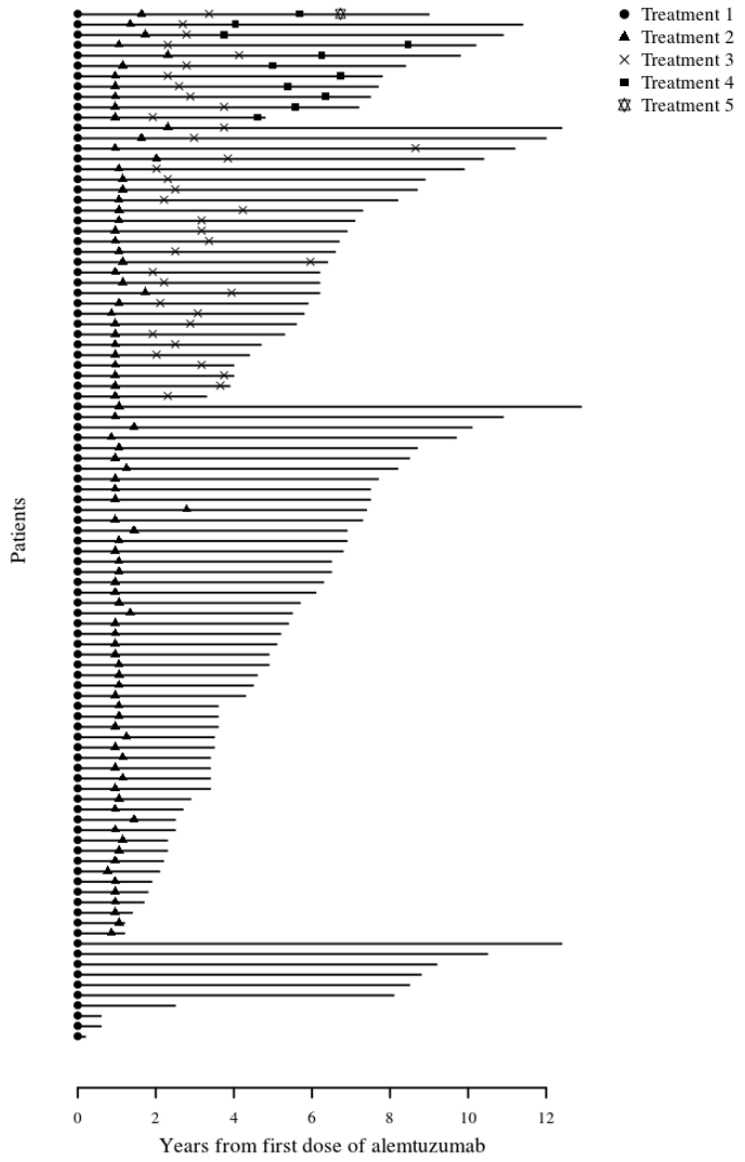


Figure 3.1. Temporal variation in retreatment rates. Horizontal lines represent duration of follow-up for each individual patient from first dose of alemtuzumab. Successive treatments are indicated by the different symbols along each line as indicated in the figure legend.

3.3.3 Relapses

One hundred patients experienced a total of 766 relapses of which 170 (22%) followed the initial treatment cycle. The mean pre-treatment annualised relapse rate (ARR) was 2.1 (median 1.8). Following first treatment cycle the ARR reduced to 0.2 (median 0.1) ($p<0.0001$) (Figure 3.2).

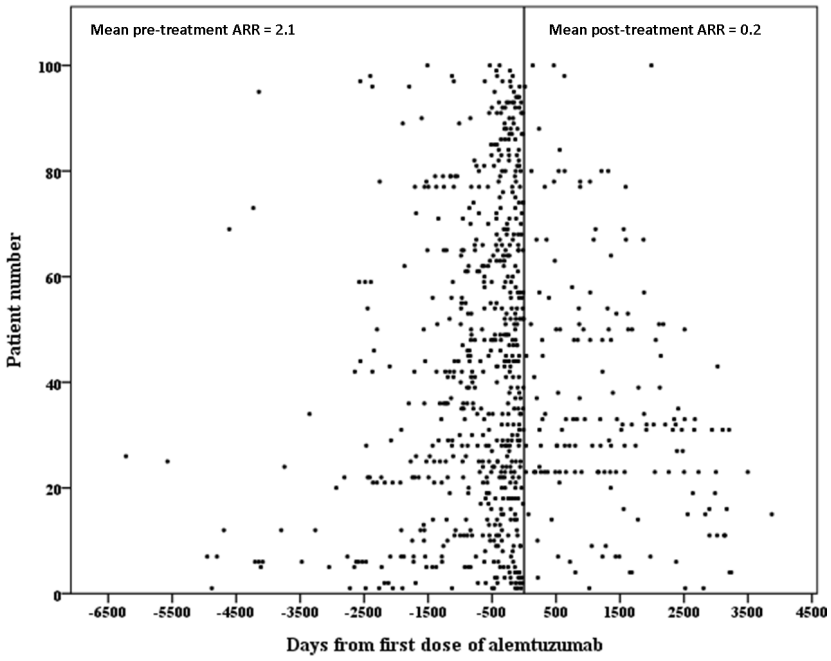


Figure 3.2. Pre- and post-treatment relapses for patients treated with alemtuzumab. Each individual patient is represented across a horizontal line from the y axis. The vertical line represents the start of the first treatment. Pre-treatment relapses (black dots) are shown to the left of the vertical line. Post-treatment relapses are shown to the right of the line.

A small number of patients were unresponsive to treatment and continued to experience frequent clinical relapses: 4 patients had ≥10 post treatment relapses and had a post treatment ARR of >1. The reduction in ARR was sustained over follow-up of up to 8 years (Figure 3.3).

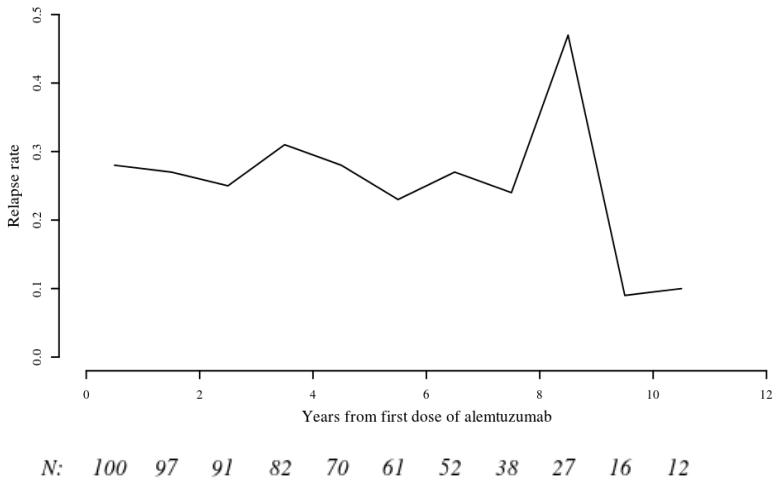


Figure 3.3. Annualized relapse rate by year of follow-up. Mean annualized relapse rate was calculated for each year post first dose of alemtuzumab. The numbers of patients (*N*) for each year - 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10 and 10-11 are shown.

3.3.4 Disability

Disability in MS is rated by the Expanded Disability Status Score (EDSS).[326] This score ranges from 0 (no disability) to 10 (death) and is utilised in clinical trials to assess disability outcomes. In this study, mean baseline EDSS was 4.0. Mean change in EDSS from treatment baseline was +0.14. For 2 patients who died of non-MS related causes, the last EDSS recorded in life was selected as their final EDSS assessment. A negative change in EDSS was seen for each of the first 3 years of follow-up and then again towards the later years of follow-up although the numbers were small in these latter groups (Figure 3.4). 27% had a SAD although none had developed this status within 2 years of follow-up. 25% patients achieved sustained reduction in disability (SRD). Twelve patients (12%) were considered to have developed secondary progressive disease a mean of 3.8 years after initial treatment.

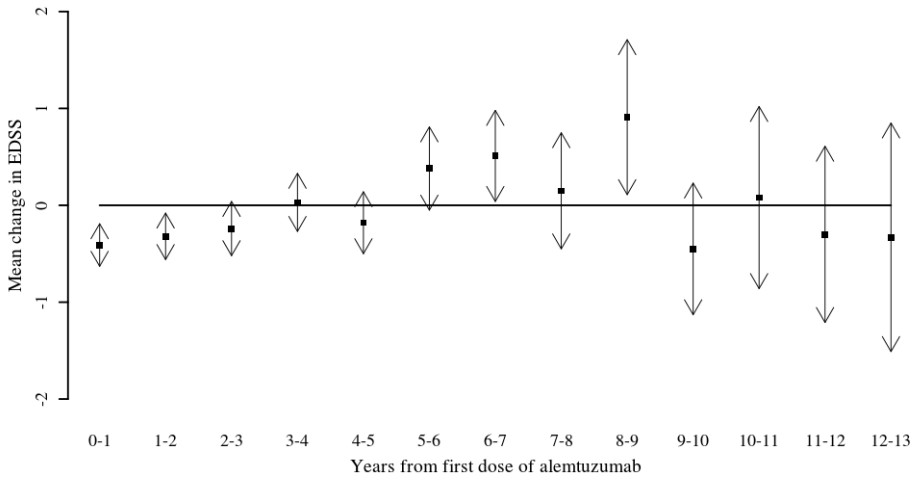


Figure 3.4. Mean change in EDSS by year of follow-up. Patients were grouped by duration of follow-up. Mean change in EDSS in these groups was calculated by comparing the latest EDSS to baseline. Black dots represent the mean. Arrow heads represent the lower and upper 95% confidence intervals.

3.3.5 Adverse events

3.3.5.1 Infusion reactions

87% patients experienced early infusion related adverse events, which occurred despite concomitant steroid use but tended to be mild and responded to conservative treatment.

3.3.5.2 Acquired autoimmune disease

Fifty-one AID diagnoses were made in a total of 47 patients. As noted in previous studies, the thyroid gland was the most common site of autoimmunity with 35% patients affected. Three patients developed idiopathic thrombocytopaenic purpura (ITP) and 13 other separate autoimmune disorders were diagnosed; 1 case each of haemolytic anaemia, pancytopaenia, autoimmune hepatitis, type II diabetes mellitus, and anti-phospholipid syndrome and 2 cases each of alopecia, neutropaenia, autoimmune alveolitis and vitiligo. Mean time to development of AID was 995 days (median 898, range 30 – 3180 days, Figure 3.5) following first treatment and a mean of 578 days (median 394, range 0 – 3180 days) after the most recent treatment.

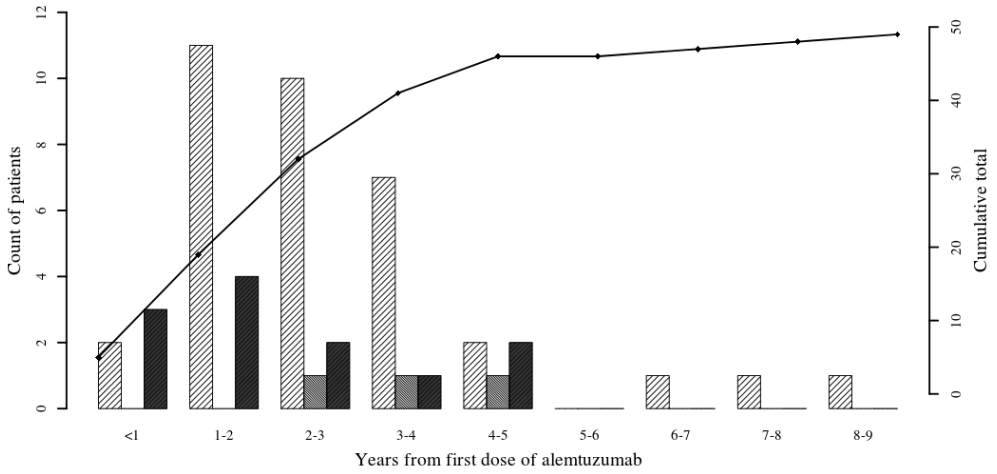


Figure 3.5. Risk of developing autoimmune disease by duration of follow-up. Onset of autoimmune disease is demonstrated in relation to years from first dose of alemtuzumab. Lined columns = thyroid autoimmune disease; grey columns = idiopathic thrombocytopaenic purpura; black columns = other autoimmune conditions.

The risk of developing secondary autoimmunity was greatest in the first 5 years of follow-up and reduced after this time. No autoimmune kidney disease was observed in this cohort. In addition to the reported 3 cases of ITP, a transient infusion related thrombocytopaenia was observed in 2 patients but resolved without intervention.

A total of 34 different novel auto-antibodies (excluding thyroid receptor and anti-TPO antibodies) were detected in 30 different patients during the period of follow-up; 13 ANA, 9 ANCA, 3 anti-smooth muscle antibodies, 2 anti-centromere antibodies, 2

parietal cell antibodies, 1 rheumatoid factor, 1 anti-dsDNA, 1 anti-cardiolipin, 1 Beta 2 Glycoprotein 1 antibody and 1 anti-GBM antibody. Importantly, the patient who developed anti-GBM antibodies had normal renal function throughout the course of treatment. The majority of these detected auto-antibodies were transient with only 4 antibodies persisting after subsequent testing. These included 1 anti-centromere antibody, 2 ANCA and 1 anti-cardiolipin antibody.

3.3.5.3 Infections

Forty-two documented infections occurred in 23 patients. All infections were mild or moderate in severity and responded to standard treatment. Urinary tract infections were most common (12%). Eight (8%) patients developed herpes zoster and six (6%) respiratory tract infections. Other diagnoses were less common and included; influenza (3%), pityriasis (2%), sinusitis (2%), tonsillitis (2%), genital herpes simplex (1%), conjunctivitis (1%), mastitis (1%), mumps (1%), acute cholangitis (1%) and cellulitis (1%). One patient (1%) developed cryptosporidium infection during a hospital inpatient stay for a surgical operation.

3.3.5.4 Pre-malignant/malignant conditions

Ten patients developed pre-malignant or malignant conditions during the period of follow-up. Five patients (5%) developed cervical dysplasia, 3 patients (3%) were identified with a low level IgG paraprotein or monoclonal gammopathy of uncertain significance (one of which was also diagnosed with meningioma) and 2 patients were diagnosed with basal cell carcinoma.

3.3.5.5 Pregnancy

Thirteen pregnancies were recorded in twelve women (18%). Two pregnancies resulted in miscarriage and one was terminated. The child of one patient who developed thyroid AID following treatment experienced transient neonatal hyperthyroidism.

3.6 Discussion

Alemtuzumab has had encouraging results in both clinical trials and open-label studies but long-term follow up data is required to confirm its efficacy and safety. Tuohy et

al[299] have recently published long-term results of a cohort of patients treated in open-label studies in Cambridge, UK but additional data from other centres is also required to understand practical application and longer-term adverse events in routine clinical practice.

3.6.1. Disability outcomes and durability of treatment

In the phase II (CAMMS223) and phase III trials (CARE-MSI and CARE-MSII), alemtuzumab was shown to reduce the ARR by 74%, 55% and 49.4% respectively. The findings in this study confirm that up to a mean 6.1 year follow-up the percentage reduction in ARR is maintained and in this cohort was 90%. In particular, the treatment seems to be durable in relation to relapses up to 8 years following treatment. After this time the ARR reduces but the number of patients in this group is small. Four patients clearly did not respond to treatment with ≥10 post treatment relapses and a combined post treatment ARR of >1 and represent an interesting sub-group which may warrant more detailed analysis of disease biology. However, these data are commensurate with the long-term efficacy outcomes of the Cambridge cohort where 52% received the standard 2 cycles of treatment, 36% received 3 cycles, 8% 4 cycles and 1 patient 5 cycles.[299] In our dataset the trend for requiring re-treatment increased over time implying that the majority of patients are likely to require further treatment cycles. So far 40% of patients have required retreatment. An important practical consideration was that significant cognitive deficits were identified as a barrier to informed consent and adherence to long term monitoring protocols and we have now altered our local selection criteria to offer alternative treatments for these patients. Three (3%) patients were also intolerant of treatment as a result of severe infusion reactions or pancytopaenia following infusion.

Previous studies have suggested an expectation of an improvement in EDSS from baseline following treatment with alemtuzumab when compared with an active comparator (interferon beta 1-a). In the CAMMS223, phase II study, the mean change in EDSS from baseline was -0.39 ($p<0.001$),(15) CARE-MSI -0.14 ($p=0.97$),(16) CARE-MSII -0.17 ($p<0.0001$)(17) and CAMMS223 5 year follow-up -0.3 ($p=0.0002$).(27) Although the improvement in EDSS in the CAMMS223 cohort achieved statistical significance over 5 years of follow-up, this significance was not observed when months

36-60 only were analysed. This would imply that the proposed effect on disability was short-lived and not sustained. Conversely our cohort experienced an overall worsening in EDSS score of +0.14 from treatment baseline. The previous phase II and two phase III trials have shown rates of SAD in the alemtuzumab treatment groups of 9%, 8% and 13% at 3, 3, and 2 years respectively. Similarly 11% of patients had SAD in the CAMMS223 5-year follow-up study. We identified a much higher proportion of patients who had SAD over 6 years follow-up so that 27% of patients were deemed to have progressed in our cohort. These results however were comparable to the levels of SAD found in the open-label Cambridge long-term follow-up study with 32% of patients found to have SAD over a median 7-year follow-up.[299] Only 27% of patients had a SRD in our cohort compared to 43.5% in the Cambridge cohort.[299] Differences in disability outcomes may in part be explained by the fact that in our cohort there were a larger number of EDSS assessors, as would be expected in a 'real-world' clinical setting, but may have introduced some increased variability. Although the mean change in EDSS score was +0.14, we would still consider this an encouraging outcome given the particularly aggressive disease profile of this cohort of patients.

3.6.2 Adverse events

3.6.2.1 Secondary autoimmune disease

The most significant adverse event of alemtuzumab treatment is secondary AID. Secondary thyroid autoimmunity has previously been shown to be unaffected by the cumulative dose, dosage interval or dosage frequency suggesting that total risk is acquired at the time of first dose.[333] 47% of patients in this study developed AID, with 35% developing thyroid autoimmunity.[9, 240, 241, 300, 303, 333] Rates of ITP (3%) were also comparable to published data. Other AIDs were seen at lower frequencies but without a control group for comparison, the relationship of these to alemtuzumab treatment is difficult to confirm. This risk of developing secondary AID appears to be maximum in the first 5 years following initial treatment, with only three cases (all thyroid AID) seen after this time. This would seem to suggest that autoimmune surveillance should be continued for a minimum of 5 years after first treatment cycle. This is commensurate with the current monitoring guidelines of 4 years after the last dose of alemtuzumab.

Although 34 novel antibodies were detected during post treatment monitoring only 4 of these persisted and have not so far been associated with relevant disease.

3.6.2.2 Infections

Despite alemtuzumab causing profound and prolonged lymphopaenia, serious infections are rare. This is thought to be due to the relative preservation of the innate immune system, haemopoetic stem cells and the nature of subsequent immune reconstitution.[311] In addition, as a result of the interval between treatments, lymphocyte repopulation occurs. Most infections following alemtuzumab treatment are mild to moderate and respond to conventional therapies. We observed similar findings in this study with urinary, respiratory and herpes zoster infections being most common. One case of cryptosporidium infection was associated with an inpatient stay for a surgical procedure. We are unaware of any serious infections occurring in our cohort.

3.6.2.3 Malignancy

Within the clinical trials, the rate of pre-malignant or malignant conditions was 0.5-2.8% although the studies were not powered in such a way as to detect small changes as compared with interferon beta-1a. Outside of trials, one case of malignant melanoma has been reported[334] and a further patient developed Castleman's disease (a prelymphomatous condition) and is now in remission following R-CHOP chemotherapy.[325] We observed pre-malignant/malignant conditions in 10% of patients. In particular, we are aware of 5 female patients who developed cervical dysplasia (5%). The occurrence of cervical dysplasia may be affected by immunosuppression[335] and these data perhaps suggest that stringent pre- and post-treatment cervical screening should be performed and is now included as routine in our protocols. In addition to these findings, an IgG paraprotein or MGUS was also detected on screening in 3 patients. The significance of this is difficult to ascertain at present, but is a novel finding in MS patients receiving alemtuzumab, although persistent paraproteinaemias have previously been reported following alemtuzumab therapy in the context of stem cell transplantation.[336]

3.6.2.4 Pregnancy

The pregnancy rate of 18% in this cohort despite advice on appropriate contraception, may reflect the positive effect on quality of life following alemtuzumab that many patients reported, leaving them more confident to start families. No unexpected adverse pregnancy outcomes have so far been observed in this cohort.

3.6.3 5-year follow-up outcomes of phase III clinical trials

Similar to the results obtained in this open label cohort, the recently published follow-up data from the phase III extension study has demonstrated marked durability over 5 years.[337, 338] For patients enrolled in CARE-MSI and CARE-MSII the low ARR was maintained in year 3 (0.19 and 0.22 respectively) to year 5 (0.15 and 0.18). For years 0-5, 80% patients in CARE-MSI and 75% patients in CARE-MSII were free from 6-month SAD. Impressively, 82% and 77% patients respectively had stable or improved EDSS scores after 5 years and 33% and 43% patients experienced SRD in years 0-5.[337, 338]

In the extension study, radiological changes also appear to have durability after 5 years of follow-up. Median rate of BV loss decreased progressively over 4 years in CARE-MSI and remained low in year 5 (Year 1: -0.59%, Year 2: -0.25%, Year 3: -0.19%, Year 4: -0.15%, Year 5: -0.20%). Similarly, median rate of BV loss progressively slowed over 3 years in CARE-MS II and remained low in Years 4 and 5 (Year 1: -0.48%, Year 2: -0.22%, Year 3: -0.10%, Year 4: -0.19%, Year 5: -0.07%). Strikingly, the majority of patients (69% in CARE-MSI and 60% in CARE-MSII) had not received further courses of alemtuzumab treatment since month 12.[337, 338] Durability of MRI outcomes have also been shown in the extension study with respect to gadolinium (Gd)-enhancing lesions, new/enlarging T2 or new T1 lesions. In years 3, 4 and 5 after initial treatment the proportion of patients free of the aforementioned measures were similar to those in year 2 (i.e. the end of the original phase III studies). In addition, most patients were free of MRI activity in each of years 3, 4 and 5.[337, 338] No new safety concerns were highlighted from these follow-up studies.

3.6.4 Conclusion

In conclusion, this follow-up study in a highly selected group of MS patients with poor prognostic indicators treated with alemtuzumab in routine clinical practice confirms a

durable effect on relapse rates but no improvement in disability. AID has affected nearly half of the cohort to date but this figure is likely to rise with longer follow-up. Unexpected findings included 3 cases of a low level IgG paraprotein and 5 cases of cervical dysplasia. Although the lack of a comparative control group does not allow definitive conclusions to be drawn, it will be important to monitor these in larger post-marketing surveillance studies. However, alemtuzumab appears to be an effective treatment for relapsing MS in routine clinical practice, and its side-effects for the most part are predictable and treatable. The clinical efficacy of alemtuzumab and its unique mechanism of action also help to confirm a central role for T cells in MS disease pathogenesis.

Chapter 4

Immunophenotyping of cerebrospinal fluid-resident T cells in Multiple sclerosis

4.1 Introduction

Genome wide association studies (GWAS),[42] histology,[47] animal models[181], *in vitro* experiments,[63] and response to therapeutics[325] provide evidence for the role of T cells in Multiple sclerosis (MS). Although T cells constitute the most common cell type present in cerebrospinal fluid (CSF), both in MS patients and controls,[92] the definitive immunophenotype of these cells has not been consistently reported. It is therefore important to understand what T cell subpopulations are present in CSF and whether there is any difference between MS patients and controls. Firstly, this will help to further understand disease pathogenesis and the role for T cells and secondly to be able to identify certain cellular subpopulations as therapeutic targets.

4.1.1 T-cell differentiation

Following development, T cells are released from the thymus as naïve T cells (T_N) specific for a given peptide/MHC (pMHC) antigen. After activating in response to their cognate pMHC antigen these cells expand and differentiate into effector cells in order to destroy their target.[96] The expression of different cell surface markers allows identification of the stage of differentiation. The C-C chemokine receptor 7 (CCR7), which mediates homing to lymph nodes[96] can be used in combination with CD45RA, a tyrosine phosphatase[339] to define subsets of T cells based on their stage of differentiation (Figure 4.1).[96] $CCR7^+$ $CD45RA^-$ T cells are called central memory (T_{CM}) T cells due to their potential to home to secondary lymphoid tissues. Conversely, $CCR7^-$ $CD45RA^-$ T cells are called effector memory (T_{EM}) T cells because of their effector function and their potential to home to peripheral lymphoid tissues.[96] Further subsets have been defined including T cells with stem cell-like properties, termed stem cell memory T cells (T_{SCM}), which precede T_{CM} cells in the differentiation pathway.[96] Transitional memory T cells (T_{TM}) represent a further subgroup, which are more

differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells. $CCR7^-$ cells that are CD45RA positive, represent cells with low proliferative and functional capacity, indicating terminal differentiation. These cells are termed terminal effector cells (T_{TE} or T_{TEMRA}).[96] Figure 4.1 outlines the expression of CCR7, CD45RA and other cell surface proteins, which help to define these subsets. Phenotypic, functional and gene expression properties of these T cell subsets supports a linear progression of differentiation in humans (T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, T_{TEMRA}). In addition, the expression of other proteins can be used to help determine the differentiation stage or functional properties of these cells.[96] T_N cells are antigen inexperienced and more dependent on co-stimuluatory signals compared with memory T cells. T_N and T_{CM} T cells home to secondary lymphoid organs whereas T_{EM} and T_{EMRA} T cells home to peripheral sites where they exert their effector functions.[340]

In this study, I have focused on determining the frequency of the four main T cell subsets based on the expression of CCR7 and CD45RA; T_N ($CD45RA^+CCR7^+$), T_{CM} ($CD45RA^-CCR7^+$) , T_{EM} ($CD45RA^-CCR7^-$) and T_{TEMRA} ($CD45RA^+CCR7^-$).[93] Of note, as well as CD45RA, CD45RO can be used to determine differentiation state. Cells expressing CD45RO are considered to be of 'memory' phenotype i.e. either T_{CM}, T_{TM} or T_{EM}.[96]

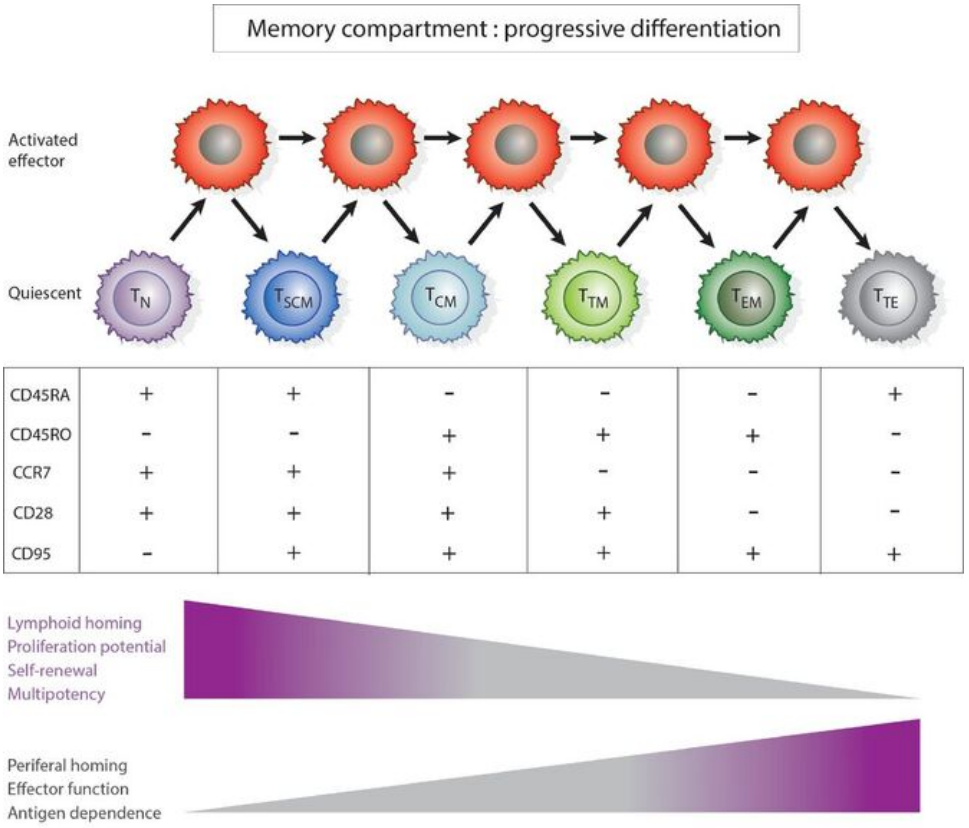


Figure 4.1. Stages of T cell differentiation. Different stages of T cell differentiation can be identified based on the expression of specific cell surface markers. The positive or negative expression of CD45RA, CD45R0, CCR7, CD28, and CD95 identifies six major subsets of T cells. While differentiating, memory T cells lose or acquire specific functions. Following encounter with antigen, these quiescent T cells develop into effectors. When the antigen is cleared, surviving effector T cells return to a quiescent memory state.[96] Figure adapted from Mahnke et al.[96]

4.1.2 The immunophenotype of CSF-resident T-cells: inconsistencies in the literature

In CSF, $CD4^+$ T cells outnumber $CD8^+$ T cells[91, 92], with a significant increase of $CD4^+$ T cells in CSF from MS patients compared with controls.[95] An increased CSF $CD4^+/CD8^+$ ratio has also been observed in MS and inflammatory neurological diseases compared with non-inflammatory disease.[93, 94] The immunophenotype of CSF-resident T cells in MS has been further characterised in several studies although the difficulty in some cases of obtaining CSF from healthy volunteers or a control population makes interpretation of the results difficult. The results of these studies have often been inconsistent with different T cell subpopulations predominating, and either significant

or no significant differences reported compared with controls (summarised in Table 4.1).

Following activation and differentiation in response to antigenic stimuli, T cells acquire an effector memory (or effector memory-RA) phenotype. If $CD8^+$ T cells are pathogenic in MS, it would be reasonable to expect differentiated $CD8^+$ T cells in the CSF. Consistent with this, previous studies have demonstrated that effector memory T cells are the most predominant subtype in the CSF and enriched (compared with peripheral blood) in patients with MS.[79, 93, 97]

When comparing effector memory populations between the CSF and blood, Jilek et al demonstrated that the majority of CSF-resident $CD4^+$ T cells in MS had a central or effector memory phenotype. In this study, the majority of CSF-resident $CD8^+$ T cells also had an effector memory phenotype. The proportion of effector memory T cells was higher in the $CD8^+$ T cell compartment than the $CD4^+$ T cell compartment, with a higher proportion in patients with active disease i.e. relapsing-remitting MS (RRMS)/possible MS.[97] However, in this study, patients with other neurological diseases (OND) also demonstrated a CSF enrichment with highly differentiated cells.[97] In subsequent studies, effector memory T cells were shown to be the predominant population in the CSF[79, 93] with a higher percentage of effector memory T cells present in the CSF compared to patients with non-inflammatory neurological disease.[93] In the study by Ifergan et al, no control CSF was available for comparison.[79]

Conversely, memory ($CD45RO^+$) T cells have been shown to be the predominant CSF population in MS[91, 100, 102] with either no differences observed between MS and control groups[91], a higher proportion in the $CD8^+$ T cell compartment,[102] or no control CSF for comparison.[100] A reduced frequency of memory $CD8^+$ T cells in the CSF of MS patients compared with healthy volunteers has also been observed.[101]

Other studies have shown an enrichment of central memory T cells in both MS and non-inflammatory neurological disease.[50, 98, 99] In one study of 84 individuals without history of neurological disease or cancer undergoing routine surgery, central memory T cells were the predominant population in CSF-resident T cells.[103]

4.1.3 Aims and objectives

To help clarify the immunophenotype of CSF-resident T cells in MS, we performed, to our knowledge, the most in-depth phenotypic analysis to date using polychromatic flow cytometry. Recent advances have extended the boundaries of flow cytometric analysis through new developments in instrumentation and fluorochrome technology, enabling the simultaneous and independent measurement of up to 18 cell surface markers.[290, 291] MS CSF-resident T cell populations were compared to those from patients with idiopathic intracranial hypertension (IIH) and OND patients as controls.

Author	Year	Cell type	Markers	Tissue	No. of MS Pts.	No. of controls	Findings
Svenningsson et al[91]	1993	$CD3^+$	CD45RO CD45RA other markers*	CSF & Blood	11	6 AM 16 HV	Majority of CSF T cells have a memory phenotype ($CD45RO^+$). No differences among all patient groups. Lower levels of VLA-6 expression in MS and AM CSF compared with HV.
Svenningsson et al[101]	1995	$CD4^+$ $CD8^+$	CD45RO CD29	CSF & Blood	21	15 HV	Reduced frequency of memory ($CD45RO^+$) $CD8^+$ T cells in blood and CSF of MS compared with controls.
Vrethem et al[102]	1998	$CD4^+$ $CD8^+$	CD45RO CD45RA	CSF & Blood	28	13 meningitis 16 OND 16 HV	Majority of CSF-resident $CD4^+$ and $CD8^+$ T cells have a memory phenotype ($CD45RO^+$) in all groups. Higher proportion of $CD8^+$ memory T cells in MS compared to controls.
Giunti et al[98]	2003	$CD4^+$ $CD8^+$	CD45RO CCR7 CD27 Other CXCR/CCR	CSF & Blood	21	14 OIND	Majority of CSF-resident $CD4^+$ and $CD8^+$T cells have a central memory phenotype ($CD45RO^+CCR7^+CD27^+$) Increased expression of CXCR3 and CCR5 in both groups. No differences between MS patients and controls.
Kivisakk et al[50]	2003	$CD4^+$ $CD8^+$	CD45RA CD27 CCR7 CD69 L-selectin	CSF & Blood	0	69 NIND	Majority of CSF-resident $CD4^+$ and $CD8^+$ T cells have a central memory ($CD45RA^-CD27^+$) phenotype. CSF-resident T cells expressed high levels of CCR7 and L-selectin.
Kivisakk et al[99]	2004	$CD4^+$	CD45RO CD27 CCR7	CSF & Blood	25	29 NIND 2 OIND	Majaroity of CSF-resident $CD4^+$ T cells have a central memory ($CCR7^+CD45RO^+CD27^+$) phenotpye. No difference compared with NIND.
Okuda et al[100]	2005	$CD4^+$ $CD8^+$	CD45RO CD25	CSF & Blood	39	21 HV (blood only)	Majority of CSF-resident $CD4^+$ and $CD8^+$ T cells have a memory phenotype ($CD45RO^+$). No control CSF for comparison.
Jilek et al[97]	2007	$CD4^+$ $CD8^+$	CD45RA CCR7	CSF & Blood	33 MS/poss. MS	19 OND	Majority of CSF-resident $CD4^+$ T cells have a central ($CCR7^-CD45RA^-$) or effector memory ($CCR7^-CD45RA^-$) phenotype. Majority of CSF-resident $CD8^+$ T cells have an effector memory ($CCR7^-CD45RA^-$) phenotype. Enrichment of highly differentiated ($CCR7^-CD45RA^+$) T cells in CSF compared with blood. Higher in $CD8^+$ than $CD4^+$ T cells. Higher in RRMS/Possible MS patients vs. PPMS/OND patients.
Ifergan et al[79]	2011	$CD4^+$ $CD8^+$	CCR7 CD62L (L-selectin)	CSF & Blood	17	10 HV (blood only)	$CD8^+$ only commented on. Majority of CSF-resident $CD8^+$ T cells have an effector memory ($CCR7^-CD62L^-$) phenotype.
de Graaf et al[103]	2011	$CD4^+$ $CD8^+$	CD45RA CD 27/28	CSF & Blood	0	84 non-neurological disease	Majority of CSF-resident $CD4^+$ and $CD8^+$ T cells have a central memory ($CD45RA^-CD27/28^+$) phenotype.
Mullen et al[93]	2012	$CD4^+$ $CD8^+$	CD45RA CCR7	CSF & Blood	37 MS 11 poss. MS	51 OIND 43 OND	Majority of CSF-resident $CD4^+$ and $CD8^+$ T cells have an effector memory ($CCR7^-CD45RA^-$) phenotype. Higher percentage of CSF-resident effector memory T cells ($CD4^+$ and $CD8^+$) in inflammatory diseases compared with non-inflammatory controls.

Table 4.1. Immunophenotyping studies of CSF-resident T cells in Multiple sclerosis. MS=Multiple sclerosis; OIND=Other inflammatory neurological disease; OND=Other neurological disease; NIND, Non-inflammatory neurological disease; CSF=Cerebrospinal fluid; HV=Healthy volunteers; AM=Aseptic meningitis; RRMS=Relapsing remitting Multiple sclerosis; PPMS=Primary progressive Multiple sclerosis. *other adhesion/activation markers

4.2 Methods

Patients attending for diagnostic lumbar puncture for investigation of Multiple sclerosis were consented under pre-existing ethical agreements. In addition, patients being investigated or treated for idiopathic intracranial hypertension and other neurological diseases were included in the study. As the majority of patients were attending for diagnostic investigations, subsequent clinical case note review was required to confirm eventual diagnoses. No patients had received prior disease modifying therapy. Up to 10ml cerebrospinal fluid (CSF) was obtained and processed within 1 hour of collection (Figure 4.2). Twenty millilitres of peripheral blood was obtained immediately after lumbar puncture, which was then processed and stored for further experiments (discussed in later chapters of this thesis).

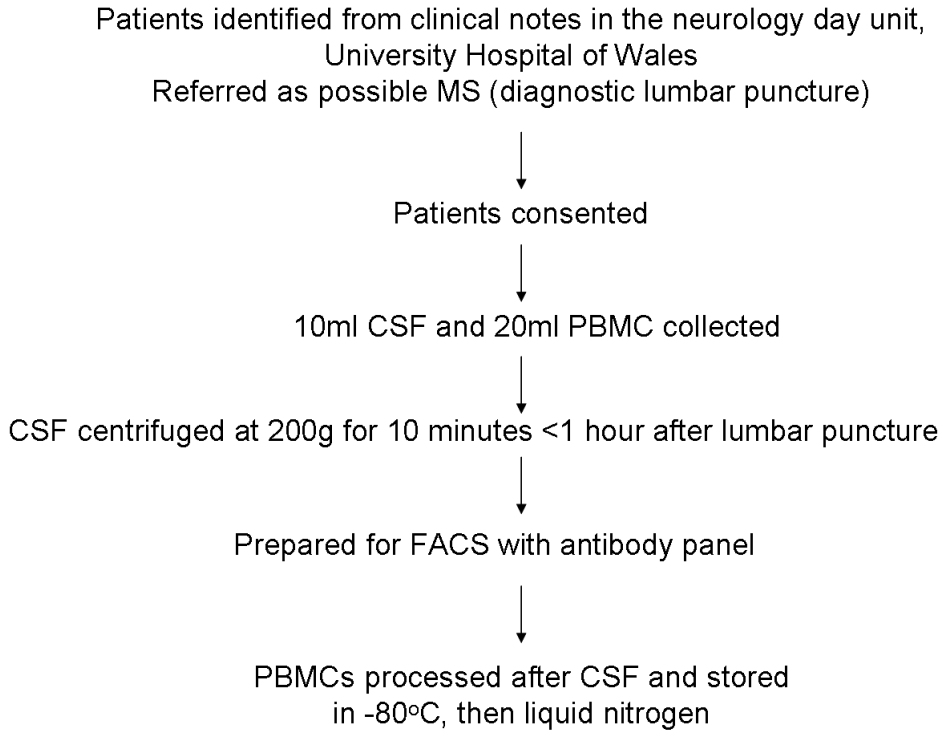


Figure 4.2. Flow chart for collection, handling and analysis of clinical samples.

CSF was centrifuged at 2000 rpm for 10 minutes and the cell pellet resuspended in 50µL phosphate buffered saline (dPBS, Sigma-Aldrich, Gillingham, UK). Cells were then stained with aqua (Thermofisher, Waltham, MA, USA) for 10 minutes at room temperature and subsequently incubated at 4°C for 20 minutes with the following antibody panel CD14 V500, CD19 V500, CD3 APC-H7, CD8 BV711, CD4 PECy5.5, CD27

Qdot605, CD45RA ECD, CD57 FITC, CD95 PE-Cy5, CCR7 PE-Cy7, CD127 BV421, CD49d APC and PD-1/CD279 PE or Pan γδ PE. Of note, the CSF stain for patients LC20552 and LJ20639 used different antibodies for CD127, CD95 and CD27. For LC20552 – PE, APC and PECy5 respectively; for LJ20639 - Pacific blue, PE and PECy5 respectively. In addition, CD49d was not used for these samples (For a full list of antibodies used for each patient, see Appendix, Section 8.1).

During incubation, corresponding compensation tubes were set up. Firstly, 8 drops of anti-mouse Ig κ/negative control compensation particles (BD, Oxford, UK) were added to 800µL PBS in a FACS tube. 50µL of this solution was then added to each individual compensation FACS tube. The same amount of corresponding antibody used in the cell stain was then added to the relevant tube. Antibodies were left to stain for 10 minutes at room temperature when 150µL PBS was then added. For the CCR7 PE-Cy7 compensation tube 50µL of anti-rat Ig κ/negative control compensation particles (BD, Oxford, UK) was used instead. Following CSF incubation, 1ml PBS was added then cells centrifuged for 2 minutes at 2000 rpm. Supernatant was subsequently discarded and cells resuspended in 100µL PBS in preparation for data acquisition and cell sorting. $CD4^+$ and $CD8^+$ T cells were sorted using a BD FACSAria II (BD, Oxford, UK) into RNAlater (Ambion, Thermofisher, Waltham, MA, USA). After sorting, cells were centrifuged at 13,000 rpm for 2 minutes then stored at -80^{o}C until required. Analysis of acquired flow cytometry data was performed using Flowjo software with cell populations compared using the Kruskall-Wallis test with Dunn's multiple comparison test using Prism software when 3 groups were compared together. When only 2 groups were compared, the Mann-Whitney test was utilised. Fluorescence minus ones (FMOs) were analysed on peripheral blood in order to set gates in Flowjo. It was not possible to perform FMOs on CSF due to the limited number of cells available. Figures 4.3 - 4.5 demonstrate a typical flow cytometry gating strategy.

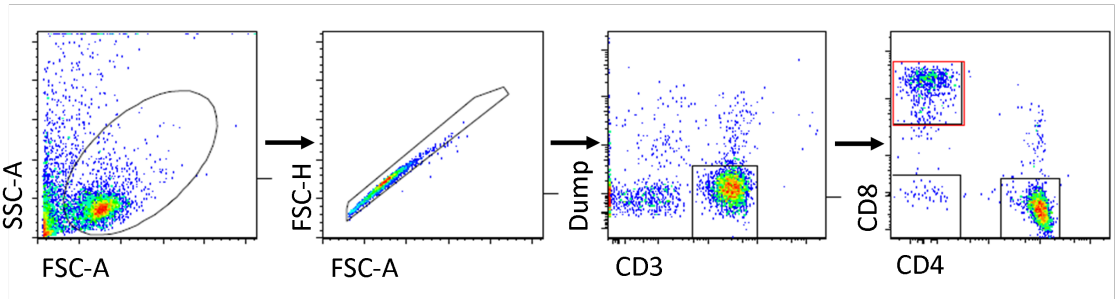


Figure 4.3. Flow cytometric analysis and sorting of CSF-resident CD4$^+$ and CD8$^+$ T-cell populations. The figure depicts a typical flow cytometry sort report from a patient with MS. CSF was spun at 2000 rpm for 10 mins, the supernatant discarded and the remaining cells stained with the following polychromatic flow panel: CD14 V500, CD19 V500, CD3 APC-H7, CD8 BV711, CD4 PECy5.5, CD27 Qdot605, CD45RA ECD, CD57 FITC, CD95 PE-Cy5, CCR7 PE-Cy7, CD127 BV421, CD49d APC and PD-1/CD279 PE or Pan γδ PE. Data were acquired on a custom-built 20-parameter BD FACSAria II flow cytometer.

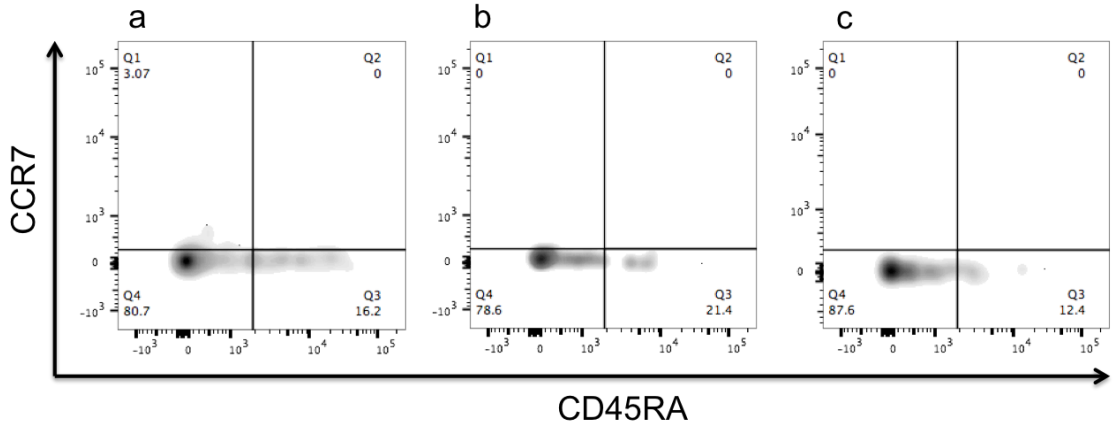


Figure 4.4. Flow cytometric gating strategy to determine stage of cell differentiation (CD45RA vs. CCR7). Examples of gating strategy for CD8$^+$ T cells from selected patients from each patient group. (a) MS, (b) IIH, (c) OND. Cells were prepared as discussed in the methods and legend of figure 4.3.

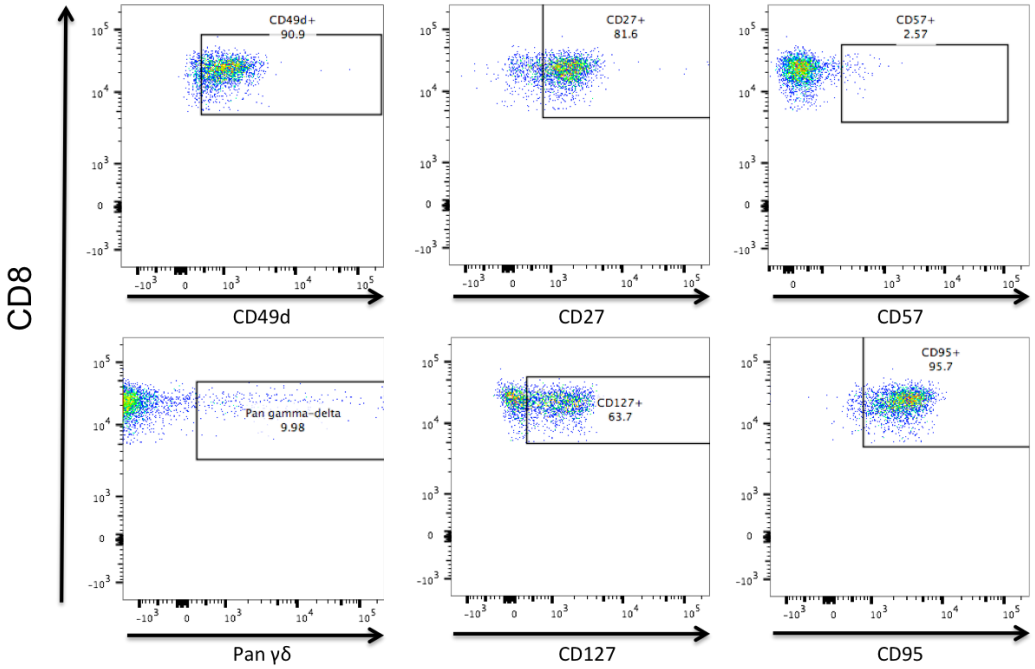


Figure 4.5. Flow cytometric gating strategy to determine expression of individual cell surface proteins. Example of gating strategy used for $CD8^+$ T cells from patient AL22847. Cells were prepared as discussed in the methods and legend of figure 4.3.

4.3 Results

4.3.1 Patient cohort

46 CSF samples were collected; 21 MS/clinically isolated syndrome (CIS) – hereafter referred to as 'MS'; 14 IIH; 11 OND. Of the initial 21 MS CSF samples collected, 1 sample was not sorted due to minimal cells being present (DD22299) and the results of one sample were not saved in error (LS20460). Of the 14 IIH samples collected, 1 was not included in the analysis as it was macroscopically contaminated with peripheral blood (VE25562). Four OND samples were not included in the analysis; 1 due to deficient antibody staining (SE29703), 1 due to a mechanical fault with the FACS Aria II (DL37517), 1 because no live cells were present (JJ37566) and 1 because of visible macroscopic blood (JP24822). Figure 4.6 summarises the samples collected and available for analysis. Tables 4.2 - 4.4 outlines patient demographics for each group, the volume of CSF collected and the number of sorted cells. The MS group contained 8 male and 13 female patients. In the IIH group, all patients were female with 3 male

and 8 females in the OND group. The median age at lumbar puncture was 47.6 years (range 20 – 68.7) in the MS group, compared with 28.6 (21.2 – 45.8) and 45.1 (21.9 – 55.1) in the IIH and OND groups respectively.

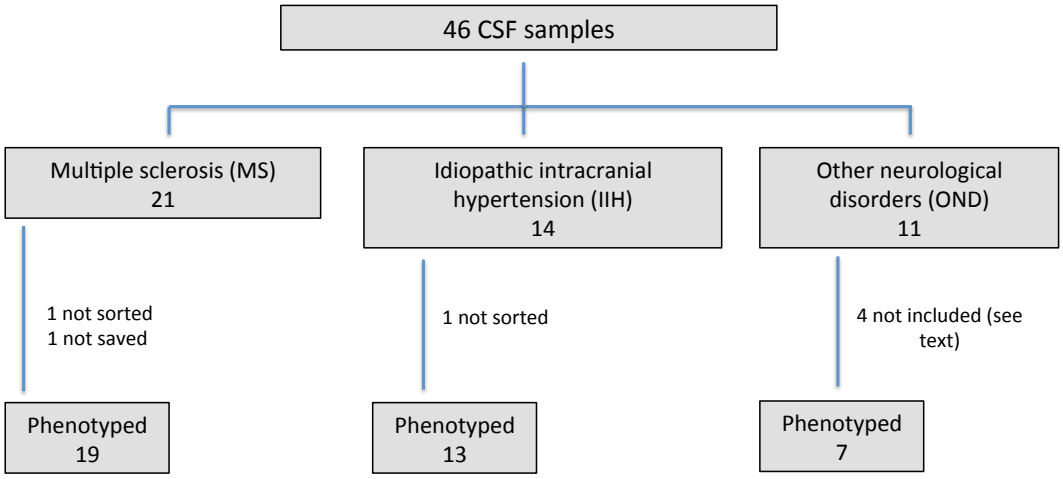


Figure 4.6. CSF samples collected and available for immunophenotyping.

Patient ID	Sex	Age at LP (yrs)	Diagnosis	Course	Vol. CSF (ml)	No. of $CD4^+$ cells	No. of $CD4^+$ cells/ml	No. of $CD8^+$ cells	No. of $CD8^+$ cells/ml
LC20552	F	42.6	MS	RR	7	5078	725.4	618	88.3
LJ20639	F	55.7	MS	PP	10	4923	492.3	361	36.1
LS20460*	F	58.6	MS	RR	10	-	-	-	-
MJ19588	M	39.9	MS	SPR	10	5807	580.7	707	70.7
EB21510	F	35.6	MS	RR	10	6111	611.1	686	68.6
KG19967	F	34	MS	SP	10	1601	160.1	237	23.7
LH18836	F	29.8	MS	RR	9.5	12180	1282.1	719	75.7
NW21326	F	43.1	MS	RR	10	538	53.8	116	11.6
MW21576	M	57.4	MS	PP	10	1877	187.7	272	27.2
CS21983	F	56.1	MS	RR	10	2114	211.4	587	58.7
AL28847	M	51.8	CIS	-	10	6485	648.5	2533	253.3
MK21405	M	63.3	MS	RR	10	3662	366.2	554	55.4
SA23376	F	20	MS	RR	4	4159	1039.8	911	227.8
RM22664	M	31	MS	RR	10	32095	3209.5	4196	419.6
TL22789	F	47.6	MS	RR	10	4985	498.5	743	74.3
CT25364	F	51.5	MS	PP	6	1774	295.7	256	42.7
HD21265	F	32.6	MS	RR	10	1472	147.2	47	4.7
MH21407	M	41.2	MS	RR	10	6884	688.4	452	45.2
RW21309	M	68.7	MS	SP	10	547	54.7	196	19.6
DD22299*	M	57.6	MS	PP	10	-	-	-	-
CG41964	F	51.2	MS	PP	10	5428	542.8	456	45.6

Table 4.2. Demographics of Multiple sclerosis/clinically isolated syndrome patients included in the study. N.B.*denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1. Abbreviations: RR, relapsing remitting; PP, primary progressive; SP, secondary progressive; SPR, secondary progressive with relapses.

Patient ID	Sex	Age at LP (yrs)	Diagnosis	Vol. CSF (ml)	No. of $CD4^+$ cells	No. of $CD4^+$ cells/ml	No. of $CD8^+$ cells	No. of $CD8^+$ cells/ml
EC21870	F	27.9	IIH	10	500	50	57	5.7
HS25204	F	28.1	IIH	10	1075	107.5	85	8.5
LH25311	F	24.1	IIH	10	962	96.2	302	30.2
ML25308	F	21.2	IIH	10	970	97	130	13
SW25353	F	31	IIH	10	4147	414.7	261	26.1
RY21758	F	27.2	IIH	10	737	73.7	42	4.2
DC37877	F	28.1	IIH	10	94	9.4	18	1.8
ES37889	F	33	IIH	10	409	40.9	9	0.9
FC24414	F	43.7	IIH	10	11	1.1	3	0.3
VE25562*	F	27.6	IIH	7.5	-	-	-	-
CC40712	F	45.8	IIH	10	2258	225.8	171	17.1
RC41200	F	29.9	IIH	8	507	63.4	84	10.5
KA38079	F	29	IIH	10.5	3487	332.1	195	18.6
CC41471	F	32.7	IIH	10	655	65.5	61	6.1

Table 4.3. Demographics of idiopathic intracranial hypertension patients included in the study. N.B. *denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1.

Patient ID	Sex	Age at LP (yrs)	Diagnosis	Vol. CSF (ml)	No. of $CD4^+$ cells	No. of $CD4^+$ cells/ml	No. of $CD8^+$ cells	No. of $CD8^+$ cells/ml
SE29703*	M	26.9	Autoimmune encephalitis	10	2541	254.1	303	30.3
JG33488	F	51.6	Normal pressure hydrocephalus	10	742	74.2	69	6.9
ND37140	F	45.1	Fibromyalgia	10	523	52.3	121	12.1
DL37517*	M	45.1	Guillain-Barré syndrome	10	-	-	-	-
CS19395	F	47.3	Cerebrovascular disease	9	54	6	8	0.9
AG20355	F	51.8	Visual field defect of unknown aetiology	5	427	85.4	77	15.4
JM25229	M	31	Pseudopappiloedema - drusen/OSA	10	2672	267.2	499	49.9
AB25236	F	21.9	Migraine	11	3996	363.3	398	36.2
CJ26014	F	28.9	Migraine	10	849	84.9	118	11.8
JJ37566*	F	26	Migraine	10	79	7.9	22	2.2
JP24822*	F	55.1	Small vessel disease	9	217	24.1	88	9.8

Table 4.4. Demographics of other neurological disease patients included in the study. N.B. *denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1.

4.3.2 Cellular constituents of cerebrospinal fluid

$CD4^+$ T cells significantly outnumbered $CD8^+$ T cells across all patient groups (Tables 4.2 – 4.4, & Figure 4.7). Within the MS group a mean of 620.8 $CD4^+$ T cells/ml was observed. This contrasted with means of 121.3 and 109.1 for the IIH and OND groups respectively. The mean number of $CD8^+$ T cells in the MS group was 86.8 $CD8^+$ T cells/ml compared with 11 $CD8^+$ T cells/ml in the IIH groups and 19 $CD8^+$ T cells/ml for the OND patients. Figure 4.8 gives an overview of the numbers of $CD4^+$ and $CD8^+$ T cells per ml of CSF.

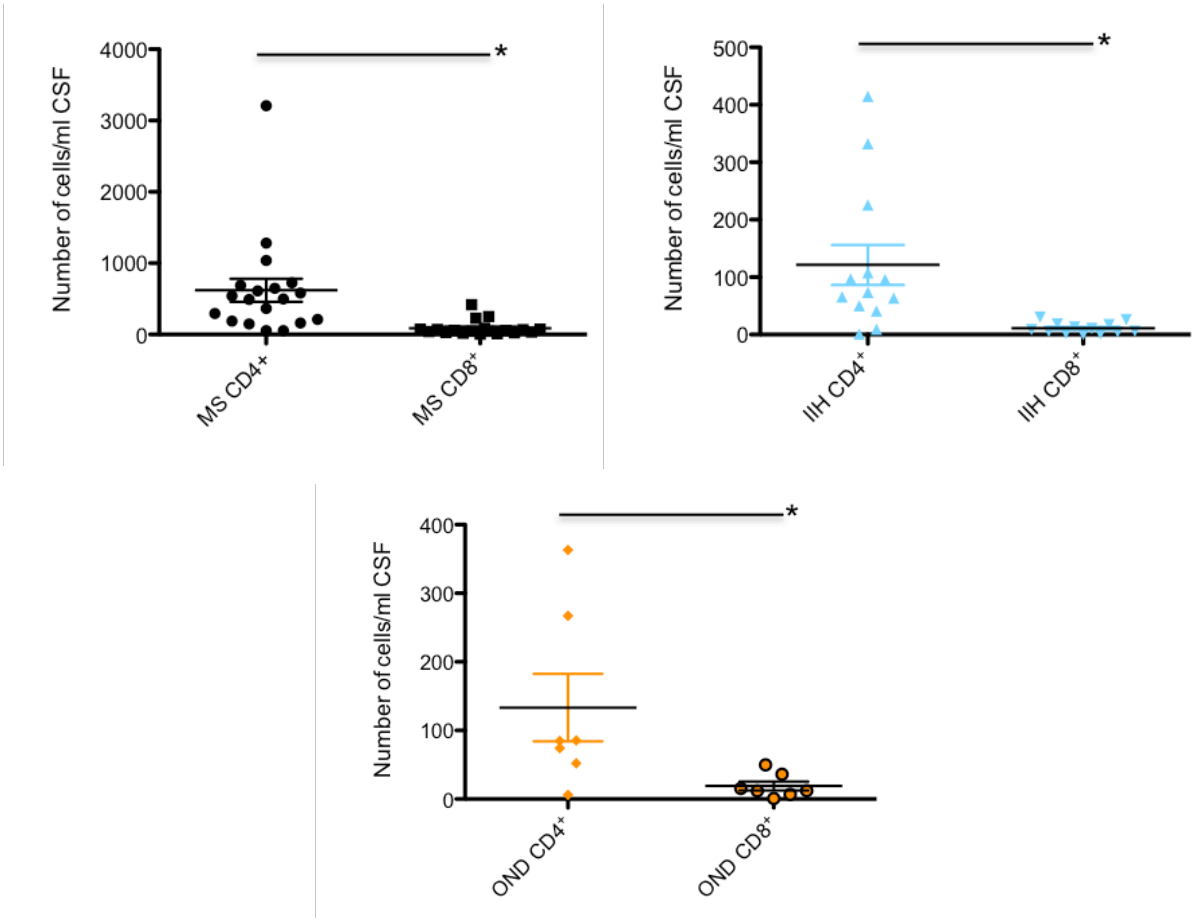


Figure 4.7. Number of $CD4^+$ and $CD8^+$ T cells/ml of CSF collected by patient group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

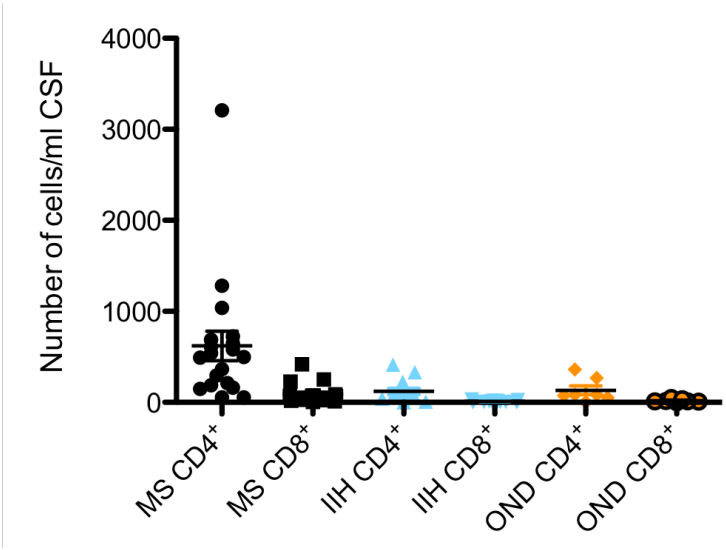


Figure 4.8. Number of CD4⁺ and CD8⁺ T cells/ml of CSF collected. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.

The number of CD4⁺ T cells/ml in MS was significantly higher ($p<0.05$) than the number of CD4⁺ T cells within the IIH and OND patient groups (Figure 4.9). Similarly, the number of CD8⁺ T cells in the MS group was significantly higher than CD8⁺ T cells in the IIH and OND group (Figure 4.10).

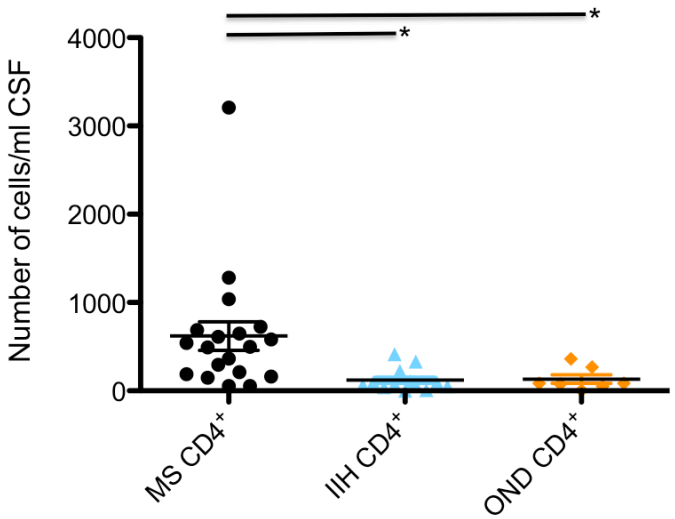


Figure 4.9. Number of CD4⁺ T cells/ml CSF across all patient groups. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

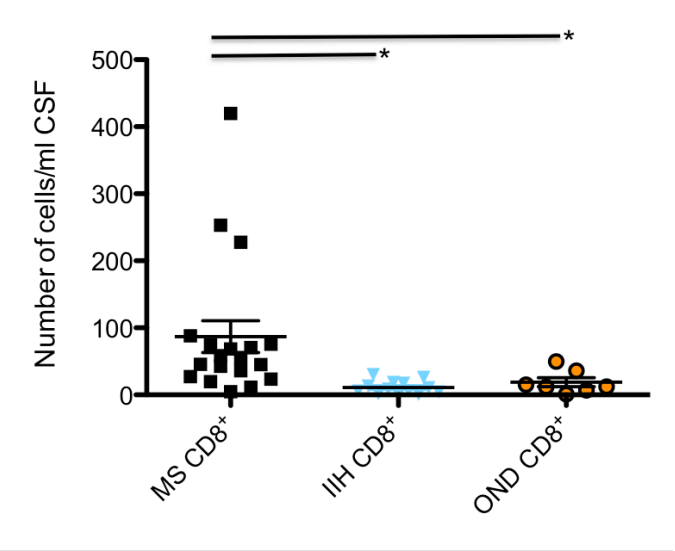


Figure 4.10. Number of CD8⁺ T cells/ml CSF across all patient groups. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

With respect to the percentage of $CD4^+$ and $CD8^+$ T cells within the $CD3^+$ T cell population, $CD4^+$ T cells were the dominant population making up a mean 81.4%, 78.5% and 79.1% $CD3^+$ cells across MS, IIH and OND groups respectively. $CD8^+$ T cells contributed 12.3%, 10% and 12% across the MS, IIH and OND groups (summarised in Figure 4.11).

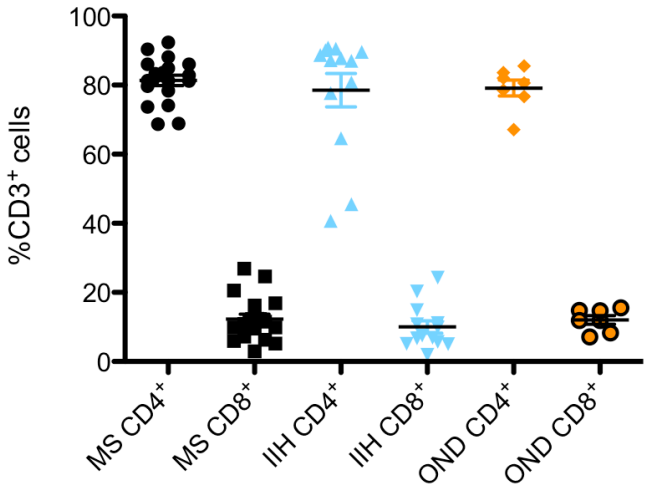


Figure 4.11. Percentage of $CD3^+$ cells that are either $CD4^+$ or $CD8^+$ across all patient groups. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

No significant differences were observed between the percentage of $CD3^+$ T cells, which were either $CD4^+$ or $CD8^+$ in all the different patient groups (Figure 4.12).

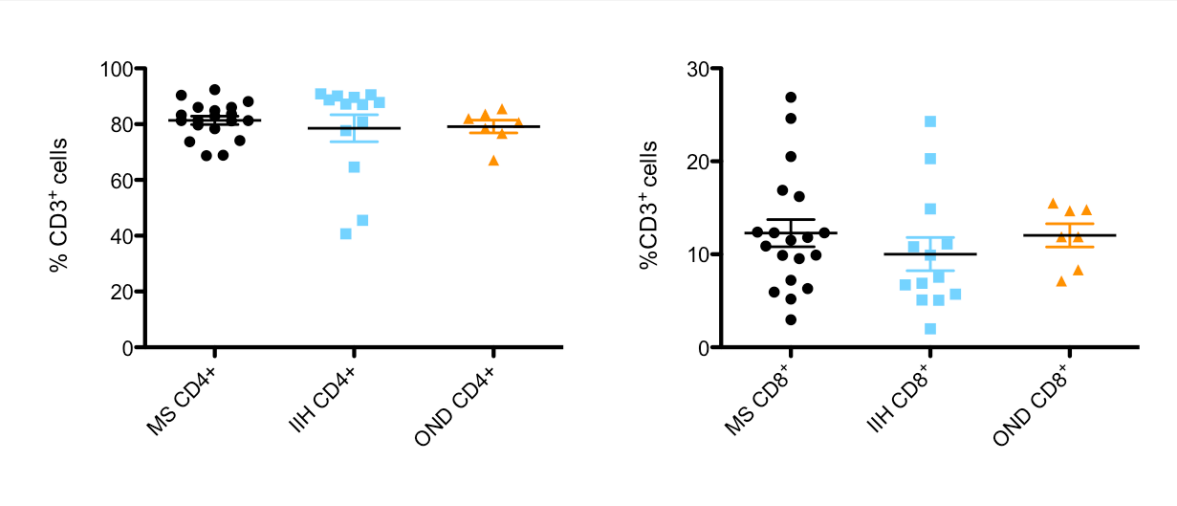


Figure 4.12. Percentage of $CD3^+$ cells that were either $CD4^+$ or $CD8^+$ across all patient groups. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

The percentage of $CD4^+$ T cells in the MS group was significantly higher than the percentage of $CD8^+$ T cells observed in this group. Similarly, the percentage of $CD4^+$ T cells in the IIH group outnumbered $CD8^+$ T cells in the IIH group. In addition, OND $CD4^+$ T cells outnumbered OND $CD8^+$ T cells (Figure 4.13).

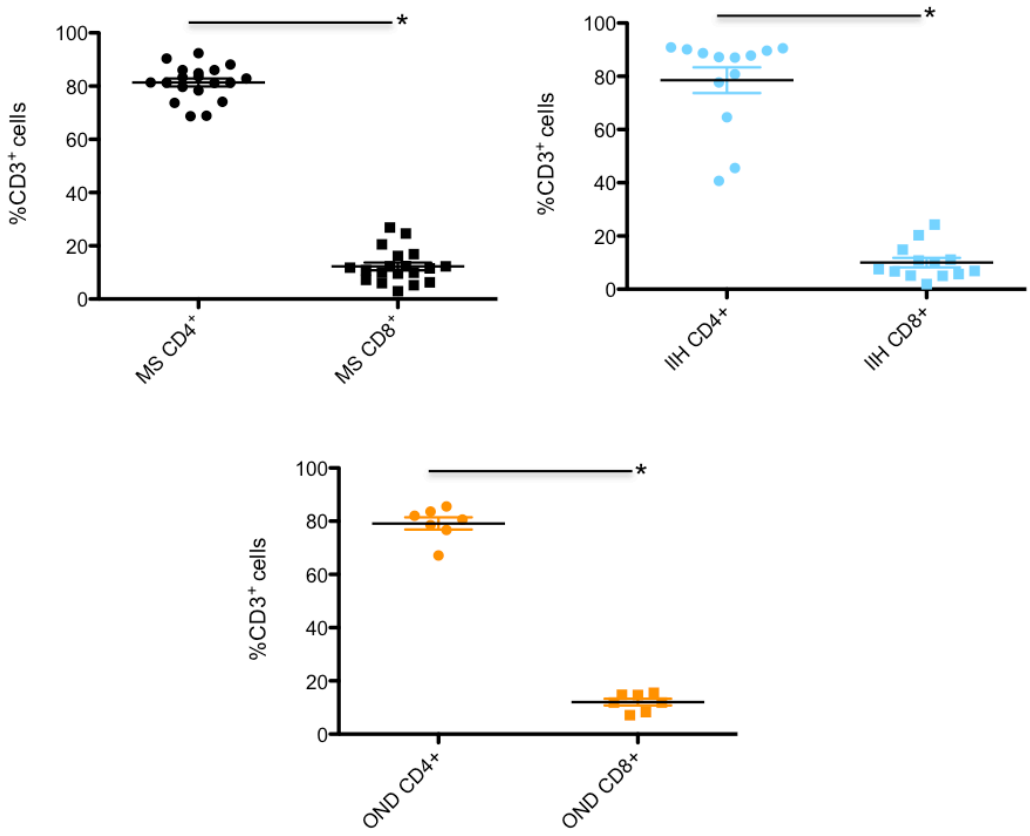


Figure 4.13. Percentage of CD3⁺ cells that were CD4⁺ vs. CD8⁺ across all patient groups. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

4.3.3 Expression of cell surface markers on CSF-resident CD4⁺ T cells

Following determination of the number and percentage of CD4⁺ and CD8⁺ T cells present, further analysis investigated the expression of a variety of cell surface markers. Figure 4.14 gives an overview of the expression of these markers on CD4⁺ T cells.

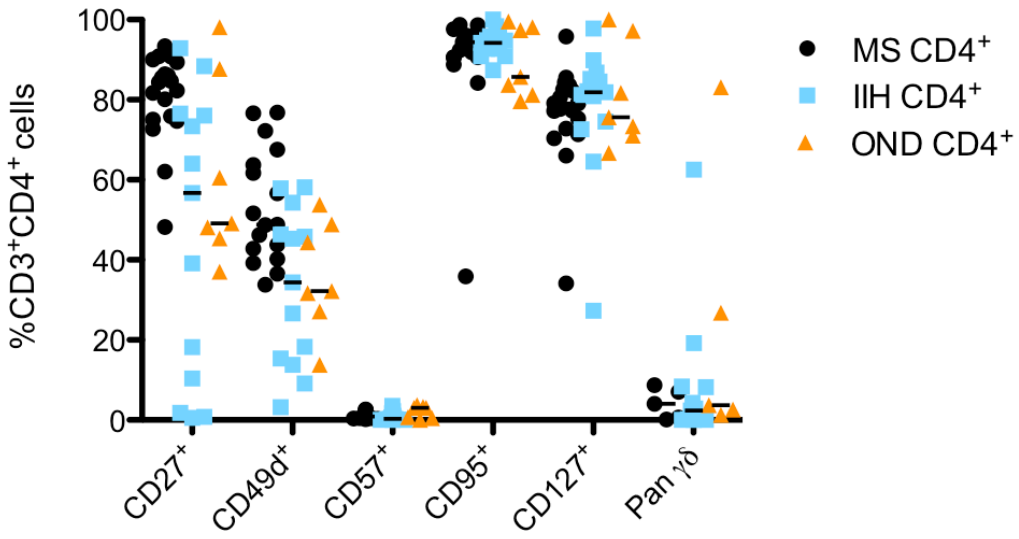


Figure 4.14. Expression of cell surface markers on CD4⁺ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

When comparing individual cell surface markers (Figure 4.15) only $CD4^+CD49d^+$ and $CD4^+CD27^+$ T cells were significantly higher in the MS group compared with IIH $CD4^+$ T cells ($p<0.05$). Otherwise, no differences were observed between the cell surface marker expression in all three groups. $CD4^+$ T cells appear to be of $CD27^+CD49^+CD57^-CD95^+CD127^+$ phenotype. In a subset of MS patients (n=12), PD-1 expression was analysed with a mean expression of 4.6% of $CD4^+$ T cells.

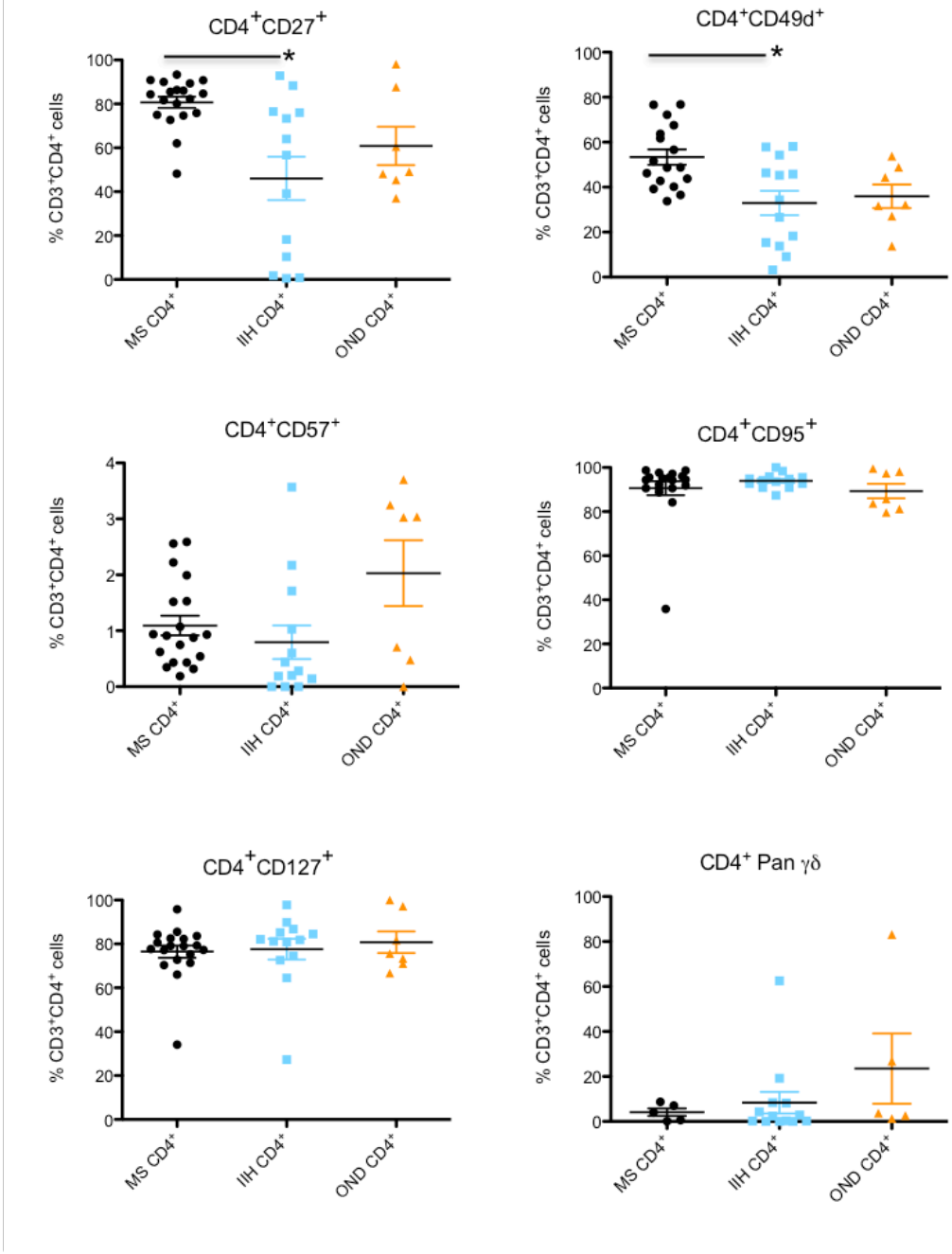


Figure 4.15. Expression of individual cell surface markers on CD4⁺ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

4.3.4 Expression of cell surface markers on CSF-resident CD8$^+$ T cells.

Figure 4.16 gives an overview of the expression of cell surface markers on CD8$^+$ T cells.

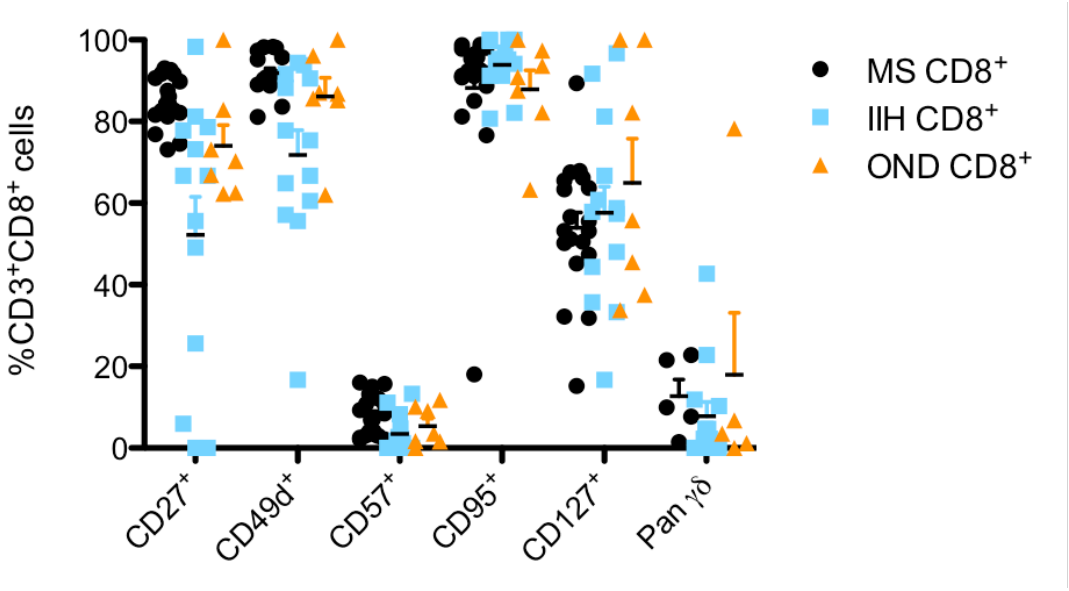


Figure 4.16. Expression of cell surface markers on CD8$^+$ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

In contrast to CD4$^+$ T cells, more differences were observed in CD8$^+$ CSF-resident T cells. In particular, expression of CD27, CD49d and CD57 by CSF-resident CD8$^+$ T cells was significantly higher amongst MS patients than IIH controls (Figure 4.17). However, across all groups, the majority of CSF-resident CD8$^+$ T cells in our study were CD27$^+$CD49$^+$CD57$^-$CD95$^+$ with CD127 expressed more variably when compared with the CD4$^+$ population. A mean of 2.4% CD8$^+$ T cells in the MS group expressed PD-1 (n=10).

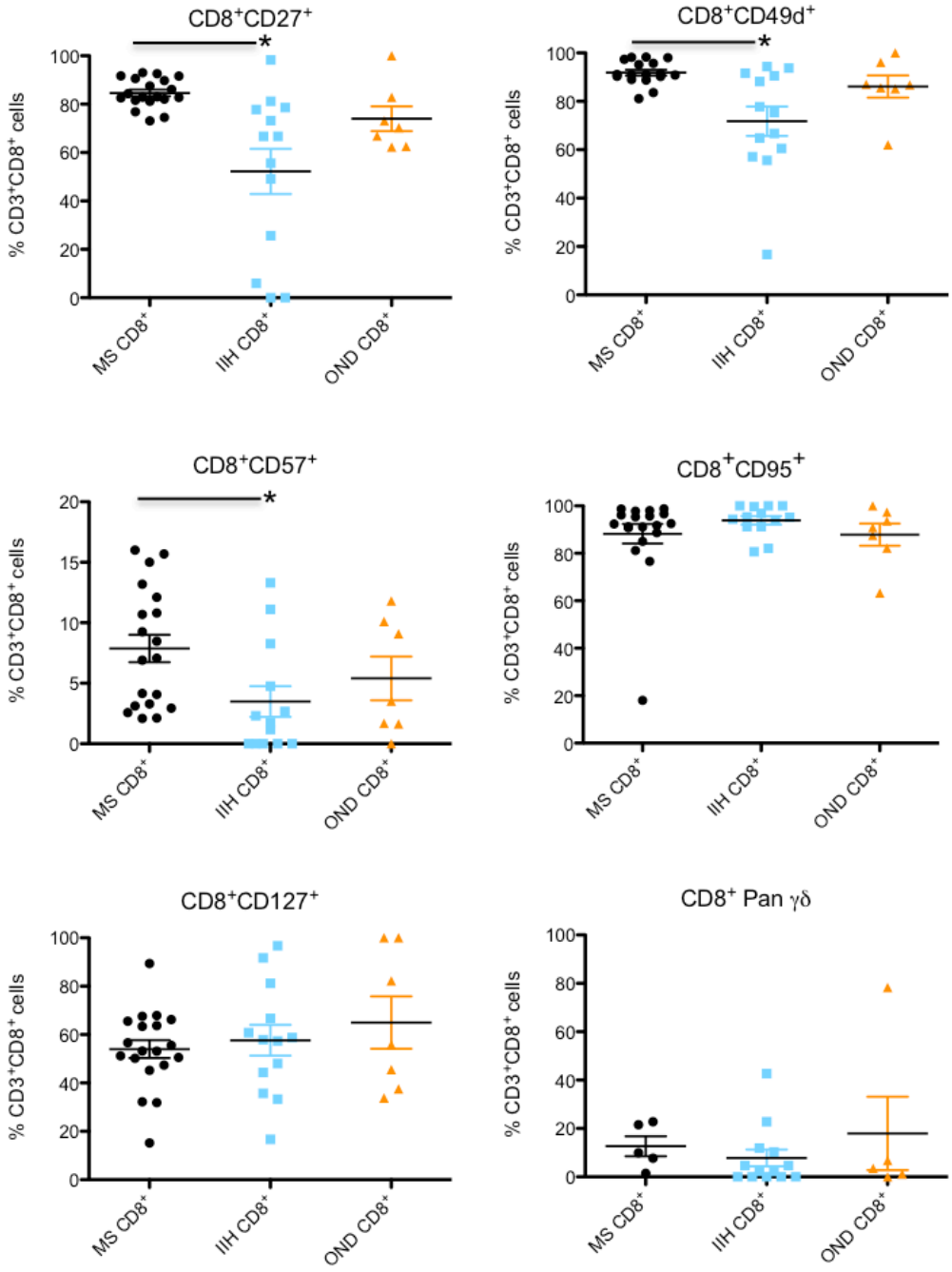


Figure 4.17. Expression of individual cell surface markers on CD8⁺ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = p<0.05

4.3.5 Differentiation of CSF-resident CD4$^+$ T cells

The majority of cells present in the CD4$^+$ T cell population were of the effector memory subtype as determined by the expression of CCR7 and CD45RA (CCR7$^-$CD45RA$^-$) (Figure 4.18).

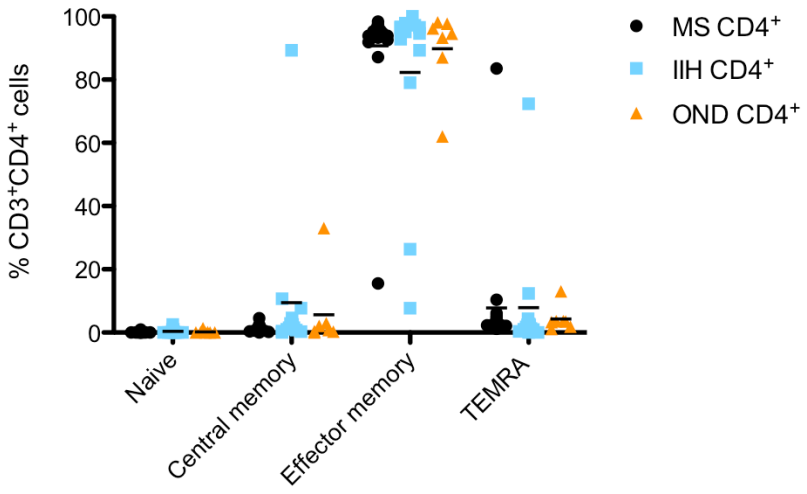


Figure 4.18. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) CD4$^+$ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.

The mean percentage of naïve CD4$^+$ T cells for the MS, IIH and OND groups was 0.1%, 0.4% and 0.2% respectively. For central memory cells the mean percentages were 1.4%, 9.5% and 5.7% for the MS, IIH and OND groups respectively. Effector memory percentages were: MS, 90.7%; IIH, 82.3%; OND 89.8% and for effector memory-RA CD4$^+$ T cells: MS, 7.8%; IIH, 7.9% and OND, 4.3%. No significant differences were observed between patient groups for the percentage of naïve, central memory, effector memory or effector memory-RA cells present (Figure 4.19).

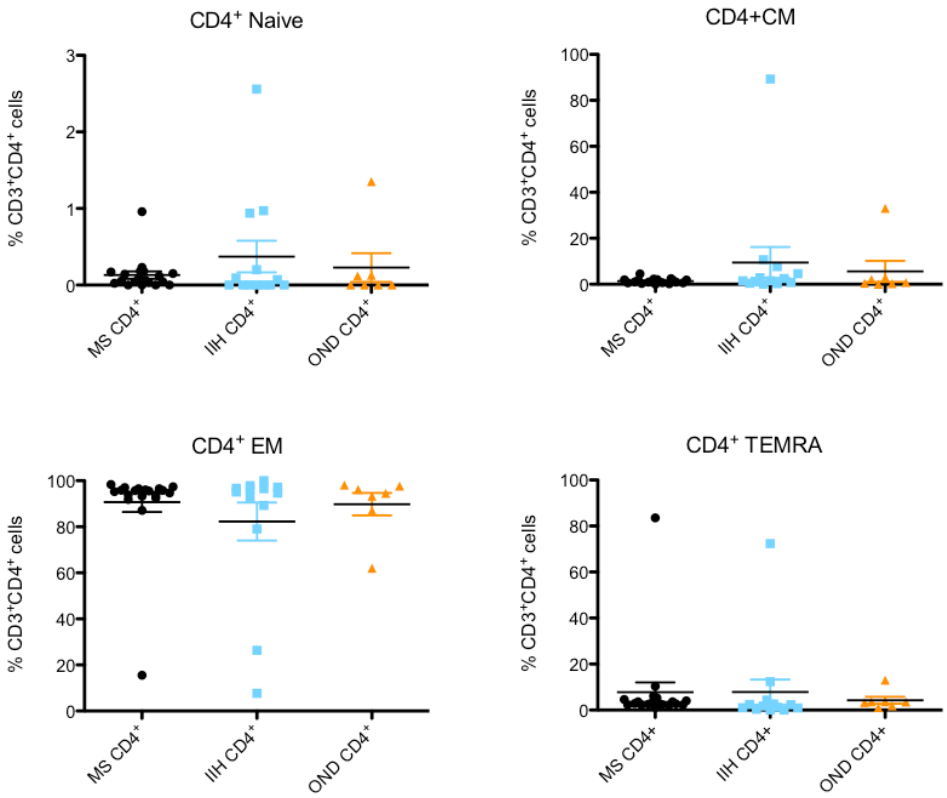


Figure 4.19. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) CD4⁺ T cells displayed by patient group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.

4.3.6 Differentiation of CSF-resident CD8⁺ T cells

As with CD4⁺ CSF-resident T cells, the majority of cells present in the CD8⁺ T cell population were of the effector memory phenotype (Figure 4.20).

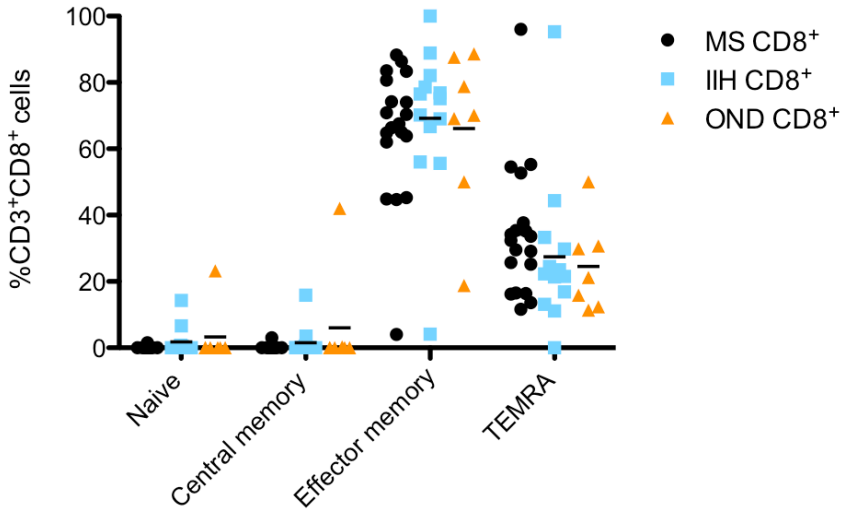


Figure 4.20. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) CD8⁺ T cells. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.

The mean percentage of naïve CD8⁺ T cells for the MS, IIH and OND groups was 0.1%, 1.8% and 3.3% respectively. For central memory cells the mean percentages were 0.3%, 1.5% and 6% for the MS, IIH and OND groups respectively. Effector memory percentages were: MS, 65.3%; IIH, 69.2; OND 66.1% and for effector memory-RA cells: MS, 34.3%; IIH, 27.5% and OND, 24.5%. No significant differences were observed between patient groups for the percentage of naïve, central memory, effector memory or effector memory-RA cells present (Figure 4.21).

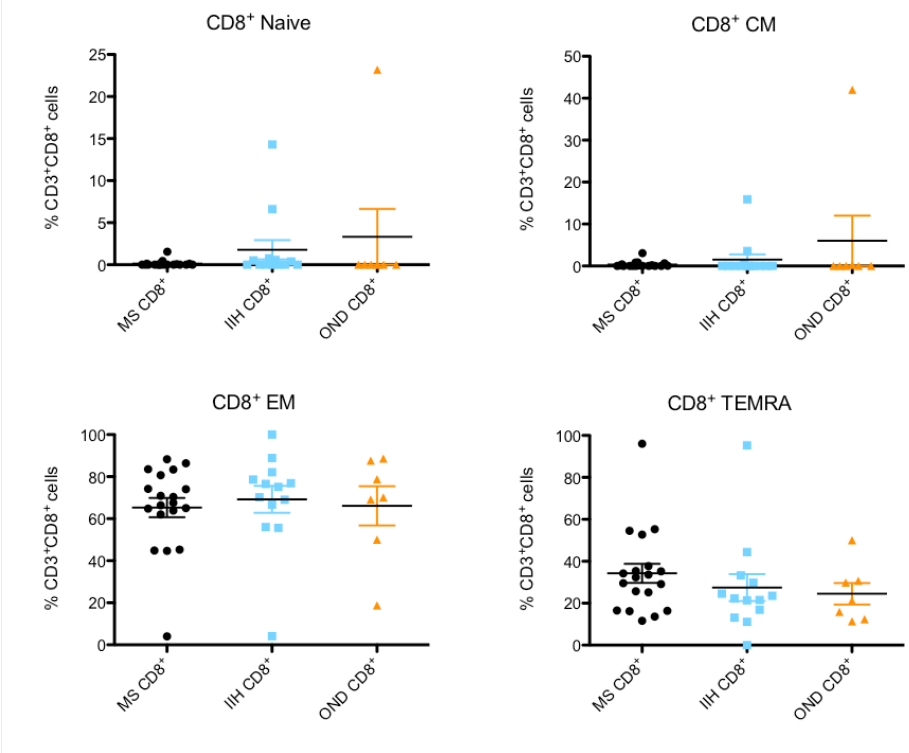


Figure 4.21. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) CD8⁺ T cells displayed by patient group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.

Interestingly, in the MS group the percentage of naïve, central memory and effector memory cells in the CD4⁺ T cell compartment was significantly higher than in the CD8⁺ T cell compartment. However, this was reversed for T_{EMRA} cells whereby the percentage of CD8⁺ T_{EMRA} cells was significantly higher (Figure 4.22).

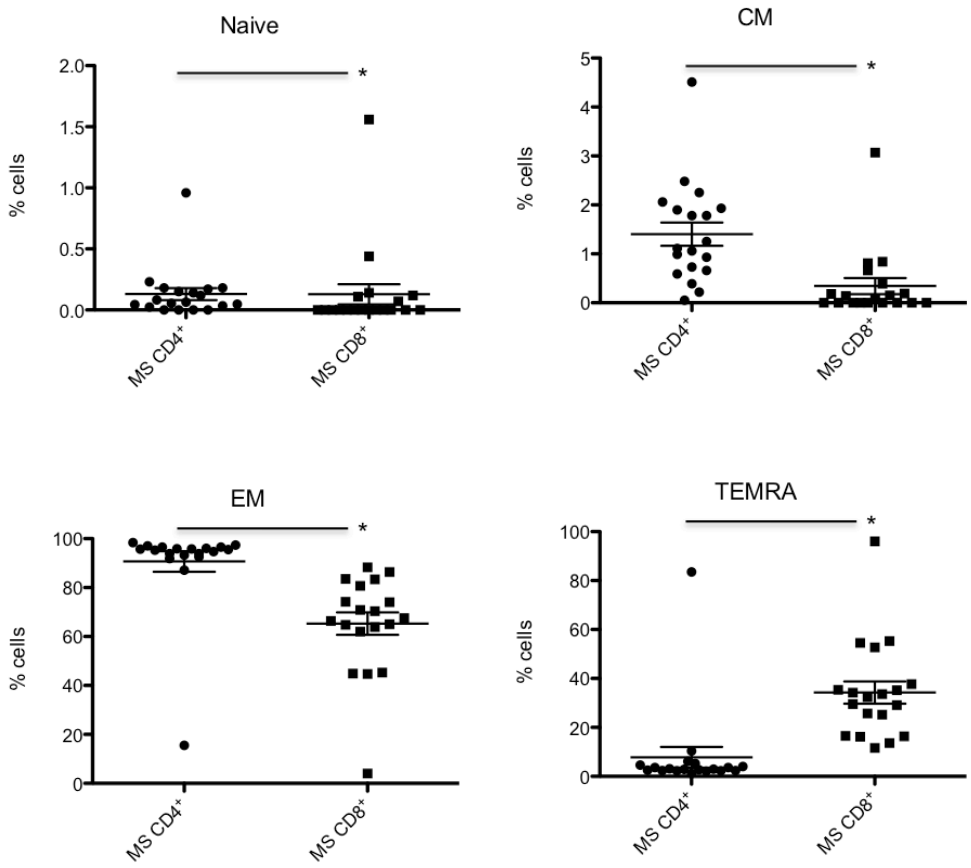


Figure 4.22. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) cells for the $CD4^+$ and $CD8^+$ compartments in the MS group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

In the IIH group, the percentage of central memory and effector memory cells in the $CD4^+$ T cell compartment outnumbered the percentage observed in the $CD8^+$ T cell compartment, although again the percentage of T_{EMRA} cells in the $CD8^+$ T cell compartment outnumbered the percentage of T_{EMRA} cells observed within the $CD4^+$ T cell compartment (Figure 4.23).

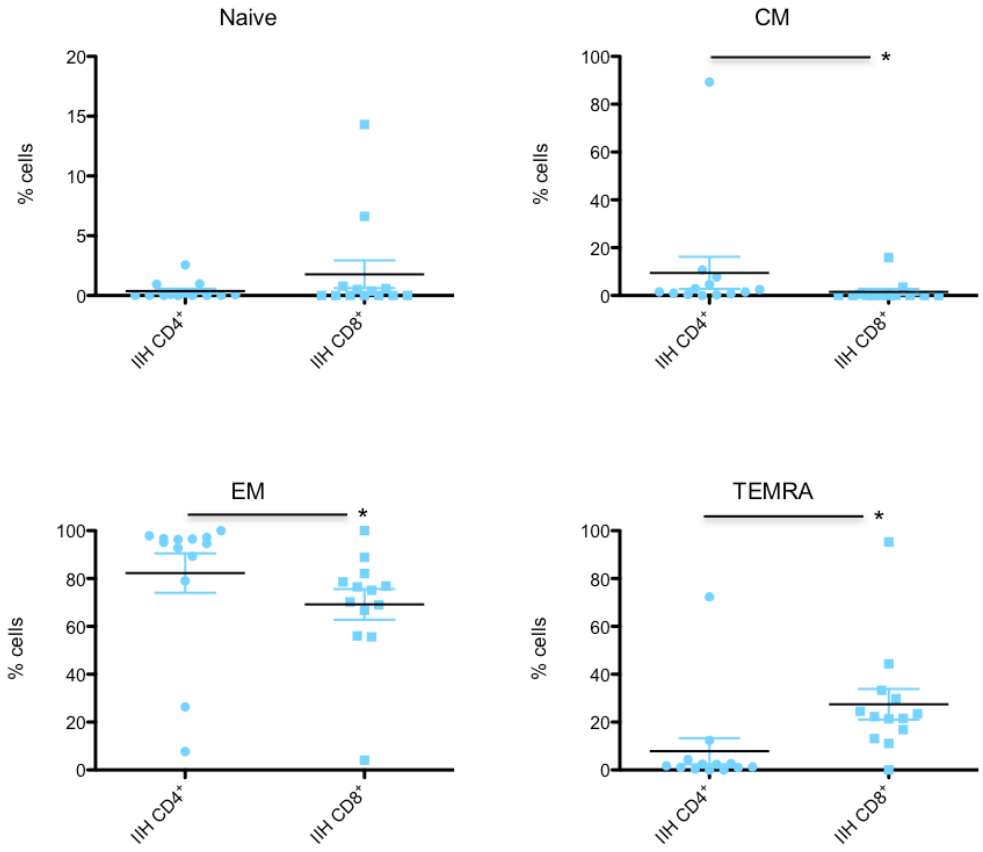


Figure 4.23. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) cells for the $CD4^+$ and $CD8^+$ compartments in the IIH group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

In OND patients, the percentage of effector memory cells within the $CD4^+$ T cell compartment was significantly higher than in the $CD8^+$ T cell compartment, with the percentage of T_{EMRA} cells in the $CD8^+$ T cell compartment again being significantly higher than that observed within the $CD4^+$ T cell compartment (Figure 4.24).

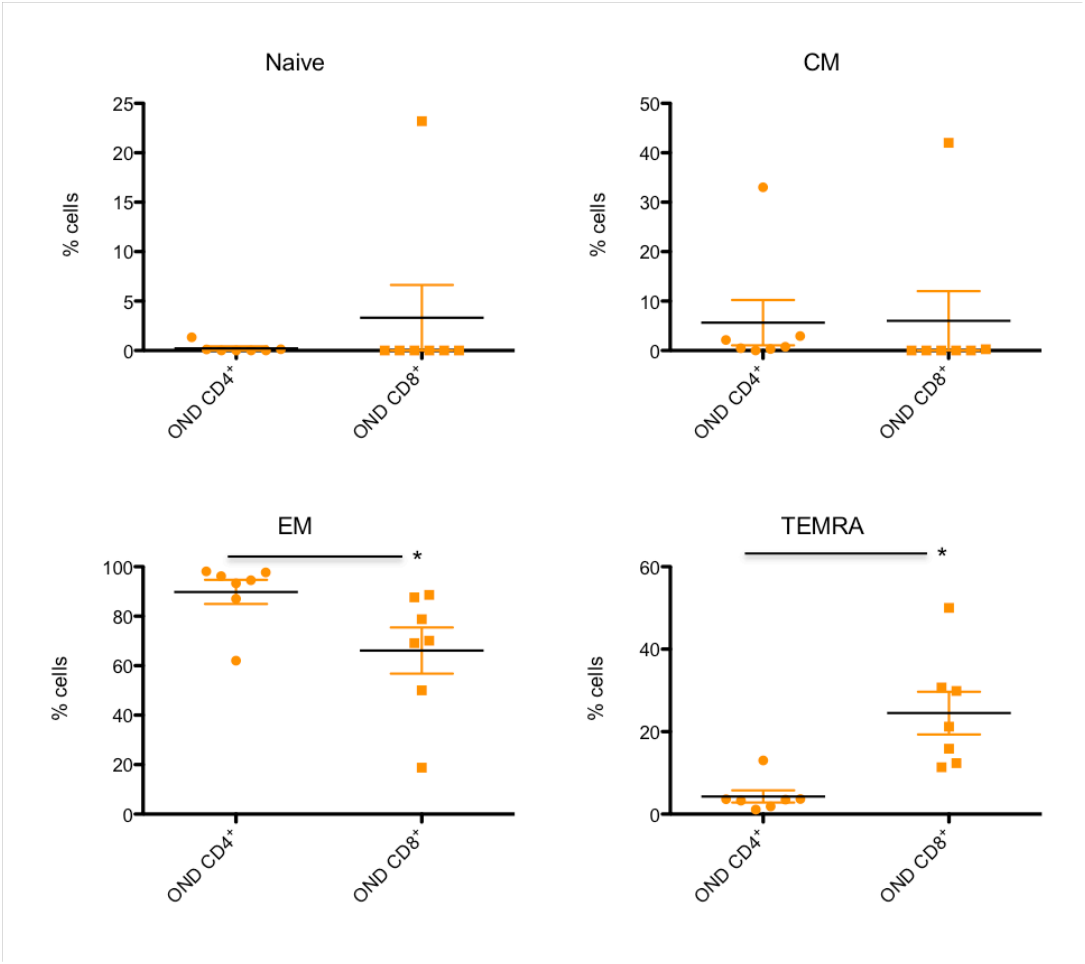


Figure 4.24. Percentage of naïve, central memory, effector memory and effector memory-RA (T_{EMRA}) cells for the $CD4^+$ and $CD8^+$ compartments in the OND group. Cells were prepared as discussed in the methods and legend of figure 4.3. Data was analysed with Flowjo software and Graphpad prism.* = $p<0.05$

4.4 Discussion

As Multiple sclerosis is considered to be of inflammatory aetiology, it is of central importance to understand the complex milieu of CSF-resident cells present in the disease. In particular, as $CD4^+$ and $CD8^+$ T cells have been demonstrated to play a key role in disease pathogenesis, developing a clear picture of their immunophenotype is crucial. Results of previous studies have yielded inconsistent findings with respect to the prevalence of different cell populations present in CSF and in some cases have been hampered by the lack of CSF from control populations or by the use of a limited number of phenotypic markers. When control populations have been studied this has also varied between studies, therefore making comparisons difficult.

Expression of CCR7 and CD45RA helps to identify naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and effector memory-RA ($CD45RA^+CCR7^-$) T cells.[93] In previous studies, $CD4^+$ and $CD8^+$ effector memory T cells have been demonstrated to be the predominant CSF-resident population in MS[79, 93, 97] although memory, and specifically central memory T cells were the dominant population in other studies (Table 4.1).[50, 91, 98-102] Central memory T cell populations have also been shown to be the predominant T cell population in controls including those without history of neurological disease attending for routine surgery.[50, 98, 99, 103]

Although effector memory or central memory T cells have been shown to be enriched in MS CSF, this is not exclusive to this group of patients (Table 4.1). For example, Mullen et al demonstrated that patients with MS had a higher percentage of effector memory T cells in the CSF compared with non-inflammatory controls, but this was also observed in patients with other inflammatory central nervous system disorders (OIND).[93] Giunti et al demonstrated a similar observation albeit with central memory cells being increased in the CSF of patients with MS and OIND.[98] Kivisakk et al showed an enrichment of central memory $CD4^+$ and $CD8^+$ T cells in the CSF in patients with non-inflammatory neurological disorders (NIND) and in MS $CD4^+$ T cells, with no difference when compared with NIND.[50, 99] Similarly in a relatively large study by de Graaf et al which involved patients attending for routine surgery, a predominance of central memory T cells was observed in CSF-resident $CD4^+$ and $CD8^+$ T cells.[103] Svenningsson et al[91] did not show any differences between MS patients, other

neurological disorders (OND) or healthy volunteers, where the majority of CSF-resident T cells were shown to be of memory phenotype ($CD45RO^+$). Other studies have shown differences between MS CSF and controls[97, 101, 102] or been hampered by the lack of control CSF.[79, 100]

In another recent study investigating differences in phenotype, function and reactivity between peripheral blood, CSF, and normal appearing white matter (NAWM) versus white matter lesions (WML) in 27 patients with MS,[104] the distribution of $CD8^+$ naive, central memory, effector memory and T_{EMRA} cells in these compartments resembled data on white matter and CSF under 'normal' CSF conditions.[103, 341] Central memory T cells predominated in the CSF and effector memory T cells were enriched in the NAWM and WML. Contrary to normal CNS conditions, effector memory $CD8^+$ T cells In MS lesions expressed a cytotoxic effector phenotype indicative of local antigenic stimulation.[104] However, a limitation of this study was that all samples were taken post mortem and therefore may not reflect the situation during life. In addition, all patients had a long progressive disease course and therefore the findings may not be relevant to earlier, relapsing forms of the disease. There were also no control groups, with previous studies used as comparisons. Of note, earlier studies that demonstrated memory or central memory T cells as the dominant population CSF-resident T cell population present used different cell markers to determine differentiation status such as CD45RO rather than the more accepted CCR7, CD45RA used now.[50, 91, 98-102]

Because of the lack of differences between MS and control groups, some authors have suggested that CSF-resident T cells could have a similar immunophenotype in both patients and controls, representative of generic CNS immune surveillance.[50, 91] Indeed, with recent advances in our understanding of CNS lymphatic drainage,[21, 22] increased attention is being paid to normal immune surveillance of the CNS. The immune-privileged status of the CNS is being revaluated and it is now becoming accepted that constant immune monitoring is being performed. As such, similarities or differences between MS and controls will be important in understanding normal homeostasis as well as MS disease pathogenesis.

In addition to variations in patient numbers and whether control groups were used or not, when defining CSF-resident T cell populations, previous studies have used a limited number of different phenotypic markers (Table 4.1). In this study, 8 different cell surface markers were used; CCR7, CD45RA, CD27, CD57, CD127, CD49, CD95 and Pan γδ. Programmed cell death protein 1 (PD-1) was also used in a subset of MS patients. In addition to CCR7 and CD45RA, the expression of these other cell surface markers in a polychromatic flow cytometry panel helps to confirm the population of cells present. CD27 is a costimulatory molecule, the expression of which is gradually lost as cells differentiate.[96] The function of CD57 is unknown but expression increases as cells differentiate.[96] The IL-7 receptor CD127 increases in expression when cells differentiate from a naïve to central memory phenotype and is then lost again as cells differentiate further.[96] CD95 (Fas), a death receptor is expressed by all memory T cells, after they have differentiated from a naïve state.[96] CD49d (α-4 integrin) is the main adhesion molecule involved in lymphocyte trafficking to the CNS.[342] PD-1 is expressed on activated T cells, B cells, and myeloid cells, and its expression is enhanced by classic programmed cell death. PD-1 and its ligand, programmed cell death ligand 1 (PD-L1), interact to downregulate the activation of T cells in autoimmune disease, chronic infection, and cancer.[343]

In this study, idiopathic intracranial hypertension (IIH) patients constitute the largest of two control groups. After retrospective clinical case note review some patients investigated for a potential diagnosis of CIS/MS were deemed not to have inflammatory neurological disease. Along with other recruited cases, these patients formed a second control group of other neurological disorders (OND). IIH is a disorder characterised by raised intracranial pressure that is most prevalent in obese females between the ages of 20-44[344]. Clinically it can present with headaches, visual loss, pulsatile tinnitus, and back and neck pain.[345] After appropriate neuroimaging, lumbar puncture is used to confirm raised intracranial pressure and may subsequently be used to relieve further episodes of headache or visual disturbance. Because of the necessity for repeated lumbar punctures and due to the patients being largely sex- and age matched to patients with MS, IIH makes an attractive control group. Although adipose tissue is now considered to be metabolically active,[346] with one small study of eight patients with IIH having higher levels of chemokine ligand 2 (CCL2) compared with

controls in CSF[347] there is little other evidence to consider the aetiology of IIH to be inflammatory in origin. In particular, it is largely thought to be a disorder of CSF dynamics either due to increased CSF production, malabsorption of CSF, increased venous sinus pressure or by a combination of these three factors.[345]

Our results demonstrate that the number of $CD4^+$ T cells/ml outnumber $CD8^+$ T cells/ml across all patient groups. The number of $CD4^+$ and $CD8^+$ T cells/ml were also higher in the MS compared with the IIH and OND groups. With regard to CCR7 and CD45RA expression, the majority of both $CD4^+$ and $CD8^+$ CSF-resident T cells were $CCR7^-$ $CD45RA^-$ therefore being of an effector memory phenotype. The other phenotypic markers in the panel demonstrated $CD4^+$ T cells to be $CD27^+CD49^+CD57^-CD95^+CD127^+$. $CD8^+$ T cells demonstrated similar expression albeit with more variable expression of CD127. Interestingly, T_{EMRA} cells were significantly higher in $CD8^+$ T cells as compared with $CD4^+$ T cells in all patient groups. This may indicate $CD8^+$ T cells to be further differentiated than $CD4^+$ T cells. The lack of expression of CD57 was surprising as this would be expected to be more highly expressed in differentiated cells. CD27 and CD49d expression was higher in the MS $CD4^+$ population compared with IIH $CD4^+$ T cells. Within $CD8^+$ T cells, the expression of CD27, CD49d and CD57 were all significantly higher in the MS group as compared with IIH. Pan γδ expression was low across all patients groups as was PD-1 expression in a subset of MS patients. The significantly higher expression of CD49d suggests greater homing activity of CSF-resident T cells in MS. The higher levels of CD27 may be relevant to regulatory activity, with expression of this molecule shown to correlate with regulatory activity.[348]

Interestingly, apart from these small differences, no other significant differences in differentiation status were observed across all three different patient groups. This is intriguing as CSF-resident memory T cells in MS are generally thought to represent a pathogenic subset. With the control groups in our study consisting of patients with IIH and other non-inflammatory diseases it appears that these differentiated effector memory cells are a constant and may represent normal immune surveillance in the CNS. Although PD-1 was expressed at a low level across our patients, a recent study of peripheral blood $CD8^+$ T cells has demonstrated high PD-1 expression in $CD57^+$ $CD8^+$ T cells in patients with stable MS as opposed to those with active disease.[349] Therefore,

despite not being in clinical relapse, this suggests that our patients may have had vigorous, subclinical disease activity.

T cells and other cells of the adaptive and innate immune systems are required to eliminate both pathogenic self and foreign antigens from the CNS. These cells may either be activated in the periphery or more centrally in CNS draining lymph nodes. These cells are then able to remove potentially pathogenic agents.[16] The importance of CNS immune surveillance is demonstrated by the risk of a potential fatal brain infection, progressive multifocal leucoencephalopathy (PML) (caused by John Cunningham (JC) virus) following administration of natalizumab.[261] Natalizumab, a monoclonal antibody against VLA-4 is licensed for use in relapsing remitting MS and prevents lymphocyte egress into the CNS and therefore reducing T cell mediated viral immune surveillance.

If the immunophenotype of CSF-resident T cells is not unique to MS, then the question of how these cells are pathogenic arises. Despite very few differences in immunophenotype, there were significantly more $CD4^+$ and $CD8^+$ T cells/ml in patients with MS compared with IIH, which may be contributory. This would be consistent with the increased trafficking across the blood-brain barrier and blood-CSF barrier observed in MS.[20] With these findings, it seems difficult to apportion blame for MS pathogenesis purely to the differentiation status of CSF-resident T cells. It is likely that differentiated, effector T cells are a natural component of the CNS acquired immune system but clearly a pathogenic subset must still be present to cause disease. As well as being activated (either peripherally or centrally) by their cognate antigen, differences in T cell function, cytokine responsiveness, cytokine production and homeostatic proliferation may also contribute to an individuals risk for MS.[3, 350] In addition, perhaps the increased numbers of activated T cells tip the balance from homeostasis to disease.

There may be an alternative explanation for the difference in T cell immunophenotype between our study and others demonstrating central memory T cells as the predominant cell type. Instead of being reflective of the normal CNS immune surveillance apparatus, it could be argued that the control samples in our study also

have an inflammatory basis. Autoimmune encephalitis and Guillain-Barre syndrome are well recognised to be autoimmune in nature, with the immune system also having a role to play in IIH,[347] migraine[351] and cerebrovascular disease.[352]

In summary, CSF-resident $CD4^+$ and $CD8^+$ T cells have a similar phenotype across patients with MS, IIH and other neurological diseases. The majority of these cells have an effector memory phenotype suggesting they are antigen experienced and primed to elicit a rapid and robust response to target cells expressing specific pMHC molecules, resulting in their destruction. However, the observed T cell phenotype is likely to be a universal observation consistent with normal CNS immune surveillance and that additional factors are likely to contribute to disease pathogenesis. A limitation of this study was the lack of comparison with both peripheral blood and central nervous system tissue. Although peripheral blood was collected for additional experiments, brain or spinal cord tissue was not available in this study. If CNS tissue was available, it would be of obvious interest to investigate the detailed immunophenotype of T cells resident in the brain both of patients with MS and in controls and could be an avenue for future enquiry. Although previous studies have also analysed blood and CSF simultaneously, a recent study has indicated that blood samples are not representative of the CSF. A poor correlation between blood and CSF of 14 different immune cell subtypes in different inflammatory and non-inflammatory disorders was observed.[111] Therefore, it seems of importance to ensure future studies are aimed at CSF and CNS tissue, rather than focusing on the more easily accessible peripheral blood. Of note, we did not specifically examine for regulatory T cell populations in our samples, which may have contributed to the cellular populations present. It should also be noted that the median age at lumbar puncture was higher in the MS group compared with the IIH group. As intra-CNS inflammation is thought to decrease with older age, this should also be considered when interpreting the overall results.

If the phenotype of CSF-resident T cells is the same across different patient groups, a key question raised would be how best to identify pathogenic T cell subsets from CSF or CNS tissue and whether the increased numbers of T cells is non-specific infiltration. Further characterisation of these CSF-resident T cells is important to understand if the significantly increased cell numbers observed is pathologically or clinically relevant.

This is of great importance with regard to future therapeutic drug design. Rather than using general immunosuppressants or immunomodulators that is current practice, knowledge of pathogenic T cell populations present in individual patients could lead to personalised T cell targeted medication. As such we performed TCR repertoire analysis of these T cell populations to investigate this further and is discussed in the following chapter.

Chapter 5

Analysis of the CSF-resident T cell receptor repertoire in Multiple sclerosis

5.1 Introduction

5.1.1 T cell receptor structure and development

T cells are characterised by the presence of the co-receptor molecules CD4 or CD8. Central to the interaction between T cells and antigen presented in combination with major histocompatibility complexes (MHC) is the T cell receptor (TCR). The majority of TCRs are heterodimers comprised of two subunit chains (α- and β-), which both contain constant and variable domains (Figure 5.1).

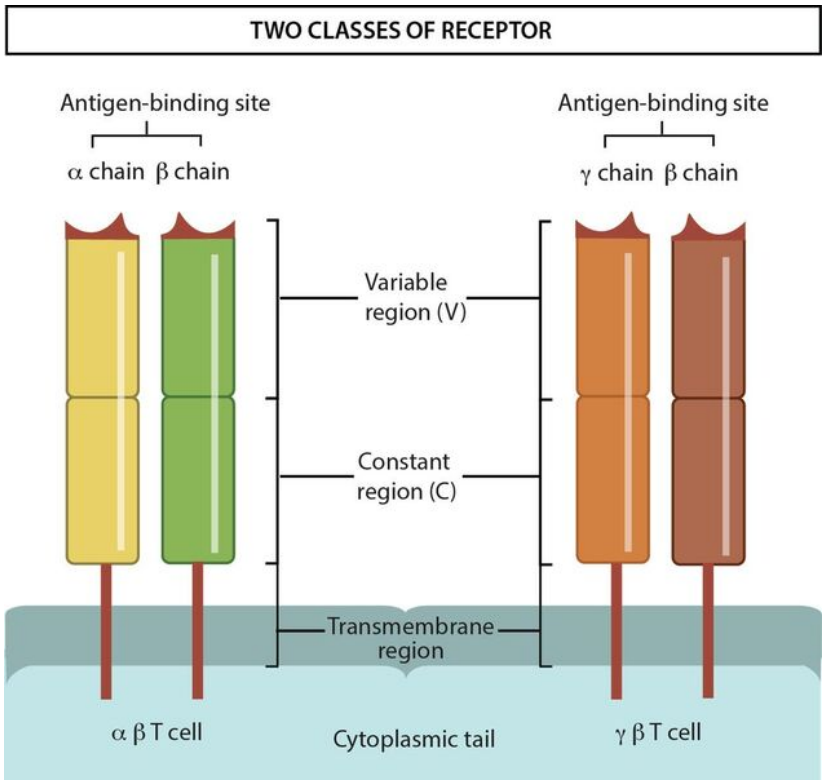


Figure 5.1. Two different classes of T cell receptors are determined by the presence of α and β, or γ and δ chains. Figure adapted from The Immune System, Parham P, Garland Science, 2009.[15]

Because of the need for a large diversity of TCRs, given the number of potential pathogens, the immune system utilises a system for diversification. TCR diversity is generated during the early stages of T cell development. During cell division, T cell progenitors undergo extensive gene recombination between the variable (V-) and junctional (J-) segments, and the V-, diversity (D-) and J- segments, in the TCR- α and TCR- β genes respectively. The region of TCR-β that spans the V-D and D-J junctions is known as the complementarity determining region 3 (CDR3) and is unique to each TCR-β variant (Figure 5.2). Following somatic diversification, T cells that lack sufficient affinity for MHC molecules and those that recognise self-antigens are eliminated (positive and negative selection respectively).[14]

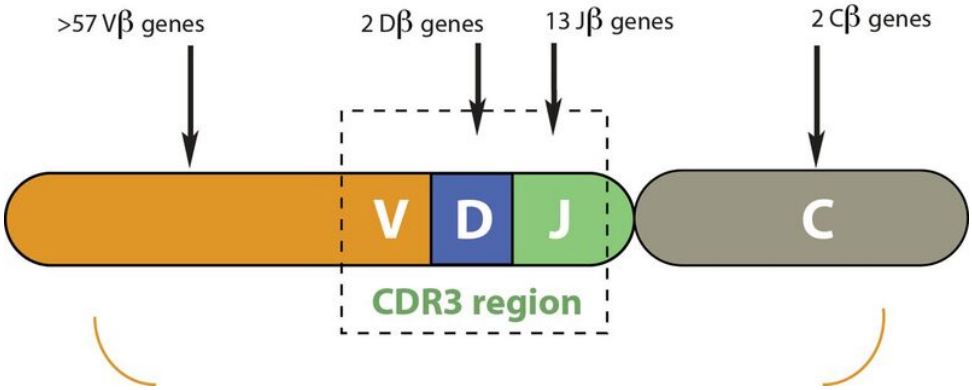


Figure 5.2. Complementarity determining region 3 (CDR3) region of the TCR-β chain.

5.1.2 T cell clonal expansion

Following activation, $CD8^+$ T cells clonally expand and deliver a range of effector functions. After clearance of the initial infection, a small proportion of these expanded cells survive and persist as a memory population.[34] The previous chapter in this thesis has demonstrated that cerebrospinal fluid (CSF)-resident T cells in Multiple sclerosis (MS), idiopathic intracranial hypertension (IIH) and other neurological diseases (OND) are mainly of an effector memory phenotype. In keeping with a differentiated, effector phenotype, clonal expansion of this cellular subset would demonstrate prior activation and response to an antigenic stimulus.

Although T cells and other constituents of the immune system have been implicated in MS pathogenesis[2] and causative agents have been suggested[151], the pathogenic stimulus and antigenic targets are currently unknown. Having an in depth knowledge

of clonally expanded T cell populations in MS and investigating their antigenic targets would therefore be fundamental in understanding disease pathogenesis, identifying novel therapeutic targets and developing new drugs.

5.1.3 Evidence for T cell clonal expansion in Multiple sclerosis

Many early studies of T cell clonal expansions in MS did not differentiate between $CD4^+$ and $CD8^+$ T cells (see table 5.1 for a full summary of all studies). More recent studies have reported monoclonal or oligoclonal expansions in the $CD8^+$ T cell repertoire of MS patients, which has not been observed in the $CD4^+$ T-cell repertoire.[51, 53, 54, 127, 130] As such it has been suggested that the expanded $CD8^+$ T-cell population might be central to MS pathogenesis. Several authors have stated that determining the antigen specificity of these expanded $CD8^+$ T-cell clonotypes is a research priority for the future.[353] However, many of these studies have been comprised of a small number of MS patients, a lack of control populations and if controls are included then limited access to sample material other than peripheral blood (i.e. no CSF and CNS samples).[51, 53, 54, 127, 130] Therefore, although a consensus seems to have arisen in the literature suggesting that $CD8^+$ T cell clonal expansions in MS are pathogenic, the limitations of these studies should heed caution in over interpretation. Nevertheless, understanding T cell clonal expansions, and in particular T cell receptor (TCR) repertoires and their role in MS disease pathogenesis needs further attention and analysis.

Following earlier studies, Babbe et al performed Vβ polymerase chain reaction (PCR) and subsequent sequencing of single cells isolated from inflammatory brain lesions from two MS patients, with blood analysed by CDR3 spectratyping and sequencing. Oligoclonal expansions were seen in the $CD8^+$ T cell repertoire in brain lesions, which were not observed in the $CD4^+$ T cell repertoire. Interestingly, the same clonal expansions were observed in the blood of one MS patient at two separate time points.[51] The same clonal expansions seen in the $CD8^+$ T cell repertoire of the brain were subsequently observed in the CSF and blood in a follow-up study, even in one sample taken 7 years after the original brain biopsy.[54] Other studies have also reported an oligoclonal expansion in the $CD8^+$ T cell repertoire. Jacobsen et al observed a skewing of the TCRVβ repertoire in the CSF-resident $CD8^+$ T cell repertoire in MS patients although no control CSF in this study was available for comparison. In this

study, no difference was seen in the peripheral blood between patients and controls.[130] Complementary determining region 3-length distribution (CDR3-LD) alteration has also been shown to be significantly higher in MS patients, and more prominent in the $CD8^+$ T cell population.[128] Junker et al identified identical T cell clones in separate brain regions in 4 MS patients although the TCR repertoire was private to each patient. Some TCR sequences in this study were identified to be expressed by $CD8^+$ T cells.[52] However, control brains also showed oligoclonal expansions. In a more recent study of TCRVβ clonality in blood, CSF and brain from 3 patients with MS, $CD8^+$ T cell clones were shown to exhibit strong sharing between the 3 repertoires, especially between the CSF and brain lesions.[53] Again, control samples of blood only were available for comparison. In another study of peripheral blood, a clonal dominance of myelin proteolipid protein (PLP)-specific $CD8^+$ T cells was seen in patients with MS. However, in countenance to this, clonal dominance within myelin basic protein (MBP)-specific $CD8^+$ T cells was observed in healthy controls but not in patients with MS.[137] Other studies have demonstrated oligoclonal expansions in MS patients but without defining whether the expansions belong to the $CD4^+$ or $CD8^+$ T cell population.[120-127, 129, 134] Another recent study using deep sequencing technology demonstrated a significantly higher frequency of clonal expansions in MS blood and CSF compared with controls, although cells were not sorted into $CD4^+$ and $CD8^+$ populations.[138]

Although these studies make a convincing case for $CD8^+$ T cell clonal expansions being pathogenic, other studies suggest caution in over interpreting these data. Early studies demonstrated no oligoclonal expansions in CSF-resident T cells in 2 MS patients,[131] and a polyclonal repertoire seen in active MS plaques.[132] In addition, although TCRVβ usage was shown to be skewed in blood, this was not observed in CSF or brain in another study.[133] Gran et al also demonstrated TCRVβ skewing that was present in MS patients and controls, with MS TCRVβ expansion returning to normal when analysed at a second time point.[134] In a separate study, the TCR Vβ5-JB and TCR Vβ17-JB repertoire showed a less diverse pattern in the CSF samples compared with blood not just in MS but also in patients with other neurological diseases.[135] In an interesting twin study of blood, a Gaussian distribution was observed in $CD4^+$ T cells with widely skewed TCR spectratypes in the $CD8^+$ T cell population. However, no correlation was found

between oligoclonality and disease with sequencing revealing shared TCRs between intra- and inter-pair twin members.[136]

These studies suggest that the case for $CD8^+$ T cell clonal expansion being pathogenic is far from conclusive. In particular, the main limitations of all the studies performed on $CD8^+$ T cell oligoclonality is either the lack of controls entirely or if present, the lack of access to CSF and CNS tissue to compare the $CD8^+$ T cell repertoire. In addition, many studies have examined the T cell repertoire as a whole without focus on $CD4^+$ and $CD8^+$ T cells specifically. It is also of note that $CD8^+$ T cell clonal expansions are thought to be a common feature of the human T cell repertoire[139] even in normal subjects and may be important for CNS immune surveillance.[52] $CD8^+$ T cell clonal expansions also occur with increasing age.[140] Clearly this area needs further exploration to understand the relevance of clonal T cell expansions in MS and whether it is indeed just a normal feature of the adaptive immune system. However, if these clonal T cell expansions are pathogenic then it will be important to identify the antigenic target of these cells in order to further understand MS pathogenesis and develop targeted therapies.

5.1.4 Aims and objectives

In this study, I aimed to fully characterise the TCR repertoire of CSF-resident $CD4^+$ and $CD8^+$ T cells in MS. In contrast to the majority of other studies, I also aimed to analyse TCR usage from a relatively large cohort of control patients in order to establish whether $CD8^+$ T cell clonal expansions are unique to MS patients. In depth knowledge of the TCRs that are clonally expanded would also allow identification of their target antigens, which will be explored in more detail in chapter 6 of this thesis.

Author	No. of MS	No. of Controls	Technique	Tissue studied	Findings
Rotteveel et al (1987)[131]	2	0	T cells clones grown from CSF. SB of TCR β-chain gene rearrangement.	CSF	No evidence of a clonal expansion
Oksenberg et al (1990)[120]	3	3	PCR amplification of TCRVα sequences.	Brain	Only 2-4 rearranged Vα transcipts detected in each of 3 MS brains. No Vα transcipts in controls brains. Results imply restriction of TCRVα gene expression in MS brain lesions.
Kotzin et al (1991)[122]	7	No. not available	MBP T cell clones made. β-chain (Vβ) and α-chain (Vα) variable regions analysed by PCR.	Blood	Bias for Vβ 5.2 and 6.1 in patients but not in controls.
Lee et al (1991)[121]	9	8 OND 4 HV	T cells from blood and CSF cloned before in vitro expansion. Clonotypes compared by SB analysis of TCR β and α chains.	Blood CSF (Blood only for HV)	Oligoclonal T cell clones identified in both the CSF and blood in 5/9 MS patients. No clonal expansion in controls. Common Vβ12 usage between blood and CSF in 3 MS patients. Identical clones between blood and CSF in 3 MS patients.
Birnbaum et al (1992)[133]	4	4 OND	T cell Vβ PCR.	Blood CSF Brain (2 MS patients; 1 OND)	Blood Vβ useage skewed in genes 1-8. No skewing in CSF or brain. Different Vβ expression patterns between paired blood and CSF. No disease specific pattern in CSF or blood. Brain Vβ pattern different and less heterogeneous than paired blood. Vβ12 increased in MS brains.
Wucherpfennig et al (1992)[132]	6	0	T cell Vα and Vβ PCR and SB.	Brain	Broad TCR Vα and Vβ repertoire in active lesions. Fewer TCR V genes detected in chronic plaques and control samples. Differences in the TCR repertoire between plaques from the same case. Data suggest TCR repertoire in MS plaques is polyclonal.
Monteiro et al (1996)[123]	125	Unknown (Abstract only)	$CD8^+$ T cell multiplex PCR assay for CDR3 length.	Unknown (Abstract only)	$CD8^+$ T cell clonal dominance frequent in MS patients. Increased frequency in Vβ 9, 18 and 23. High sequence diversity in clonally dominant TCRs. Identical TCR Vβ sequence from 2 different MS patients.
Gran et al (1998)[134]	40	20 HV	TCR Vβ PCR and sequencing.	Blood	Nine MS patients had expansion of one or more Vβ segments. Six MS patients had expansion of Vβ9. 3 also had expansion of Vβ 1, 11 and 22. TCR Vβ9 further analysed and found to be polyclonal. Skewed repertoire returned to normal in 5 patients assessed at 2 time points. Some TCR Vβ expansions were present in controls and not MS patients.
Lozeron et al (1998)[135]	20	11 OND	T cell Vβ PCR using Vβ5 and Vβ17 with a combination of Jβ primers. CDR3 length analysed by 'Immunoscope'.	Blood CSF	Less diverse Vβ5-Jβ and Vβ17-Jβ repertoire in CSF c/w blood in all samples. Three MS patients had expansions with identical CDR3 length in the CSF.

Table 5.1. Summary of studies investigating the clonal expansion of T cell subsets in Multiple sclerosis. SB=Southern blotting; MBP=Myelin basic protein; OND=Other neurological diseases; HV=Healthy volunteers; OIND=Other inflammatory neurological disease; PLP=Proteolipid protein; CDR3-LD=complementarity determining region 3-length distribution.

.

Author	No. of MS	No. of Controls	Technique	Tissue studied	Findings
Babbe et al (2000)[51]	2	0	Single cell T cell Vβ PCR and sequencing from MS lesions. Blood analysed by CDR3 spectratyping and sequencing.	Blood Brain	$CD8^+$ T cell population oligoclonal. 1 patient had matching $CD8^+$ clone in blood and brain. $CD4^+$ T cell population polyclonal.
Gestri et al (2001)[124]	11	10 OIND	T cell Vβ seminested PCR with heteroduplex analysis.	Blood CSF	CSF oligoclonal T cell expansions found in 13 patients with CNS inflammatory disease. Monoclonal expansions found in 5 patients.
Muraro et al (2002)[125]	4	20 HV	TCR Vβ PCR.	Blood	Expansions of TCR Vβ genes in MS patients were significantly more frequent than in controls. Expansions were predominantly oligoclonal. Expansions significantly correlated with responses to MBP and MRI disease activity.
Jacobsen et al (2002)[130]	36	75 HV	Flow cytometry with Vβ antibodies. TCR sequencing in 2 patients.	Blood CSF (Blood only for HV)	No difference in Vβ expression in blood between patients and controls. No control CSF for comparison. TCR Vβ chain expression differs between CSF and blood from MS patients and mainly in $CD8^+$ T cells. Skewing of CSF is due to expansion of $CD8^+$ T cells with similar or identical TCRs.
Matsumoto et al (2003)[126]	42	30 HV	TCR CDR3 spectratyping and sequencing.	Blood CSF (5 patients) (Blood only for HV)	Vβ5.2 and 24 significantly expanded in blood compared with controls. Vβ 5.2 most dominant in CSF but unable to compare as no control CSF.
Skulina et al (2004)[54]	2 (1 from Babbe et al)	0	CDR3 spectratyping and sequencing.	Blood CSF Brain	Follow-on from Babbe et al (2000). Several identical $CD8^+$ T cell clones found in blood, CSF and brain. One clone was present in blood 7 years after original brain biopsy.
Laplaud et al (2004)[128]	35 (includes CIS)	13 HV	TCR Vβ CDR3-LD spectratyping.	Blood	Mean % alteration of CDR3-LD significantly higher in MS compared with controls. Alterations more prominent in $CD8^+$ T cells.

Table 5.1 (continued). Summary of studies investigating the clonal expansion of T cell subsets in Multiple sclerosis.

Author	No. of MS	No. of Controls	Technique	Tissue studied	Findings
Muraro et al (2006)[129]	1	0	Vβ and CDR3 spectratyping. (No sequencing)	CSF	Relapse vs. remission in one patient with MS. Changes in Vβ gene family expression between relapse and remission.
Laplaud et al (2006)[127]	9	14 HV	Vβ CDR3 spectratyping.	Blood	Significant blood skewing in MS compared with controls (Vβ 5.2, 12, 8 and 21). TCR alterations also seen in controls. Positive correlation between the change in blood TCR biases and lesion activity.
Somma et al (2006)[136]	5 pairs of identical twins 4 pairs discordant for MS 1 pair concordant	0	TCR CDR3 spectratyping.	Blood	Gaussian distribution of $CD4^+$ T cells. Skewed TCR spectratypes for $CD8^+$ T cells. No correlation between oligoclonality and disease. Shared TCRs between intra- and inter-pair twin members.
Junker et al (2007)[52]	4	0	Vβ CDR3 spectratyping and sequencing.	Brain	Identical T cell clones detected in separate brain regions. TCR repertoire oligoclonally diverse in each brain. Some TCR sequences were from $CD8^+$ T cells. The TCR repertoire was private to each patient. Control brains also showed oligoclonal expansions.
Biegler et al (2011)[137]	3	3 HV	Short-term culture, FACS and non-biased PCR.	Blood	Clonal dominance within MBP-specific $CD8^+$ T cells in HV, but not MS. Distinct TCR Vβ usage in MBP-reactive $CD4^+$ T cells in MS. Clonal dominance of PLP-specific $CD8^+$ cells in MS.
Salou et al (2015)[53]	3	4 MS 4 HV	Vβ CDR3 spectratyping and high-throughput sequencing.	Blood CSF Brain (Blood only for controls)	Post mortem tissue. Private T cell clones ($CD8^+$). Brain $CD8^+$ TCR repertoire closer to CSF than blood. Different brain lesions had the same $CD8^+$ repertoire.
de Paula Alves Sousa (2016)[138]	5	5 IIH	Deep sequencing.	Blood CSF	TCR repertoire diversity greater in blood and CSF of MS compared with controls. Frequency of clonal expansions in MS significanlty higher in blood and CSF compared with controls. Highly expanded T cell clones enriched in MS CSF compared with blood.

Table 5.1 (continued). Summary of studies investigating the clonal expansion of T cell subsets in Multiple sclerosis.

5.2 Methods

Patients included in this section of the study were recruited as described in Chapter 4 of this thesis.

5.2.1. Clonotyping of TCR repertoires

CSF samples were sorted into $CD4^+$ and $CD8^+$ T cells by flow cytometry as described. TCR usage in these populations was analysed using a strand-switch anchored RT-PCR approach that enables quantitative characterisation of TCR gene usage without bias.[291] The clonotyping technique used for this analysis is described in detail in the materials and methods section (Chapter 2) of this thesis but will be briefly described below.

Firstly, frozen $CD4^+$ and $CD8^+$ T cell samples stored in freezing media were thawed at room temperature then centrifuged at 15000g for 7 mins at 4°C. mRNA was then extracted using Miltenyi's µMACS mRNA Isolation kit (Miltenyi Biotec, Bisley, UK) as described. cDNA was subsequently made using a SMARTer™ RACE cDNA Amplification kit (Takara Clontech, Saint-Germain-en-Laye, France) and either used immediately or stored at -80°C until required. The TCR β-chain product was then amplified by PCR. The cDNA PCR product was subsequently isolated by agarose gel electrophoresis and extracted under UV light, then extracted and cloned using the TOPO® TA Cloning® Kit for Sequencing with One Shot® MAX Efficiency® DH5α-T1R E. coli (Thermofisher, Waltham, MA, USA) as per the manufacturers instructions. Bacteria were subsequently grown and white colonies containing the CDR3 amplicon were picked into a 96 well plate and colony PCR performed. Plates were then sent for sequencing (Genewiz, South Plainfield, USA) and analysis performed using sequencher software (Gene codes corporation, Ann Arbor, USA), the IMGT (international ImMunoGeneTics information system) website, Microsoft Excel (Redmond, USA) and Graphpad prism (La Jolla, USA). Of note, sorted cell samples <100 cells were not clonotyped for all patient groups.

5.2.2. Data processing and analysis

Following sequencing, data was imported into sequencher and sequence ends trimmed. If quality scores for each individual sequence were <50%, data was excluded from the analysis. Included data was then converted to TCR sequences using the IMGT

website and exported into Microsoft Excel. TCRs were then filtered and only 'in-frame' sequences chosen for further analysis. TCR sequences were excluded if amino acid sequences were not consistent with known CDR3 regions to the particular TCRVβ region. Once determined, TCR frequencies were sorted according to the following hierarchy: 1. TCR frequency 2. CDR3 amino acid length 3. Highest TRBV 4. Highest TRBJ. Data analysis was performed in Microsoft Excel (Redmond, USA) and Graphpad prism (La Jolla, USA). TCR diversity and the degree of clonal expansion was assessed as described below. Additional analysis was also performed by Dr Vanessa Venturi (Infection Analysis Program, The Kirby Institute, UNSW Australia, Sydney, NSW 2052, Australia) and Dr Adel Rahmani (School of Mathematical Sciences Physical Sciences, University of Technology Sydney, 15 Broadway, Ultimo, NSW 2007, Australia). Dr Venturi and Dr Rahmani have expertise in using computational biology approaches to understand immunological data.

5.2.3 Samples with TCR frequencies <50 and overlapping TCRs

Of note, where initial clonotyping yielded overall TCR frequencies of <50 these samples were repeated either from the original mRNA, cDNA or gel extraction stages of the clonotyping method. When the repeat sample was >50, this was included in the analysis and the original TCR analysis discarded. If the repeat sample had <50 TCR frequencies then this patient sample was excluded from the analysis. Similarly, clonotyping samples with overlapping TCRs were repeated. Original results were discarded if the repeat samples were unsuccessful. Following, this approach, the minimum remaining sample size across all the remaining $CD4^+$ and $CD8^+$ T cell repertoires was 54 TCR sequences.

5.2.4 TCR repertoire sample diversity analysis

To investigate the diversity of both the $CD4^+$ and $CD8^+$ T cell repertoires, two different approaches were used. The first was to evaluate the number of unique (V+CDR3+J) TCR clonotypes in each TCR repertoire and the second was to determine Simpson's diversity index.[354] Simpson's diversity index provides a relative measure of the evenness of the abundances (i.e. number of copies) across the unique observations (i.e. unique TCR clonotypes) in each TCR repertoire, and ranges in value from 0 (minimal diversity) to 1 (maximal diversity).[355] To compare diversities between the MS,

IIH and OND groups, the Kruskall-Wallis test with Dunn's post-test was used. The Mann-Whitney test was used to compare diversities between the MS group and the combined IIH and OND cohorts. The Wilcoxon test was used to compare diversities between the $CD4^+$ and $CD8^+$ T cell repertoires across patients for whom both $CD4^+$ and $CD8^+$ T cell samples were available.

5.2.5 Clonal expansion analysis

To determine the degree of clonal expansion, TCRs that constitute the top 10% of each TCR repertoire were identified and their contribution to the overall repertoire was assessed. Of note, if there were fewer than 10 TCR clonotypes within a patient sample, the top clonotype was taken to represent the top 10%. In some cases with for example, only 1 TCR, this TCR was taken to contribute 100% of the repertoire; similarly if there were only 2 TCR clonotypes, then the frequency of the top TCR was taken to represent the top 10% despite it actually being the top 50%. This continued for up to 10 unique TCR clonotypes. When 3 groups were compared, the Kruskall-Wallis test with Dunn's post test was used to calculate significance. When comparing 2 groups, the Mann-Whitney test was used. In addition to this initial analysis, a cumulative clonotype frequency distribution analysis was performed by Dr Venturi and Dr Rahmani. This analysis compared the cumulative proportion of unique clonotypes against the cumulative proportion of the total repertoire.

5.2.6 TCR Vβ staining of peripheral blood

TCRVβ staining was performed where matching peripheral blood was available. Patient PBMCs were thawed and centrifuged at 1500 rpm for 5 mins. Cells were resuspended in 8ml PBS and divided between 8 FACS tubes. Tubes were then centrifuged at 2000 rpm for 2 mins and supernatant discarded. 8 µl aqua was then added to each sample and incubated for 10 mins at room temperature. After 10 mins, V beta antibodies (A-H) (IOTest® Beta Mark, Beckman Coulter, Brea, USA) were added to each of the 8 tubes along with the following antibodies (CD14, CD19, CD3, CD8, CD4, CD27, CD45, CD95, CCR7, CD127, CD49d) and incubated for 30 mins at 4°C. Following this, 1 ml PBS was added to each tube and centrifuged at 2000 rpm for 2 minutes. Supernatant was then discarded, 100 µl PBS added and samples analysed on a FACSAria II. The IOTest® Beta Mark Kit is a multi-parametric analysis tool designed for quantitative

determination of the TCRVβ repertoire of human T lymphocytes by flow cytometry. Eight vials (labeled A-H) each containing 3 different TCRVβ mAb are used. The first mAb within each labeled vial is FITC-conjugated, a second one is PE-conjugated and a third one is a mixture of a PE- and a FITC-conjugated form. The 8 vials containing mixtures of conjugated TCRVβ antibodies correspond to 24 different specificities (about 70% coverage of normal human TCR Vβ repertoire). The TCRVβ included in this assay were as follows; 4-1 4-2 4-3, 5-5, 28, 3-1, 19, 14, 5-1, 18, 30, 6-5 6-6 6-9, 6-6, 12-3 12-4, 5-6, 10-3, 20-1, 9, 11-2, 13, 2, 25-1. Data analysis was performed in Microsoft Excel and Graphpad prism.

5.3 Results

5.3.1. Inclusion and exclusion of samples

Of the 21 patient samples available in the MS patient cohort, clonotyping was performed on 20 patients. One patient sample (DD22299) was not sorted due to technical issues with the flow cytometer. Of the 20 patient samples, $CD4^+$ T cells only were clonotyped for 2 patient samples; one (CT25364) because of unsuccessful $CD8^+$ T cell clonotyping and one (HD2165) where $CD8^+$ T cell clonotyping was not performed due to a sorted cell count of <100. Of the 14 IIH patient samples, 3 samples were excluded from clonotyping analysis: 1 sample (DC37877) was lost after sorting; 1 sample (FC24414) was excluded because of contamination of the $CD4^+$ T cell sample, with the $CD8^+$ T cell sample <100 cells; 1 sample (VE25562) was excluded because it was macroscopically bloodstained after lumbar puncture. In the OND group, 3 patient samples were excluded from clonotyping analysis: 1 sample had a low number of cells in both the $CD4^+$ T cell and $CD8^+$ T cell sort (DL37517); 1 sample had no live cells (JJ37566); 1 sample was macroscopically bloodstained and therefore excluded (JP24822).

Overall, in the MS group, 18 complete ($CD4^+$ and $CD8^+$ T cell) samples were clonotyped. For 2 patient samples, $CD4^+$ T cell clonotyping only was included for analysis. In the IIH group, there were 3 complete patient samples ($CD4^+$ and $CD8^+$ T cells), 7 patient samples with results for $CD4^+$ T cells only and 1 patient sample with results for $CD8^+$ T cells only. In the OND group, there were 5 complete patient samples ($CD4^+$ and $CD8^+$ T cells) and 3 patient samples with results for $CD4^+$ T cells only. Figure 5.3 summarises the patient samples available for phenotyping and clonotyping analysis. Tables 5.2-5.4 give more details as to which samples were included or excluded and the reasons why. Due to the exclusion of samples with <100 cells, fewer $CD8^+$ T cell samples in the control groups were included for analysis.

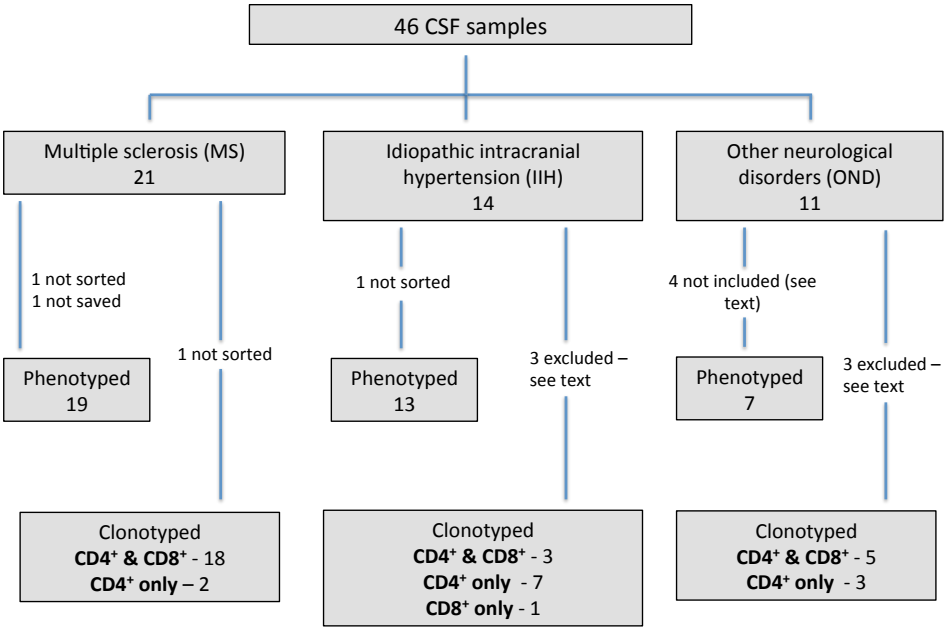


Figure 5.3. Summary overview of samples included for phenotyping and clonotyping.

Patient ID	Diagnosis	Phenotyped	Clonotyped & included in analysis		Reason for exclusion
			CD4	CD8	
LC20552	MS	Yes	Yes	Yes	N/A
LJ20639	MS	Yes	Yes	Yes	N/A
LS20460*	MS	No	Yes	Yes	N/A
MJ19588	MS	Yes	Yes	Yes	N/A
EB21510	MS	Yes	Yes	Yes	N/A
KG19967	MS	Yes	Yes	Yes	N/A
LH18836	MS	Yes	Yes	Yes	N/A
NW21326	MS	Yes	Yes	Yes	N/A
MW21576	MS	Yes	Yes	Yes	N/A
CS21983	MS	Yes	Yes	Yes	N/A
AL28847	CIS	Yes	Yes	Yes	N/A
MK21405	MS	Yes	Yes	Yes	N/A
SA23376	MS	Yes	Yes	Yes	N/A
RM22664	MS	Yes	Yes	Yes	N/A
TL22789	MS	Yes	Yes	Yes	N/A
CT25364	MS	Yes	Yes	No	Unsuccessful (CD8)
HD21265	MS	Yes	Yes	No	<100 sorted cells (CD8)
MH21407	MS	Yes	Yes	Yes	N/A
RW21309	MS	Yes	Yes	Yes	N/A
DD22299*	MS	No	No	No	Not sorted
CG41964	MS	Yes	Yes	Yes	N/A

Table 5.2. Patients included for phenotyping and clonotyping in the MS group. Reasons for exclusion in the clonotyping analysis are described in the text. N.B.*denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1.

Patient ID	Diagnosis	Phenotyped	Clonotyped & included in analysis		Reason for exclusion
			CD4	CD8	
EC21870	IIH	Yes	Yes	No	Unsuccessful (CD8)
HS25204	IIH	Yes	Yes	No	<100 sorted cells (CD8)
LH25311	IIH	Yes	Yes	Yes	N/A
ML25308	IIH	Yes	Yes	No	Clonotyping contaminated (CD8)
SW25353	IIH	Yes	Yes	Yes	N/A
RY21758	IIH	Yes	Yes	No	Unsuccessful (CD8)
DC37877	IIH	Yes	No	No	Sample lost
ES37889	IIH	Yes	Yes	No	<100 sorted cells (CD8)
FC24414	IIH	Yes	No	No	Clonotyping contaminated (CD4) <100 sorted cells (CD8)
VE25562*	IIH	No	No	No	Not sorted
CC40712	IIH	Yes	Yes	Yes	N/A
RC41200	IIH	Yes	Yes	No	<100 sorted cells (CD8)
KA38079	IIH	Yes	No	Yes	<50 clonotypes (CD4)
CC41471	IIH	Yes	Yes	No	<100 sorted cells (CD8)

Table 5.3. Patients included for phenotyping and clonotyping in the IIH group. Reasons for exclusion in the clonotyping analysis are described in the text. N.B.*denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1.

Patient ID	Diagnosis	Phenotyped	Clonotyped & included in analysis		Reason for exclusion
			CD4	CD8	
SE29703*	Autoimmune encephalitis	No	Yes	Yes	N/A
JG33488	Normal pressure hydrocephalus	Yes	Yes	No	Unsuccessful (CD8)
ND37140	Fibromyalgia	Yes	Yes	Yes	N/A
DL37517*	Guillain-Barré syndrome	No	No	No	Minimal sorted cells (CD4) <100 sorted cells (CD8)
CS19395	Cerebrovascular disease	Yes	Yes	No	Unsuccessful (CD8)
AG20355	Visual field defect of unknown aetiology	Yes	Yes	No	Clonotyping contaminated (CD8)
JM25229	Pseudopappiloedema - drusen/OSA	Yes	Yes	Yes	N/A
AB25236	Migraine	Yes	Yes	Yes	N/A
CJ26014	Migraine	Yes	Yes	Yes	N/A
JJ37566*	Migraine	No	No	No	No live cells
JP24822*	Small vessel disease	No	No	No	Bloody sample

Table 5.4. Patients included for phenotyping and clonotyping in the OND group. Reasons for exclusion in the clonotyping analysis are described in the text. N.B.*denotes patients where CSF was collected but not included in the phenotyping analysis as described in section 4.3.1.

5.3.2 Initial TCR diversity analysis

The raw data from the clonotyping analysis is displayed in Appendix, Section 8.2. Each patient sample is ordered as described in the methods: 1. TCR frequency, 2. CDR3 amino acid length, 3. Highest TRBV; and 4. Highest TRBJ. Initially, the number of unique TCR clonotypes for each patient sample was calculated. In this regard, the number of unique TCR clonotypes was significantly higher in the $CD4^+$ T cell repertoire of MS patients compared with both the IIH ($p<0.05$) and OND patient groups ($p<0.05$). No significant difference was seen between the number of unique clonotypes in the $CD4^+$ T cell repertoire between IIH and OND controls. For $CD8^+$ T cell repertoires, no significant differences were observed between the MS group and either the IIH or OND group alone, or between the IIH and OND groups. When TCR diversity in the MS patient group was compared to the IIH and OND groups pooled as one control group, significance was still observed in the $CD4^+$ T cell repertoire ($p<0.0001$). In addition, significance was also observed in the $CD8^+$ T cell repertoire ($p=0.0324$). These results are displayed in Figure 5.4. Although this analysis offered some insights into TCR repertoire diversity, additional analysis was performed in collaboration with computational biologists at the University of New South Wales and The University of Technology, Sydney, Australia. The results of these additional analyses are described in the following subsections.

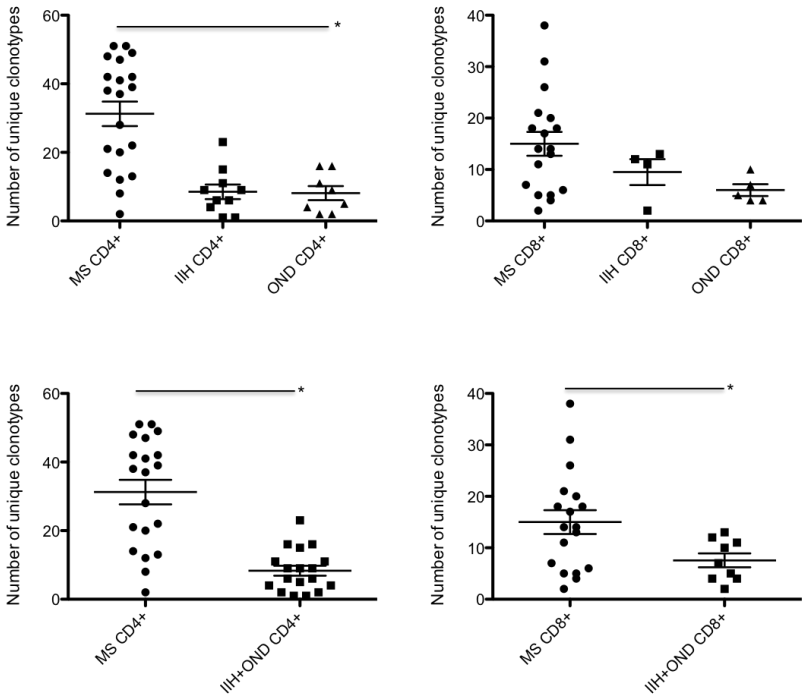


Figure 5.4. Comparison of the number of unique clonotypes in the T cell repertoires. $CD4^+$ (left panels) and $CD8^+$ (right panels). When comparing across 3 patient groups (upper panels), the Kruskall-Wallis test with Dunn's post test was used to calculate significance. When comparing 2 groups (lower panels) the Mann-Whitney test was used.

5.3.3 Additional TCR repertoire sample diversity analysis

In order to assess differences in TCR clonotype diversity between MS, IIH and OND groups, the number of unique TCR clonotypes and Simpson's diversity index were estimated for a standard sample size of 54 TCR sequences (the lowest TCR frequency across all samples) obtained per sample. This analysis accounts for differences in the numbers of sequences obtained per sample. With regard to unique TCR clonotypes, significantly higher numbers of unique TCR clonotypes were observed in the $CD4^+$ TCR repertoires in the MS group compared with both the IIH and OND groups. Similarly, the Simpson's diversity index was significantly higher for the $CD4^+$ TCR repertoires in the MS group compared with both the IIH and OND groups (Figure 5.5). Although the data suggests a trend towards a higher number of unique clonotypes and higher Simpson's diversity index within the $CD8^+$ TCR repertoire in the MS group compared to either the IIH or OND group alone, this did not reach significance.

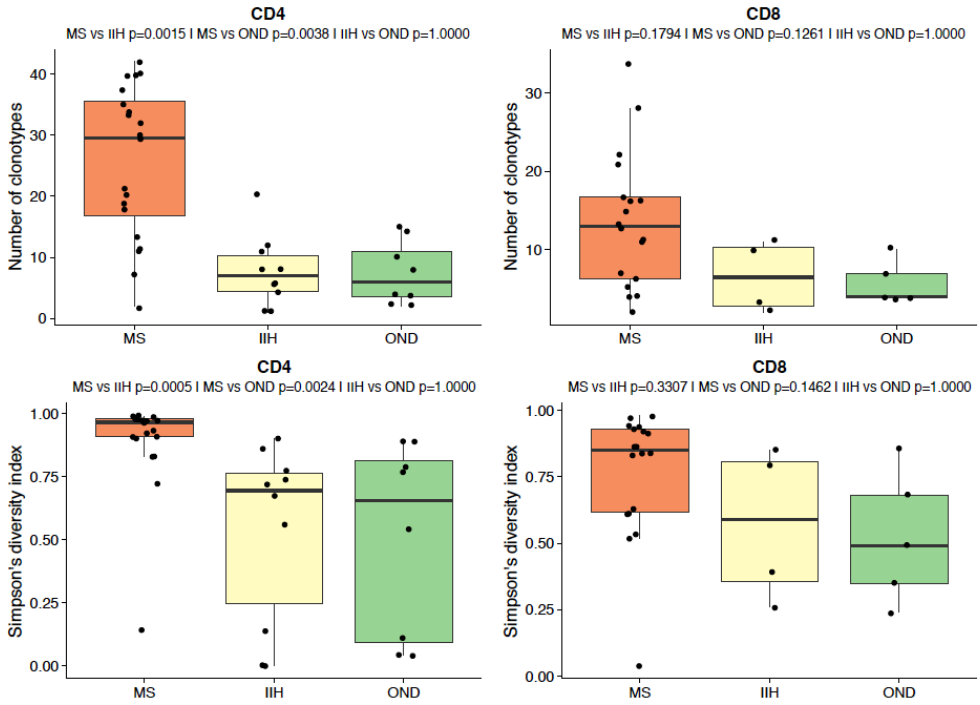


Figure 5.5. Comparison of CD4$^+$ and CD8$^+$ TCR repertoire diversity between the MS, IIH and OND groups. The upper panels demonstrate the number of unique clonotypes (V+CDR3 sequence+J) estimated for a standard sample size of 54 TCR sequences per sample for the CD4$^+$ (left panels) and CD8$^+$ (right panels) T cell populations. The lower panels demonstrate the Simpson's diversity indices estimated for a standard sample size of 54 TCR sequences per sample. Data points represent individual samples. The box plots show the inter-quartile range (IQR, shaded box) and median (horizontal line within box). Outliers are determined as data points more than 1.5x IQR below the 25th percentile or more than 1.5x IQR above the 75th percentile. The whiskers extend to the farthest non-outlier points. The TCR clonotype and TCR repertoire diversities of the MS, IIH and OND groups were compared using the Kruskall-Wallis and Dunn's multiple comparison post-tests. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

As no significant differences were observed in TCR diversity between the IIH and OND groups, the two control groups were pooled together and compared with the MS group. This analysis demonstrated significantly higher numbers of unique TCR clonotypes in both the CD4$^+$ and CD8$^+$ TCR repertoires of MS patients compared with controls (IIH and OND combined) (Figure 5.6). In addition, Simpson's diversity indices were significantly higher for both the CD4$^+$ and CD8$^+$ TCR repertoires of MS patients compared with patients in the combined control group (Figure 5.6). This additional analysis is in agreement with the initial analysis performed above (Figure 5.4).

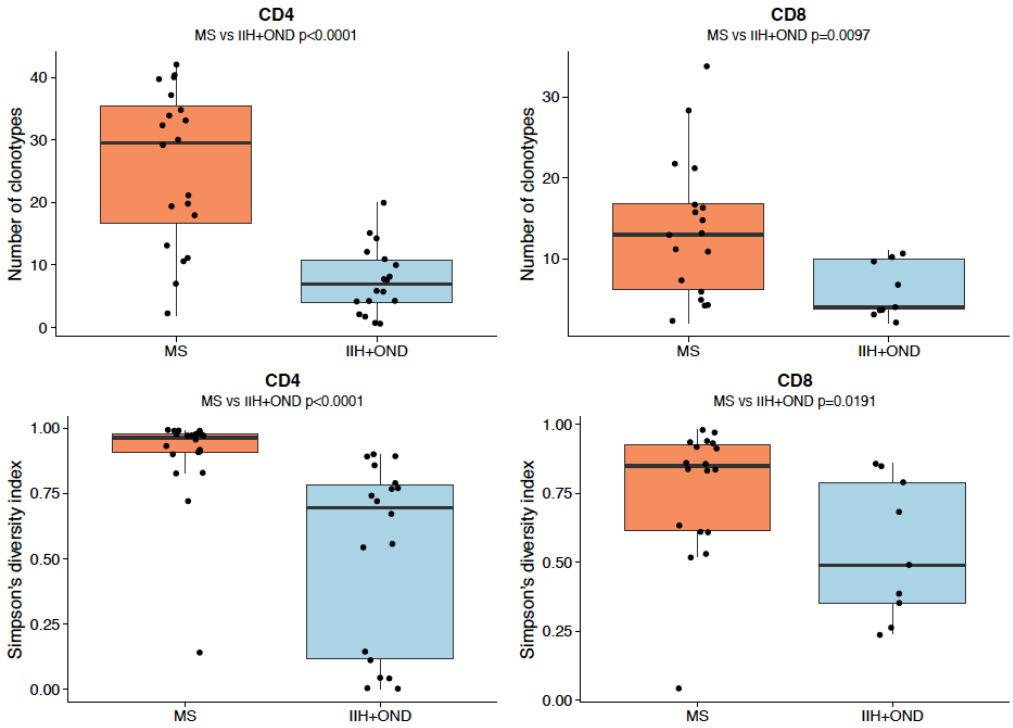


Figure 5.6. Comparison of $CD4^+$ and $CD8^+$ TCR repertoire diversity between the MS and pooled control (IIH and OND) groups. The upper panels demonstrate the number of unique clonotypes estimated for a standard sample size of 54 TCR sequences per sample of $CD4^+$ (left panels) and $CD8^+$ (right panels) T cell populations. Simpson's diversity indices are demonstrated in the lower panels, estimated for a standard sample size of 54 TCR sequences per sample. The TCR clonotype and TCR repertoire diversities of the MS and control group (IIH and OND) were compared using the Mann-Whitney test. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

5.3.4 Impact on number of sorted cells per sample on sample TCR diversity

In order to assess whether the variation in the number of sorted cells per sample has an impact on TCR diversity analysis, further analyses were performed by Dr Venturi. A significant positive correlation was observed between TCR diversity (number of unique clonotypes and Simpson's diversity index) and the number of sorted cells for both the $CD4^+$ and $CD8^+$ T cell populations in the MS group (Figure 5.7 & 5.8).

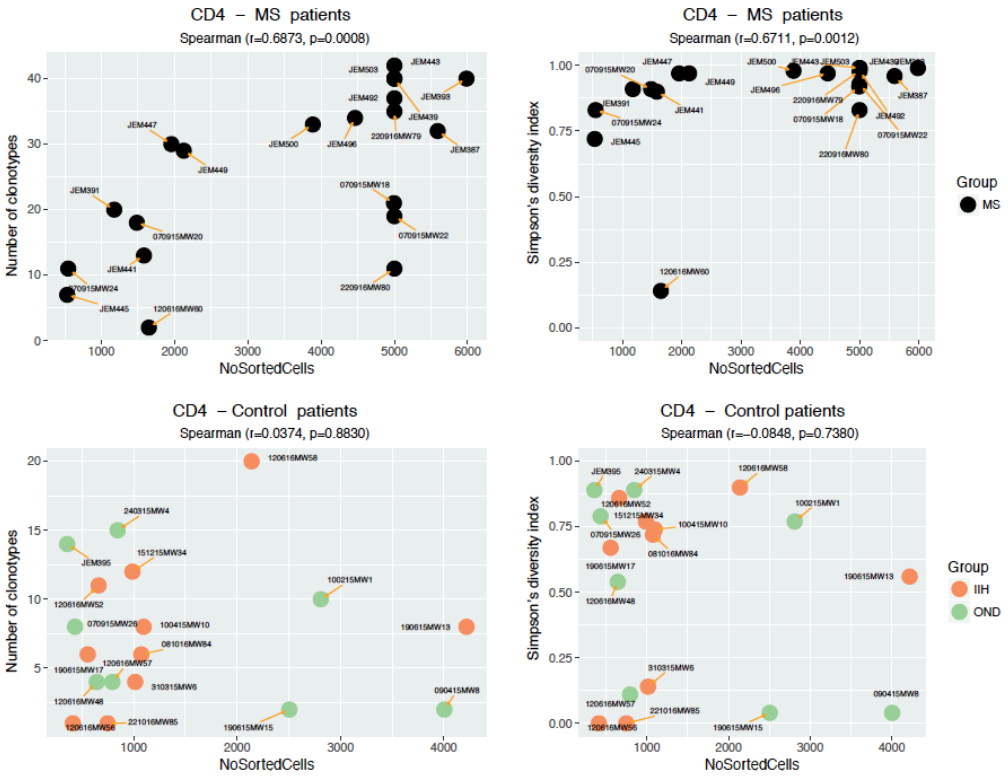


Figure 5.7. Correlation between TCR diversity and the number of sorted cells for the CD4$^+$ population.

Shown are the number of unique clonotypes (V+CDR3+J) and Simpson's diversity index estimated for a standard sample size of 54 TCR sequences versus the number of sorted cells per sample. The data points are labelled by the sample ID. The correlation between TCR diversity and number of sorted cells for the MS group (upper panels) and the control group (lower panels) was assessed using a Spearman test. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

Conversely, no significant correlations were observed between TCR diversity (number of unique clonotypes and Simpson's diversity index) and the number of sorted cells for either the CD4$^+$ or CD8$^+$ T cell populations in the control groups (Figures 5.7 & 5.8). However, there were fewer samples in total in the control groups, more CD4$^+$ samples with <1000 cells and all of the CD8$^+$ samples from control patients had <1000 cells. If the sorting of CD4$^+$ and CD8$^+$ T cells yielded a random sample of a much larger population with subsequent clonotyping revealing the TCR repertoire for a random sample of these sorted cells, then a correlation between TCR diversities (estimated for a standard-sized subsample) and the number of sorted cells would not be expected. Owing to the significant correlation in the MS group, further analysis was performed.

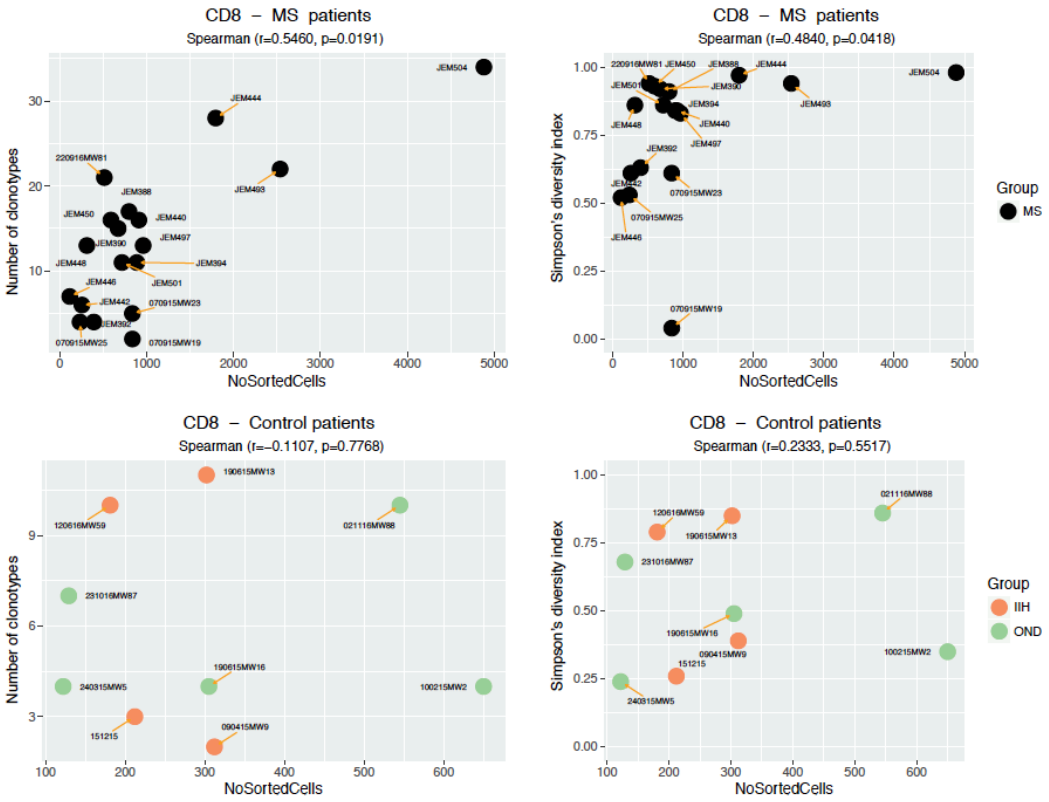


Figure 5.8. Correlation between TCR diversity and the number of sorted cells for the CD8$^+$ population.

Shown are the number of unique clonotypes (V+CDR3+J) and Simpson's diversity index estimated for a standard sample size of 54 TCR sequences versus the number of sorted cells per sample. The data points are labelled by the sample ID. The correlation between TCR diversity and number of sorted cells for the MS group (upper panels) and the control group (lower panels) was assessed using a Spearman test. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

In order to understand more fully the correlation between TCR diversity and the number of sorted cells for both CD4$^+$ and CD8$^+$ T cell populations across MS but not for control patients, the depth of clonotype sequencing for the individual TCR repertoires was examined. Species accumulation curves were used to plot the accumulation of unique TCR clonotypes for increasing-sized subsamples of sequences from the original data (Appendix, Section 8.3). From this analysis, it was observed that many of the CD4$^+$ and CD8$^+$ T cell samples for MS patients were still accumulating new unique clonotypes at a high rate as the sample size approached the total number of sequences obtained per sample. This suggests that the MS TCR repertoires are more diverse and that the small samples obtained have not captured the full extent of the population diversity i.e. the observed sample diversity most likely underestimates the TCR repertoire

diversity more for MS than control samples. This suggests that the difference in TCR diversity between MS and control group may be larger than that observed.

5.3.5 Comparison of TCR diversity between $CD4^+$ and $CD8^+$ TCR repertoires

In order to assess the differences in the TCR clonotype diversities between the $CD4^+$ and $CD8^+$ T cell populations, the number of unique clonotypes and Simpson's diversity index were estimated for a standard sample size of 54 TCR sequences per sample. Only patients with paired ($CD4^+$ and $CD8^+$) TCR repertoire data were considered for analysis. The MS and control (IIH and OND) groups were analysed separately. Following this analysis, significantly higher numbers of unique clonotypes and higher Simpson's diversity indices in the $CD4^+$ TCR repertoires compared with the $CD8^+$ TCR repertoires were observed in MS patients (Figure 5.9). No significant differences in diversity between the $CD4^+$ and $CD8^+$ TCR repertoire were observed in the combined control group (Figure 5.10).

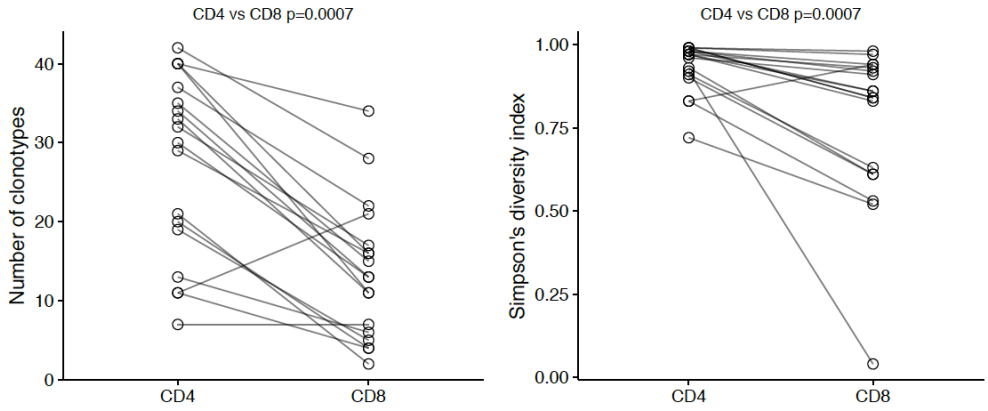


Figure 5.9. Comparison of TCR diversity between the $CD4^+$ and $CD8^+$ T cell repertoires of MS patients. The number of unique clonotypes (V+CDR3+J) estimated for a standard sample size of 54 TCR sequences per sample for the paired $CD4^+$ and $CD8^+$ T cell populations are shown in the left hand panel. Simpson's diversity indices are demonstrated in the right hand panel. The TCR clonotype and TCR repertoire diversities of the $CD4^+$ and $CD8^+$ samples were compared using the Wilcoxon test. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

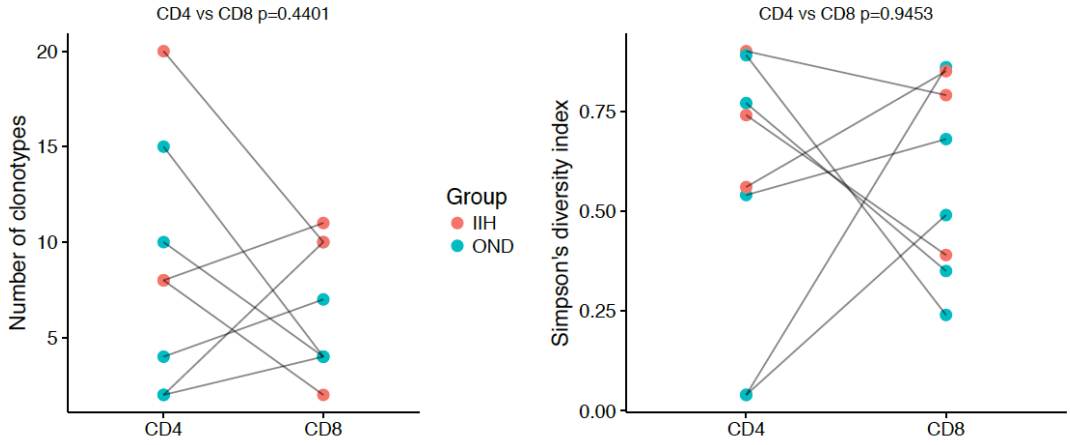


Figure 5.10. Comparison of TCR diversity between the CD4$^+$ and CD8$^+$ T cell repertoires of control patients. The number of unique clonotypes (V+CDR3+J) estimated for a standard sample size of 54 TCR sequences per sample for the paired CD4$^+$ and CD8$^+$ T cell populations are shown in left hand panel. Simpson's diversity indices are demonstrated in the right hand panel. The TCR clonotype and TCR repertoire diversities of the CD4$^+$ and CD8$^+$ samples were compared using the Wilcoxon test. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

5.3.6 TCR clonotype frequency and contribution to the overall repertoire

Figures 5.11-5.16 demonstrate the frequency of each unique TCR clonotype across all three patient groups. This preliminary analysis demonstrates that clonotype frequency across the CD4$^+$ and CD8$^+$ T cell repertoires is not evenly distributed in the MS group and that oligoclonal expansions are frequently observed, which is consistent with what has been previously described in the literature. However, interestingly a similar hierarchical structure is also observed in the IIH and OND patient groups, which is even more marked than in the MS group. In fact, the CD4$^+$ and CD8$^+$ T cell repertoires in MS patient samples are composed of a greater number of lower frequency clonotypes compared to the control groups. In contrast, CD4$^+$ and CD8$^+$ T cell repertoires in the IIH and OND patient samples are composed of a small number of higher frequency clonotypes. Therefore skewing of both the CD4$^+$ and CD8$^+$ T cell populations occurs across all three patient groups but is less marked in the MS patient group.

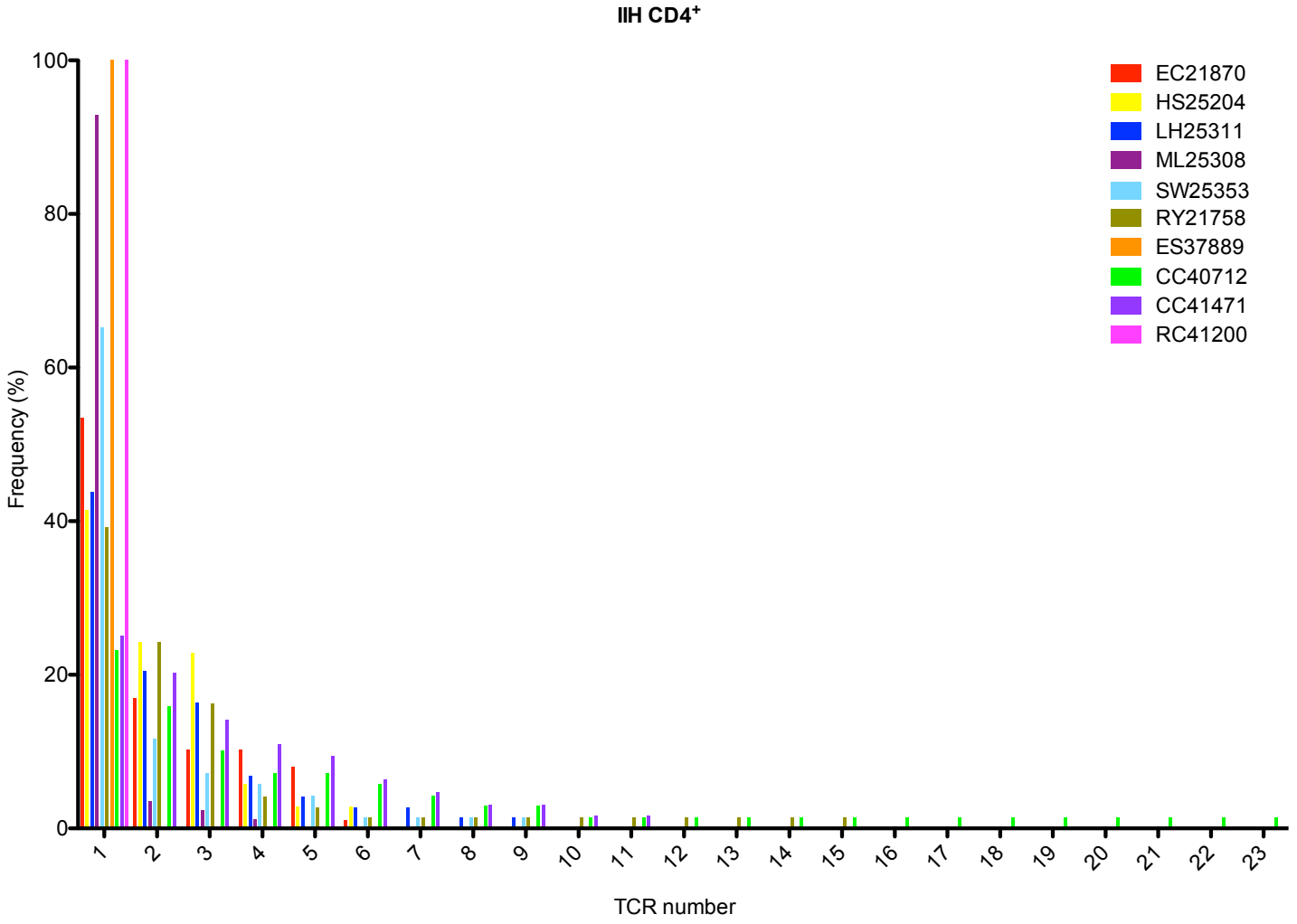


Figure 5.11. Distribution of CD4⁺ TCR clonotype frequencies within the IIH group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

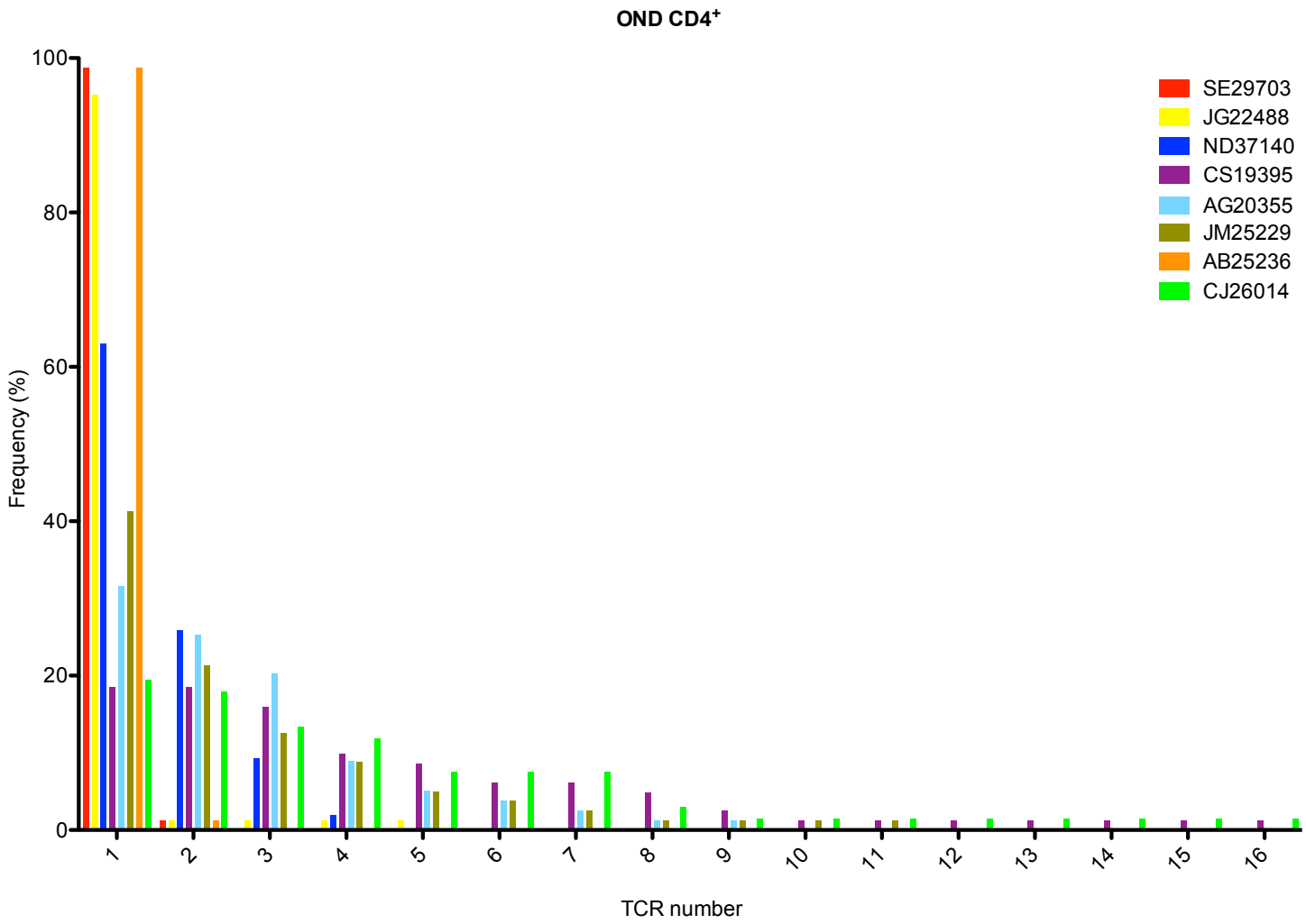


Figure 5.12. Distribution of CD4⁺ TCR clonotype frequencies within the OND group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

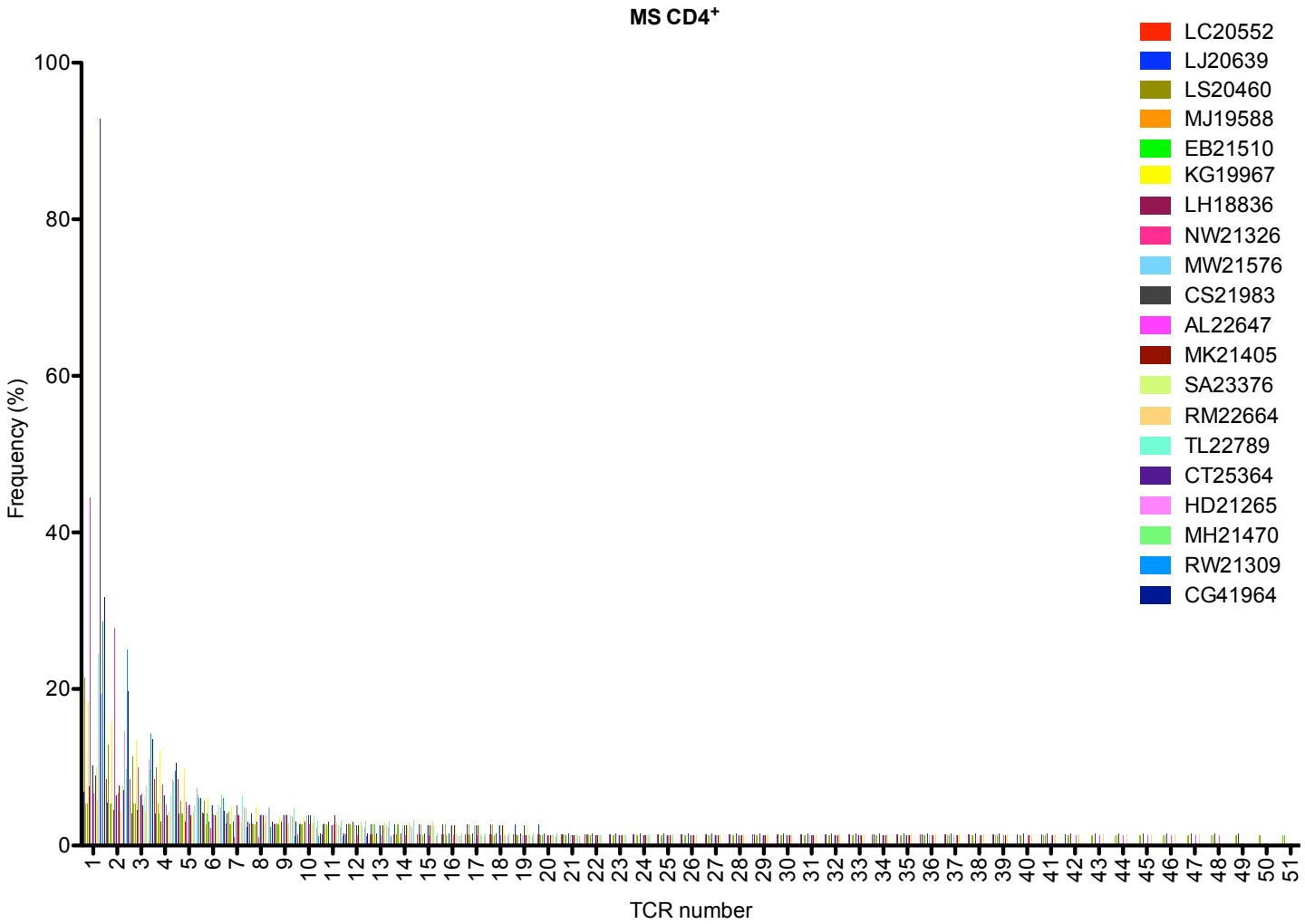


Figure 5.13. Distribution of CD4⁺ TCR clonotype frequencies within the MS group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

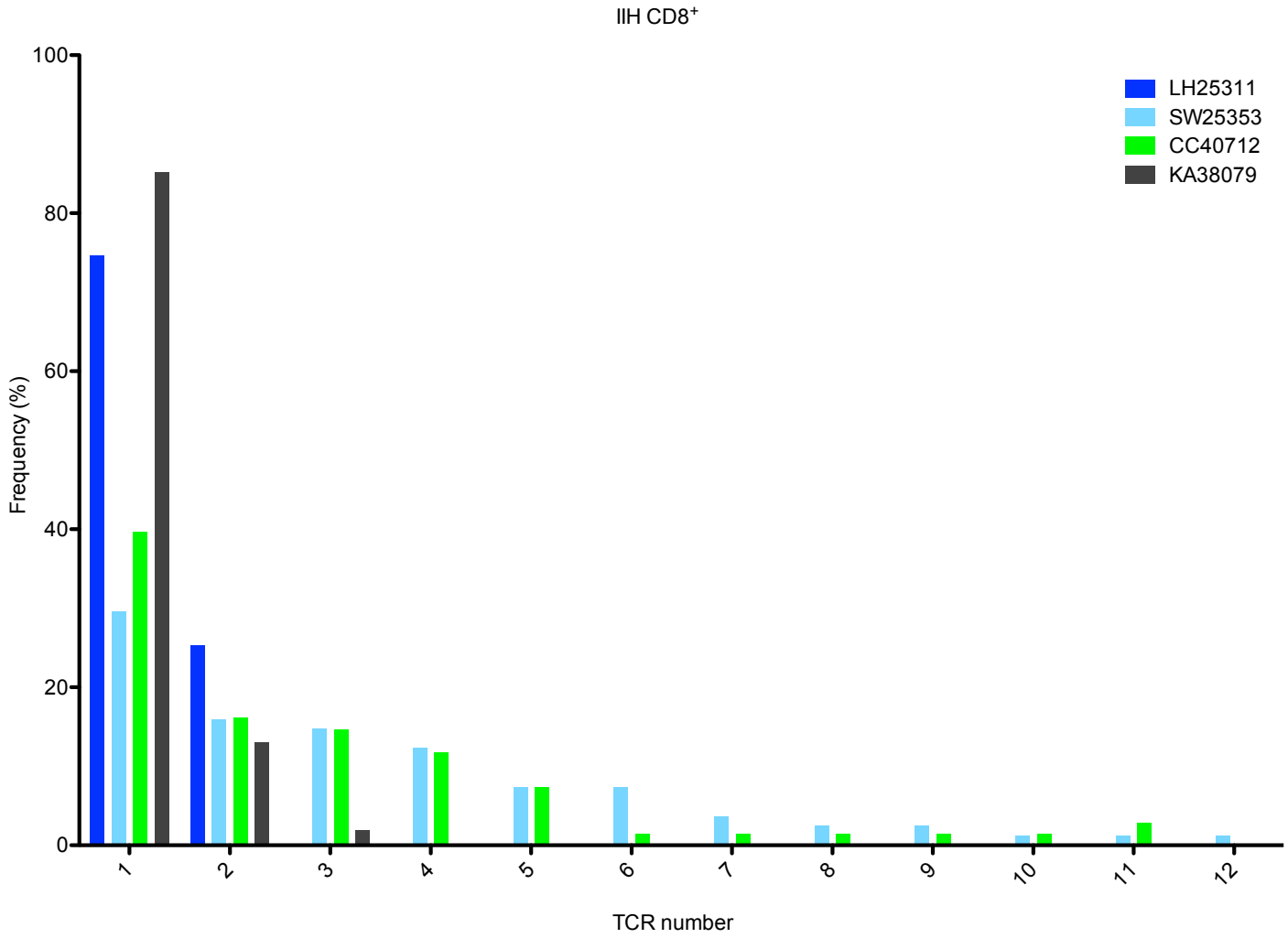


Figure 5.14. Distribution of CD8⁺ TCR clonotype frequencies within the IIH group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

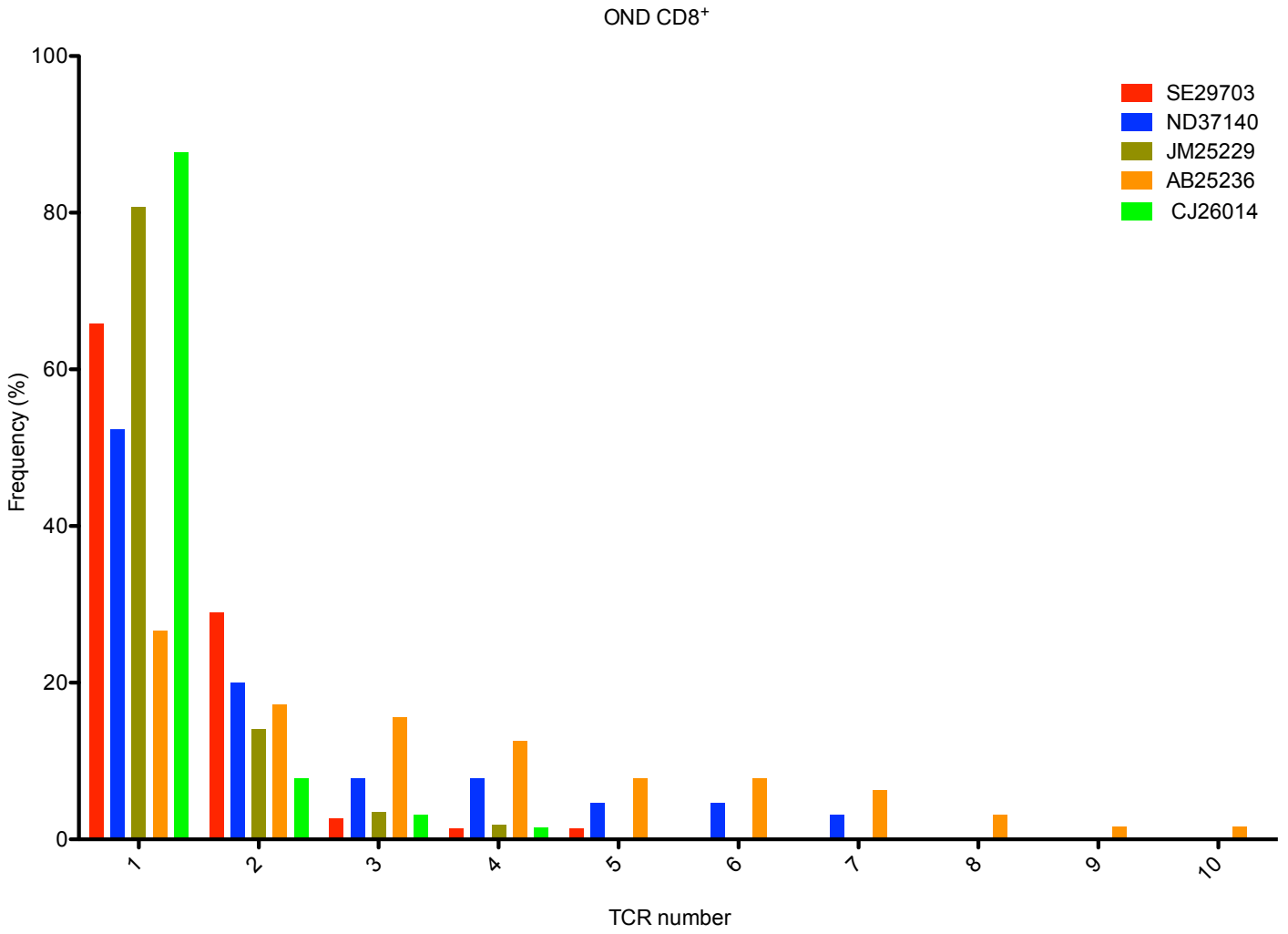


Figure 5.15. Distribution of CD8⁺ TCR clonotype frequencies within the OND group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

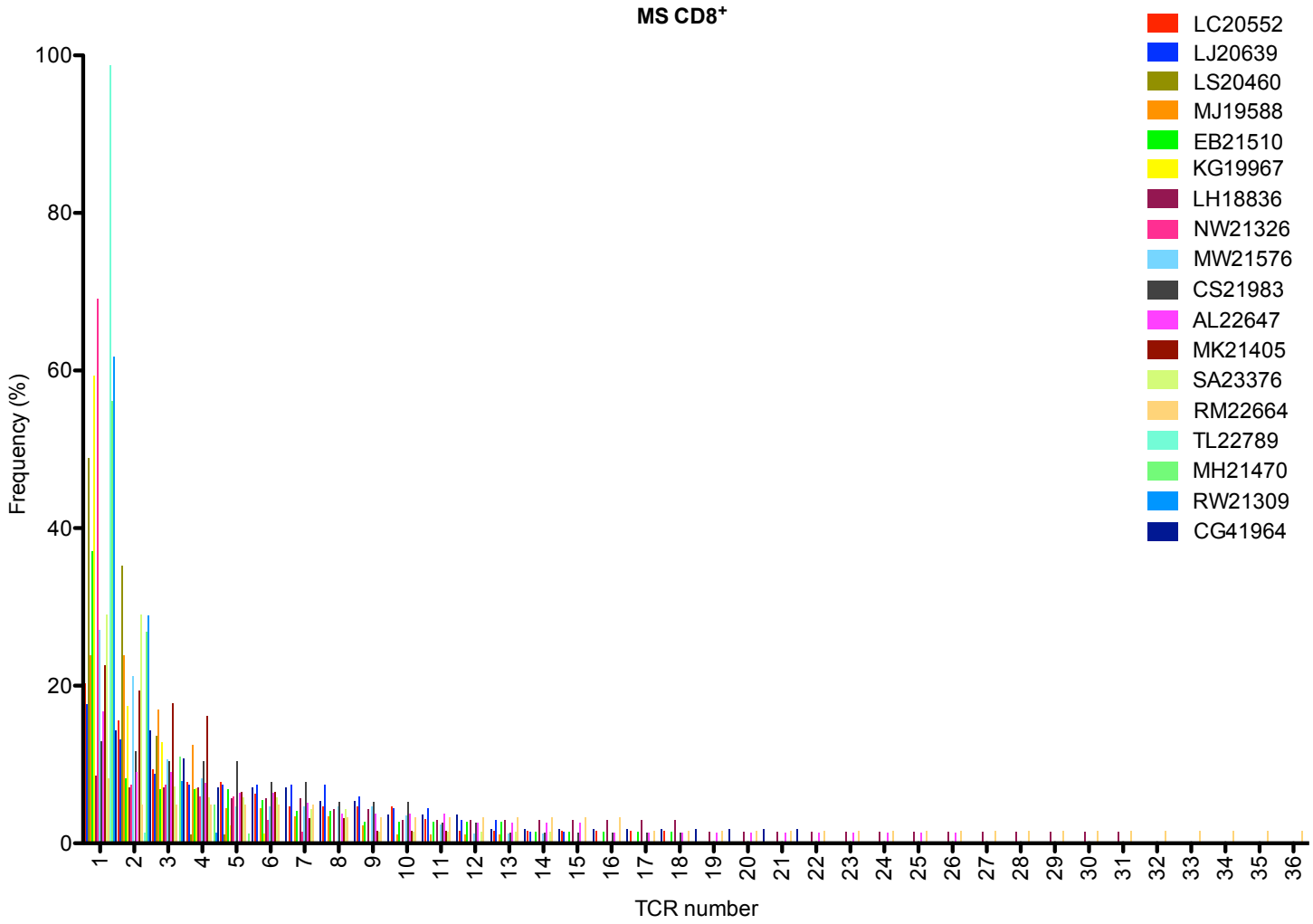


Figure 5.16. Distribution of CD8⁺ TCR clonotype frequencies within the MS group. Each unique TCR sequence was assigned a number sequentially i.e. 1, 2, 3 etc. The frequencies of these given TCR sequences were then plotted to demonstrate frequency distributions.

5.3.7 Clonal expansion analysis

It is important to understand how much each unique TCR clonotype contributes to the overall TCR repertoire in each patient. The contribution of the top 10% of TCRs in the MS group were significantly lower than the contribution of the top 10% of TCRs in the IIH ($p<0.05$) and OND groups ($p<0.05$) within the $CD4^+$ T cell repertoire. No significant differences were seen between the MS group and either of the control groups or between each of the control groups themselves in the $CD8^+$ T cell repertoire. With both control groups pooled together, significance was observed between the MS and control groups in the $CD4^+$ T cell repertoire ($p<0.0001$) and also in the $CD8^+$ T cell repertoire ($p=0.0422$). These results are displayed in Figure 5.17.

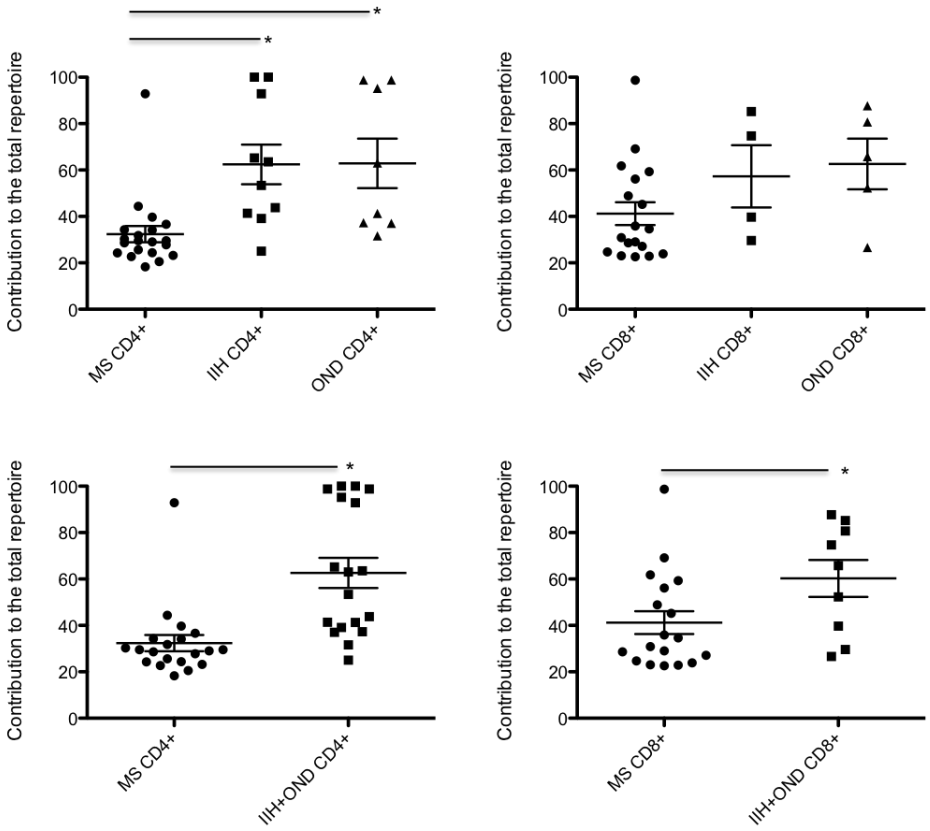


Figure 5.17. Comparison of the contribution of the top 10% TCR clonotypes to the overall TCR repertoire. $CD4^+$ (left panels) and $CD8^+$(right panels). When comparing across 3 patient groups (upper panels), the Kruskall-Wallis test with Dunn's post test was used to calculate significance. When comparing 2 groups (lower panels) the Mann-Whitney test was used.

Further to my initial analysis, in order to determine if there were any clonal expansions within the $CD4^+$ and $CD8^+$ T cell repertoires, Dr Venturi and Dr Rahmani performed a cumulative clonotype frequency distribution analysis (Appendix, Section 8.4). Here, the

top 10% of the largest clonotypes were identified and their relative contribution to the total repertoire was examined. In the MS group, the top 10% of the largest clonotypes comprised more clonotypes and collectively made a smaller contribution to the repertoire that the combined (IIH and OND) control groups. This result was stronger for $CD4^+$ than for the $CD8^+$ TCR repertoire (Figures 5.18 & 5.19). This result was in agreement with the analysis performed in Figure 5.17.

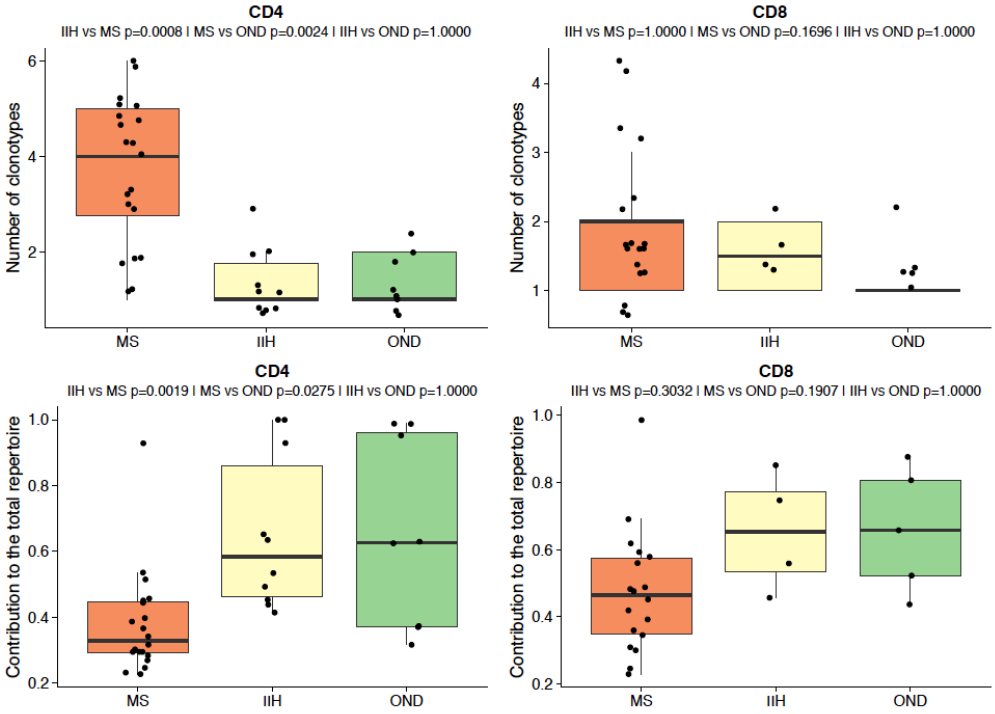


Figure 5.18. Cumulative clonotype frequency distribution analysis. The number of clonotypes comprising the top 10% of largest clonotypes within the repertoires (upper panels) and the collective relative contribution of these clonotypes to the total repertoires (lower panels) for the MS, IIH and OND patient groups. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

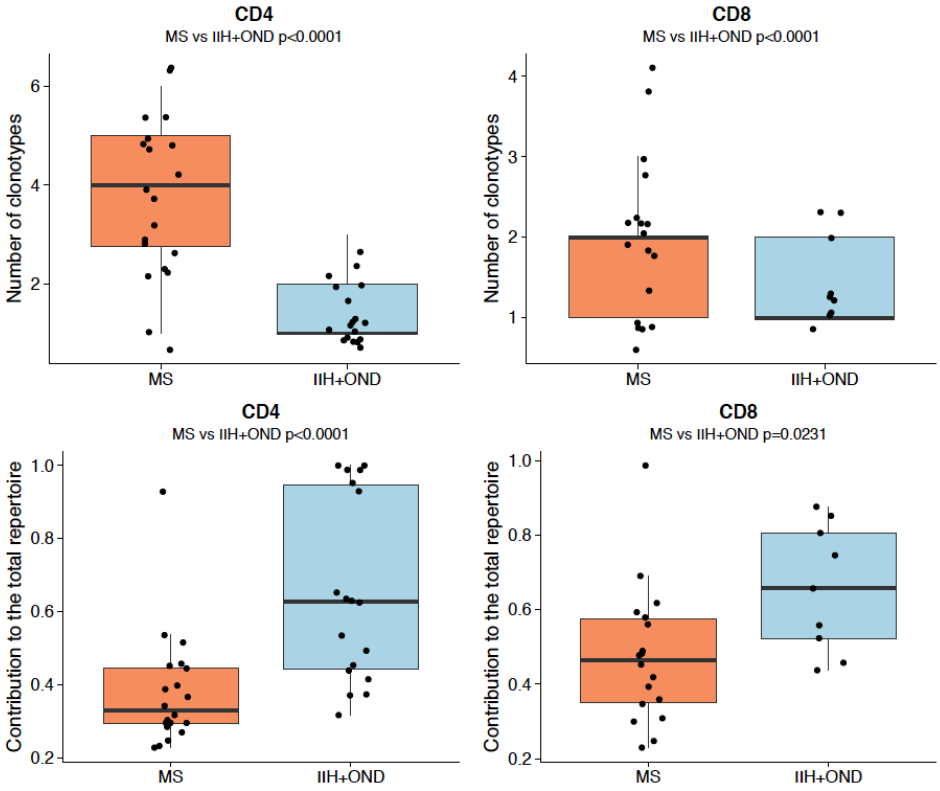


Figure 5.19. Cumulative clonotype frequency distribution analysis. The number of clonotypes comprising the top 10% of largest clonotypes within the repertoires (upper panels) and the collective relative contribution of these clonotypes to the total repertoires (lower panels) for the MS and pooled IIH and OND patient groups. Analysis performed by Dr Vanessa Venturi and Dr Adel Rahmani as described.

5.3.8 TCRVβ usage analysis of CSF-resident T cells and peripheral blood

The results of the TCRVβ screen are displayed in Appendix, Sections 8.5 and 8.6. TCRVβ screening of CSF and blood was performed for a total of 14 MS patients, 5 IIH patients and 4 OND patients for the $CD4^+$ T cell repertoire and 11 MS patients, 3 IIH patients and 3 OND patients for the $CD8^+$ T cell repertoire. Of note, TRBV 5-1, 18 and 30 stained poorly on PBMCs both with the original supplied batch, and with a replacement vial from the manufacturers. The results for TRBV 5-1, 18 and 30 were therefore excluded from the analysis. As described in Appendix, Section 8.6, some CSF TCR clonotypes were not covered by the peripheral blood TCRVβ screen and therefore direct comparison could not to be made.

CSF-resident TCRVβs >10% were highlighted in the graphs to enable easier comparison with the PBMC TCRVβ screen. The data for the $CD8^+$ T cell repertoire of patients NW21326 and RW21309 were excluded as there was a clear dominant TCRVβ expansion, which could not be detected by any of the antibodies in the TCRVβ panel. Some patients have results for $CD4^+$ T cells only as CSF-resident $CD8^+$ T cells were not clonotyped and therefore could not be compared with PBMC. Also, of note, some of the results for the PBMC TCRVβ screens total >100%. This is due to overlap in the analysis on Flowjo. Within the $CD4^+$ T cell repertoire, TCRVβ 5-1 occurred at a higher frequency within CSF-resident T cells in the MS group occurring in 10/14 patients. This TCRVβ was seen at frequencies of 3/5 in the IIH group and 1/4 in the OND group within the $CD4^+$ T cell repertoire. Within the $CD8^+$ T cell repertoire, TCRVβ 27 was the most frequently seen TCR Vβ within CSF-resident T cells within the MS group, occurring in 6/11 patients. This TCRVβ was not observed in the control populations although within the $CD8^+$ T cell repertoire only 3 patients from each control group were analysed. Initial analysis did not identify any correlation between CSF TCRVβ usage when compared with peripheral blood. In order to investigate this further, Vβ usage of different T cell subpopulations was examined – naïve ($CD45RA^+CCR7^+$), central memory (CM, $CD45RA^-CCR7^+$), effector memory (EM, $CD45RA^-CCR7^-$)), effector memory-RA (T_{EMRA}, $CD45RA^+CCR7^-$)) and all effector cells combined (All, $CCR7^-$). Similar to the initial analysis, no definitive correlations were observed between the CSF and peripheral blood compartments.

5.4 Discussion

The current literature suggests that $CD8^+$ T cells in the CNS of MS patients exhibit clonal expansion suggesting that they are antigen experienced and therefore likely to be pathogenic.[51-54, 128, 130] However, several limitations to these studies exist, suggesting caution is required before a definitive conclusion is drawn. In previous studies, the number of MS patient samples has generally been small and control populations are either lacking entirely or there is no comparison with CNS repertoires i.e. CSF or CNS tissue. It is of obvious importance and consequence to further understand the T cell repertoire within the CNS of MS patients and how this differs, if at all, from the normal population or non-MS patients. In order to try and answer this question, I performed in depth clonotyping of CSF-resident T cells to examine the TCR

repertoires in both $CD4^+$ and $CD8^+$ T cell repertoires in MS patients and non-MS patient controls. Our existing ethics covered collecting CSF from patients attending for routine diagnostic (or therapeutic in the case of IIH) lumbar punctures but not from 'normal' volunteers. We therefore endeavoured to collect CSF from patients with IIH as this was bountiful and considered to be the closest match to 'normal' CSF as possible. Patients attending for diagnostic routine lumbar puncture for neuroinflammatory disease but later determined to have an alternative diagnoses were collected as a second control group. Other patients undergoing lumbar puncture for alternative diseases were also included in this group.

The initial overview from my data (without standardisation for TCR frequency or sorted cell numbers) suggested that samples from MS patients contained more TCRs in the $CD4^+$ and $CD8^+$ T cell repertoire compared to control groups. In addition, it appeared that the $CD4^+$ and $CD8^+$ T cell repertoires were more skewed in the control groups compared with the MS group. In order to understand variations in TCR diversity further, the number of unique clonotypes and their contribution to the overall TCR repertoire was analysed. Analysis of the number of unique TCR clonotypes demonstrated that within the $CD4^+$ T cell repertoire there were significantly more unique clonotypes in the MS group compared with both the IIH and OND groups. Significance was also observed when the IIH and OND group results were pooled together. With respect to the $CD8^+$ T cell repertoire, no significance was initially observed between the MS group and either of the control groups, but significance was observed when the control groups were pooled together.

To understand how these unique clonotypes contributed to the overall TCR repertoire, the total contribution of the top 10% TCRs to the overall repertoire was analysed. As expected from the above results, the top 10% of TCRs in the MS $CD4^+$ T cell repertoire contributed significantly less to the overall TCR repertoire than those in both the IIH and OND groups. This significance also held when both control groups were pooled together. Within the $CD8^+$ T cell repertoire, no significance was seen when comparing the MS group with the IIH and OND groups separately but significance was achieved when the groups were pooled together. This initial analysis suggested that the MS $CD4^+$ T cell repertoire was more diverse with less skewing and thus less evidence of

clonal expansion. Similarly, although significance was not achieved when comparing the MS group with either control group in the MS repertoire within the $CD8^+$ T cell repertoire, significance was achieved with the combined control group, again supporting the notion of a less clonally expanded repertoire in the MS group. If these results are considered the other way around, the control groups might be considered to be demonstrating clonal expansion, with fewer TCRs in the repertoire and the top 10% contributing more to the overall repertoire. However, this initial analysis suffered from the lack of standardisation for overall TCR frequencies obtained from each sample and also for the number of sorted cells. In order to attempt to confirm these findings and the effect of cell numbers on the results, further analysis was performed by Dr Venturi, as discussed in the methods and results section of this chapter.

In order to account for the differences in overall TCR frequencies, Dr Venturi was able to standardise the data set to the minimum number of TCR frequencies observed in the patient samples. Therefore all data was standardised for a TCR frequency of 54 clonotypes. After standardisation, the results for the number of unique clonotypes were similar to the initial analysis. Within the $CD4^+$ T cell repertoire, there were significantly more unique TCR clonotypes in the MS group compared with both control groups individually as well as when combined. The initial results were also mirrored by the standardised $CD8^+$ T cell analysis. A comparison of the number of unique TCR clonotypes did not demonstrate any difference between the MS groups and the control groups individually but was significant when the data from the control groups was pooled together. This result was further confirmed by calculating Simpson's diversity index. The standardised data also yielded similar results with respect to overall contribution of individual TCR clonotypes to the TCR repertoire. The top 10% of TCRs in the $CD4^+$ T cell repertoire in the MS group contributed less to the overall repertoire than those in the control groups individually and when data from each of the control groups was pooled. Again, as in the initial analysis, no significance was observed in the $CD8^+$ T cell repertoire between the MS group and the individual control groups but was achieved when both of the control groups were combined.

In order to examine whether there was any effect of the number of sorted cells per sample on the overall TCR diversity, correlation between the numbers of sorted cells,

the number of unique TCR clonotypes and Simpson's diversity index was examined. This analysis demonstrated that significant positive correlations were present for both $CD4^+$ and $CD8^+$ T cell repertoires in the MS group. However, no correlation was found for the control groups. However, when the samples were analysed with species accumulation curves, it was found that $CD4^+$ and $CD8^+$ T cell repertoires in the MS group were still accumulating new unique clonotypes at a high rate as the sample size approached the total number of sequences obtained per sample. This suggests that the differences observed are likely to be an underestimate of TCR diversity in the MS group and that the difference in TCR diversity between MS and control groups may actually be larger and more pronounced than that observed.

In addition to the differences observed between patient groups, it is also noteworthy that when comparing the $CD4^+$ and $CD8^+$ T cell repertoires, there were significantly higher numbers of unique TCR clonotypes and higher Simpson's diversity indices in the $CD4^+$ TCR repertoire compared with the $CD8^+$ TCR repertoire of MS patients. However, no significant differences in TCR diversity between the $CD4^+$ and $CD8^+$ T cell repertoires were observed in the combined control group. Despite a difference in the $CD8^+$ and $CD4^+$ T cell repertoire in MS patients, suggesting clonal expansion in the $CD8^+$ T cell repertoire (higher number of clonotypes and higher Simpson's diversity index in the $CD4^+$ T cell population), no differences were observed between $CD4^+$ and $CD8^+$ T cells in the control groups, with $CD8^+$ T cells showing more evidence for clonal expansion in the control groups when compared with the MS group.

Taken together, these results provide evidence for an intriguing interpretation of TCR repertoires in both the 'normal population' and in MS pathogenesis. Firstly, an important observation is that within all of the patient groups there seems to be evidence for clonal expansions in both the $CD4^+$ and $CD8^+$ T cell repertoires, which is to some extent lost in the MS patient group. The IIH and OND TCR repertoires are typically more skewed, with fewer large clonotypes making a substantial contribution to the repertoires with the remainder of the repertoire being comprised of low copy number clonotypes. The MS repertoires are more diverse and the frequencies more evenly distributed across TCR clonotypes.

Although clonal expansions have been reported previously in patients with MS, the lack of control data has hindered interpretation. Although these clonal expansions in previous studies may still be pathologically relevant, the observation that they are present in control populations here is of particular importance. It may suggest that clonal expansions are a universal finding within CSF with relevance to general CNS immune surveillance and anti-viral activity similar to that found in peripheral blood.[139] T cells are essential in maintaining CNS immune surveillance,[16] identifying and destroying potential pathogens. Thus, it is logical that clonal expansions might be observed in 'normal' CSF if that is the case.

Of course, caution should also be exercised in over interpreting these results, as IIH and OND can still not be totally comparative to the 'normal' general population. In addition, lower patient numbers in the control groups and less successfully clonotyped samples may have made a contribution to the overall results. Interestingly, a recent study has examined TCR repertoires through deep sequencing technology in a small sample of MS patients against a control population of IIH. This study did demonstrate a significantly higher frequency of clonal expansions in the MS group although there were only 5 patients in each group and samples were not sorted into $CD4^+$ and $CD8^+$ T cell repertoires.[138]

If T cell clonal expansion is a feature of normal CSF immune surveillance, the question of why this would be lost in MS needs to be addressed. As discussed in chapter 4, a significantly higher number of both $CD4^+$ and $CD8^+$ T cells is observed in the CSF of MS patients compared with controls, possibly owing to the increased permeability in the blood-brain-barrier observed in MS.[2] As such, the increased influx of $CD4^+$ and $CD8^+$ T cells may 'even out' the TCR repertoire. However, one could argue that this should still result in a skewed repertoire. An alternative explanation may be that T cells from MS patients have lower activation thresholds and make more diverse responses to pMHC antigen causing a flattening of the repertoire. Genome wide association studies have identified risk variants associated with T cell activation thresholds, which may support this.[45, 356] T cells may also be reactive against multiple CNS epitopes, which may reflect the phenomenon of epitope spreading. It should be noted that this study as well as others only offers a snapshot of the CSF constituents at the time of sampling. The

results may be different if CSF sampling was undertaken at multiple time points. If this were possible, it may be postulated that different TCRs at different frequencies would be observed, reflecting normal turnover in the immune-surveillance of the CNS. Ideally, serial lumbar punctures would be performed to examine this in more detail but ethically this is difficult to justify.

I also considered the overall contribution of individual TCRVβ genes to the observed clonotypes and investigated if any of the dominant expansions in the CSF could also be seen in the peripheral blood. Interestingly, there were no real correlates between CSF TCRVβ usage and that observed in peripheral blood. In addition, the most common CSF TCRVβ usage was not consistent with that seen in the literature previously (Table 5.1).

In conclusion, $CD4^+$ and $CD8^+$ CSF T cell repertoires are skewed in all patient groups. Although, the IIH and OND repertoires are typically more skewed, with fewer large clonotypes making a substantial contribution to the repertoires and the remainder of the repertoire being comprised of low copy number clonotypes. The hierarchical structure of CSF resident T cell repertoires in all patient groups is likely to be a feature of 'normal' CNS immune-surveillance. The MS repertoires are more diverse and frequencies are more evenly distributed across clonotypes. Although this may be a result of non-specific T cell infiltration across a more permeable blood-brain-barrier present in MS patients, this would seem unlikely as a hierarchical structure is still maintained. In light of the results of recent GWAS studies it would seem much more likely to be the result of lower T-cell activation thresholds in MS patients resulting in more diverse TCR repertoires being mobilized in response to antigenic stimulus. This in turn would result in a T cell repertoire with a much higher probability of being able to react to self-antigen and drive autoimmune disease. These data have important implications for understanding normal CNS immune-surveillance and also MS disease pathogenesis. The evidence for $CD4^+$ and $CD8^+$ CSF-resident T cells being involved in MS disease pathogenesis is overwhelming, but relying on $CD8^+$ T cell clonal expansions to identify the pathogenic TCRs may not be possible if this 'evening out' of the TCR repertoire is a consistent observation in future studies. However, under the premise that MS CSF-resident $CD8^+$ T cell expansions are pathogenic, we aimed to identify the

pathogenic triggers and antigenic targets of these populations. This is discussed in more detail in chapter 6.

Chapter 6

Identifying the pathogenic triggers and antigenic targets of CSF-resident CD8$^+$ T cells in Multiple sclerosis

6.1 Introduction

6.1.1 Identifying antigenic specificity of CD8$^+$ T cells in MS

Despite the weight of evidence convincingly demonstrating that MS is an autoimmune disease, one fundamental question remains unanswered: what is the target antigen of immune cells involved in MS pathogenesis? In addition, although CD8$^+$ T cells have been shown to have an antigen-experienced phenotype and in some studies are characterized by an oligoclonal expansion, what triggers this expansion remains unknown. It is clear that MS is a clinically heterogeneous disease with patients experiencing a range of disease trajectories ranging from a relatively benign course to one of a more aggressive, rapidly disabling nature.[3] Therefore knowledge of the pathogenic triggers and antigenic targets is especially important for both disease stratification and for the design of individualised therapy.[353]

6.1.2 Candidate antigenic targets of CD8$^+$ T cells in MS

Studies to date have attempted to identify CD8$^+$ T cells specific for certain CNS resident antigens *in vitro* or by studying their potentially pathogenic effects in murine models. These studies have focused on co-culturing peripheral blood CD8$^+$ T cells with candidate antigens followed by functional assays investigating cytokine release and cytotoxicity. Antigens have included myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), glial fibrillary acidic protein (GFAP) and transaldolase.[63, 142, 143, 145, 146, 148] Whilst some of these studies demonstrate increased CD8$^+$ T cell responses in MS patients[142, 145, 148] others have not demonstrated any differences from controls.[143, 288] Two recent studies by the same group investigating CD4$^+$ and CD8$^+$ T cell responses from CSF-

derived cell lines against a variety of candidate antigens did not demonstrate any substantial T-cell reactivity in MS patients.[104, 149] In addition to *in vitro* studies, candidate antigen-specific $CD8^+$ T cells have been shown to be pathogenic in animal models. These include $CD8^+$ T cells specific to MOG,[179, 180, 357] MBP,[181], GFAP,[144] in addition to haemagglutinin[182] and ovalbumin expressed by oligodendrocytes.[183] As well as pathogenic targets, $CD8^+$ T cells may also target $CD4^+$ T cells, acting in a regulatory capacity.[147, 203, 210, 358, 359]

6.1.3 Epstein-Barr virus as a potential causative agent in MS pathogenesis

In addition to antigenic targets, the initial causative agent in MS is also unknown. Based on epidemiological,[153] serological[154, 155], CSF[156-159] and Epstein Barr Virus (EBV)-reactive T cell frequencies[163-167] there is some evidence that this virus may play a role in disease pathogenesis. Four different hypotheses exist as to its potential role in MS disease pathogenesis. Firstly, the EBV cross reactivity hypothesis proposes that T cells primed by exposure to EBV antigens cross-react with and attack CNS antigens.[360] Secondly, the EBV bystander hypothesis proposes that the CNS immune attack is primarily directed towards EBV but resulting in bystander CNS damage.[361] The third hypothesis, the αβ-crystallin (mistaken self) hypothesis proposes that exposure to infectious agents induces the expression of αβ-crystallin, a heat-shock protein, in lymphoid cells. The immune system then mistakes self, oligodendrocyte-derived αβ-crystallin for a microbial antigen resulting in demyelination.[362] The final hypothesis (the EBV infected autoreactive B cell hypothesis) proposes that in genetically susceptible individuals, EBV-infected autoreactive B cells produce pathogenic autoantibodies and provide survival signals to autoreactive T cells in the target organ.[363]

Despite these candidate antigen approaches and EBV-derived hypotheses, to date, no unbiased assessment of antigen specificity has been performed on $CD8^+$ T cells isolated from the CSF of patients with MS. It is also of note that previous studies have predominantly investigated the antigen specificity of $CD8^+$ T cells from the peripheral blood of MS patients. However, given that a poor correlation exists between blood and intrathecal T-cell phenotypes,[111] there is some doubt about how data from peripheral

blood translates to CSF.[149] In a recent article, the importance of performing an unbiased assessment of $CD8^+$ T cell antigen specificity has been highlighted recommending an approach similar to the one undertaken in this study.[353] This part of the thesis therefore aimed to determine the pathogenic triggers and antigenic targets of CSF-resident $CD8^+$ T cells without prior hypothesis as to the potential target.

6.1.4 Aims and objectives

In this chapter, we aimed to utilise combinatorial peptide library (CPL) screen technology[286] in an unbiased approach to determine the peptide specificity of an individual TCR identified from the CSF $CD8^+$ T cell repertoire of a patient with MS. In addition we utilised a more targeted approach using B95.8 EBV to determine if $CD8^+$ T cells reactive to this peptide were also present in the CSF. In response to antigenic stimuli (such as EBV), $CD8^+$ T cells can induce apoptosis via Fas and TNFα. Therefore, by utilising a TNFα capture assay, EBV-specific TCRs can be identified. TCR EBV-specificities were also analysed across the two control groups (IIH and OND). In summary, this part of the thesis aimed to utilise knowledge of TCRs from CSF-resident $CD8^+$ T cells from MS and determine their pathogenic triggers and antigenic targets.

6.2 Methods

All methods are described in detail in the materials and methods section of this thesis (Chapter 2) but will be briefly summarised here.

6.2.1 Lentiviral transfection of $CD8^+$ T cells and combinatorial peptide library screening

6.2.1.1 Cloning of donor TCR and lentivirus synthesis

Following clonotyping of CSF-resident T cells, patients with an oligoclonal expansion in the $CD8^+$ T cell population were identified. TCR α chain clonotyping was then performed and 2 patients with matching α and β chain frequencies were chosen for TCR design by Genewiz Inc (USA) and lentiviral construction. Briefly, donor TCR and pELN.003 plasmids were digested with Xbal and BamHI enzymes. Following digest isolation on a 1% agarose gel and DNA extraction, ligations were performed.

6.2.1.2 Bacterial transformation of ligation reactions

Following ligation, products were added to XL10 gold bacteria and after incubation were spread on plates and incubated overnight. The following day, colonies were picked and further cultured in LB media overnight. Miniprep was performed the following day.

6.2.1.3 Miniprep/maxiprep of amplified donor TCRs

Incubated media was centrifuged and the pellet resuspended in PBS. Miniprep (Zymo Research, Irvine, USA) was performed as per the manufacturer's instructions.

Following miniprep, DNA was quantified and sequenced to confirm the presence of amplified TCR. Following confirmation, maxiprep was performed as per the manufacturer's instructions.

6.2.1.4 $CaCl_2$ transfection for lentiviral production

Lentiviral synthesis was performed by combining pELN lentivirus vector containing donor TCR, pRSV.Rev, pVSVg, pMDLg/pRRE, 150 µl of $CaCl_2$ and pH 7.1 media. This lentiviral mix was then added to 293 T cells. Media was collected 48 hours after transfection and again at 72 hours. Collected media was centrifuged, the pellet resuspended then frozen until required.

6.2.1.5 $CD8^+$ T cell isolation and lentiviral transfection

$CD8^+$ T cells were isolated from PBMC by MACS separation as per the manufacturer's instructions instructions (Miltenyi Biotec, Bisley, UK) and incubated overnight with $CD3^+$ $CD28^+$ beads. Lentivirus was subsequently added with polybreen. Lentivirus positive cells were subsequently sorted 1 week later into T cell media and further expanded in vitro.

6.2.1.6 Sizing scan and combinatorial peptide library (CPL) screening of $CD8^+$ T cells expressing dominant CSF-resident TCRs

Individual $CD8^+$ T cells recognise antigens presented at the cell surface by major histocompatibility complex class I (MHCI) molecules. These antigens are in the form of intracellular protein-derived peptide fragments, 8-14 amino acids in length.[296]

Individual MHCI-restricted TCRs exhibit a preference for a single MHCI-peptide length.[293] What is more, each TCR is able to recognise up to 1 million different MHC-bound peptides at each preferred length.[286] It is therefore of importance to first determine the length restriction of each TCR before attempting to determine antigen specificity with CPL screens.

On day 1, $CD8^+$ T cells were washed in PSG and put in R2 overnight. On day 2, cell cultures were set up for sizing scans or CPL screens. 5 µl of either sizing scan or CPL peptide mix (at a concentration of 10mM or 1mM, respectively) per well was plated in 96 well round bottom plates (in duplicate). For the sizing scan, the following mixtures were used to define the MHCI-peptide length preference of the donor TCR: X^8, X^9, X^{10}, X^{11}, X^{12}, and X^{13} (where X is any of the 19 proteogenic L-amino acids excluding cysteine; Pepscan, Lelystad, The Netherlands). For combinatorial peptide library screens (CPL) the 8mer CPL was synthesized in a positional scanning format (Pepscan). 60,000 target cells were added to each well and plates incubated at 37°C for 1-2 hours. 30,000 $CD8^+$ T cells were then added to each well and plates incubated overnight. The following day, supernatant was harvested and MIP1-β ELISA (Duoset kit, R&D Systems Inc. Minneapolis, MN, USA) performed as per the manufacturer's instructions. Plates were read on a Biorad iMark microplate absorbance reader (Biorad, Hercules, USA). Of note, T2-A2 cells, autologous EBV-LCLs and B7 cells were initially used as targets for sizing scans. However due to the high background release of MIP-1β from autologous EBV-LCLs and B7 cells, T2-A2 cells were used as targets for the final sizing scans and CPL screens.

6.2.1.7 Analysis of combinatorial peptide library screening

Results from the CPL screen were inputted into the Warwick Systems Biology Centre webtool (http://wsbc.warwick.ac.uk/wsbcToolsWebpage/resetpass.php, University of Warwick, UK) for peptide identification.[296]

An overview of the strategy employed to determine antigen specificity via CPL screening is shown below (Figure 6.1).

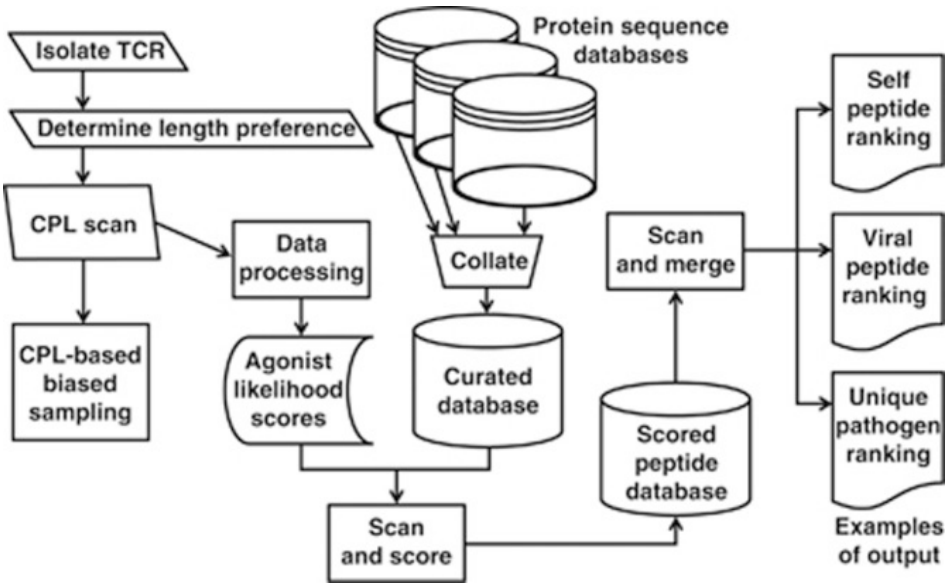


Figure 6.1. CPL-driven database screening. The peptide length preference of the isolated TCR is first determined followed by the number of peptides recognised at this preferred length. The sequence identity of these peptides is then determined. Figure taken from Szomolay et al.[296]

6.2.1.8 Assessing TCR response against chosen peptides identified by combinatorial peptide library screen

Peptides were chosen based on scores from the CPL screens (Appendix, Section 8.7) and for disease relevance (Pepscan, Lelystad, The Netherlands). Peptides were first diluted to an 8mM stock and then further diluted in PSG to a concentration of 1mM (10^{-3}M). Peptides were then diluted to concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}. 5µl of each peptide concentration was then moved to an ELISA plate and made up to final concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} following the addition of 45µl of target cells. MIP-1β ELISA was then set up in duplicate as described in section 2.3.9.10.

6.2.2 Tumour necrosis factor alpha (TNF-α) capture assay to determine antigen specificity of CSF-resident T cells

6.2.2.1 Generation of EBV stimulated T cell lines

Autologous donor PBMC were cultured with irradiated EBV-LCLs and regularly stimulated with further irradiated EBV-LCLs and IL-2.

6.2.2.2 TNFα capture assay

Where enough PBMC samples were available, TNFα capture assay was performed as outlined in detail in Materials and Methods (Chapter 2). In some instances, although the assay was performed, clonotyping of the sorted samples was unsuccessful and therefore could not be included in the analysis. For some patients, CSF-derived $CD4^+$ or $CD8^+$ T cells were not clonotyped and therefore comparison with TNF^+ PBMCs was not possible (Appendix, Section 8.8 & 8.9).

On the day before the experiment, EBV stimulated T cell lines were restimulated with EBV-LCLs. TAPI-O and anti-TNFα PECy7 was added along with 3µl each of CD28 and CD49d. Samples were then incubated overnight at 37^{o}C. Cells were then stained with aqua and the following antibodies: CD14, CD19, CD3, CD8 and CD4. Flow cytometry and cell sorting was performed of TNF^+ $CD4^+$ and $CD8^+$ populations. Following cell sorting, clonotyping and TCR analysis of these cell populations was performed as previously described.

6.2.2.3 HLA typing

Donor PBMCs were HLA typed at A, B and C loci by Proimmune (Oxford, UK).

6.3 Results

6.3.1 Patient selection for lentiviral transfection of $CD8^+$ T cells and combinatorial peptide library screening

Two patients with MS were initially chosen for lentiviral construction and $CD8^+$ T cell transfection; KG19967 and NW21326. Both patients had a large monoclonal $CD8^+$ T cell expansion that was present in both β and α chain sequencing as shown in Tables 6.1-6.4 below.

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASSYGAYNEQF	2-1	59.30	51
29-1	CSVTGQGTTEQY	2-7	17.44	15
11-2	CASSLTAGGYEQY	2-7	12.79	11
29-1	CSVSMVGTSGRYEQF	2-1	6.98	6
20-1	CSAPQGVNTGELF	2-2	2.33	2
3-1	CASSPATGNTEAF	1-1	1.16	1
			100	86

Table 6.1. TCR β chain sequencing of CSF-derived CD8$^+$ T cells for KG19967. CSF-resident T cells were sorted into CD4$^+$ and CD8$^+$ T cell populations and then clonotyped and analysed as described in chapter 2. The dominant TCR β chain was identified and paired with the dominant TCR α chain.

TRAV	CDR3	TRAJ	Freq (%)	Count
20	CAVQYNFNKFY	21	83.33	55
17	CATDKPTGNQFY	49	7.58	5
12-2	CAVNNNDMR	43	6.06	4
20	CAVQYSFNKFY	21	1.52	1
20	CAVQYNFDKFY	21	1.52	1
			100	66

Table 6.2. TCR α chain sequencing of CSF-derived CD8$^+$ T cells for KG19967. CSF-resident T cells were sorted into CD4$^+$ and CD8$^+$ T cell populations and then clonotyped and analysed as described in chapter 2. The dominant TCR β chain was identified and paired with the dominant TCR β chain.

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CASSLGGTEAF	1-1	69.12	47
19	CASSYGRAVGELF	2-2	7.35	5
5-5	CASSFWEANEQF	2-1	7.35	5
4-1	CASSQDSTPTHSNQPQH	1-5	5.88	4
7-9	CASSLPGSSYEQY	2-7	5.88	4
7-9	CAGSLGGTEAF	1-1	2.94	2
20-1	CSVQDRTYEQY	2-7	1.47	1
			100	68

Table 6.3. TCR β chain sequencing of CSF-derived CD8$^+$ T cells for NW21326. CSF-resident T cells were sorted into CD4$^+$ and CD8$^+$ T cell populations and then clonotyped and analysed as described in chapter 2. The dominant TCR β chain was identified and paired with the dominant TCR α chain.

TRAV	CDR3	TRAJ	Freq (%)	Count
14/DV4	CAMREREMNNAGNMLT	39	95.56	86
14/DV4	CATREREMNNAGNMLT	39	1.11	1
14/DV4	CAMREREMNSAGNMLT	39	1.11	1
14/DV4	CAMREREMNNAGYMLT	39	1.11	1
14/DV4	CAMREREMNNAGSMLT	39	1.11	1
			100	90

Table 6.4. TCR α chain sequencing of CSF-derived CD8$^+$ T cells for NW21326. CSF-resident T cells were sorted into CD4$^+$ and CD8$^+$ T cell populations and then clonotyped and analysed as described in chapter 2. The dominant TCR β chain was identified and paired with the dominant TCR β chain.

Following attempts at cloning of donor TCR, only KG19967 was successful and therefore taken forward for lentiviral construction and CPL screening.

6.3.2 HLA typing of KG19967

Patient KG19967 underwent full HLA typing and was heterozygous at the A, B and C loci. The patient's HLA type was as follows; HLA-A*02:01/A*30:02, HLA-B*07:02/B*51:01, C*07:02/*16:01. Initially, T2-A2 cells were used as target cells to assess whether or not the KG19967 TCR was restricted by HLA A*0201 and if so, what peptides could be recognized.

6.3.3 Sizing scan of KG19967 TCR

Sizing scan of KG19967 demonstrated a strong preference for 8mer peptides (Figure 6.2).

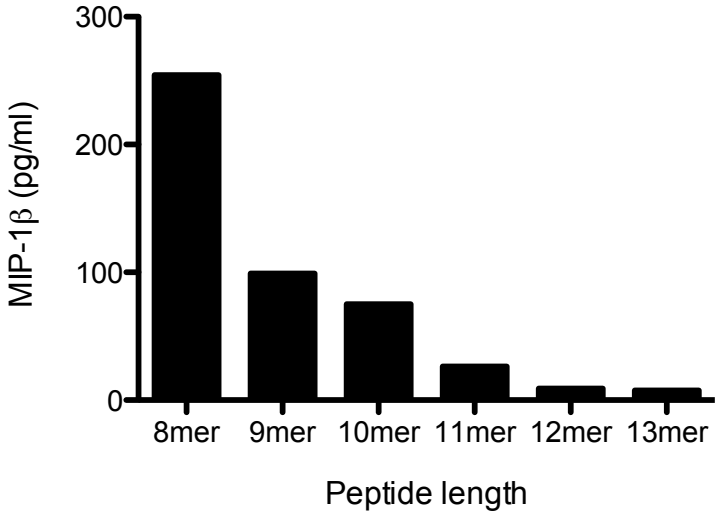


Figure 6.2. Sizing scan for KG19967 TCR demonstrating 8mer length preference. $CD8^+$ T cells expressing the dominant TCR α and β chains from patient KG19967 were washed in PSG and put in R2 overnight. On day 2, cell cultures were set up for sizing scans. 5 µl of sizing scan mix at a concentration of 10mM per well was plated in 96 well round bottom plates (in duplicate). The following mixtures were used to define the MHCI-peptide length preference of the donor TCR: X^8, X^9, X^{10}, X^{11}, X^{12}, and X^{13} (where X is any of the 19 proteogenic L-amino acids excluding cysteine). 60,000 target cells were added to each well and plates incubated at 37°C for 1-2 hours. 30,000 $CD8^+$ T cells were then added to each well and plates incubated overnight. The following day, supernatant was harvested and MIP1-β ELISA performed.

6.3.4 Combinatorial peptide library screening of KG19967 TCR

Results of the 8mer CPL screen for the KG19967 TCR are shown in figure 6.3. These results give the preferences for the TCR for each amino acid position in the 8mer peptide.

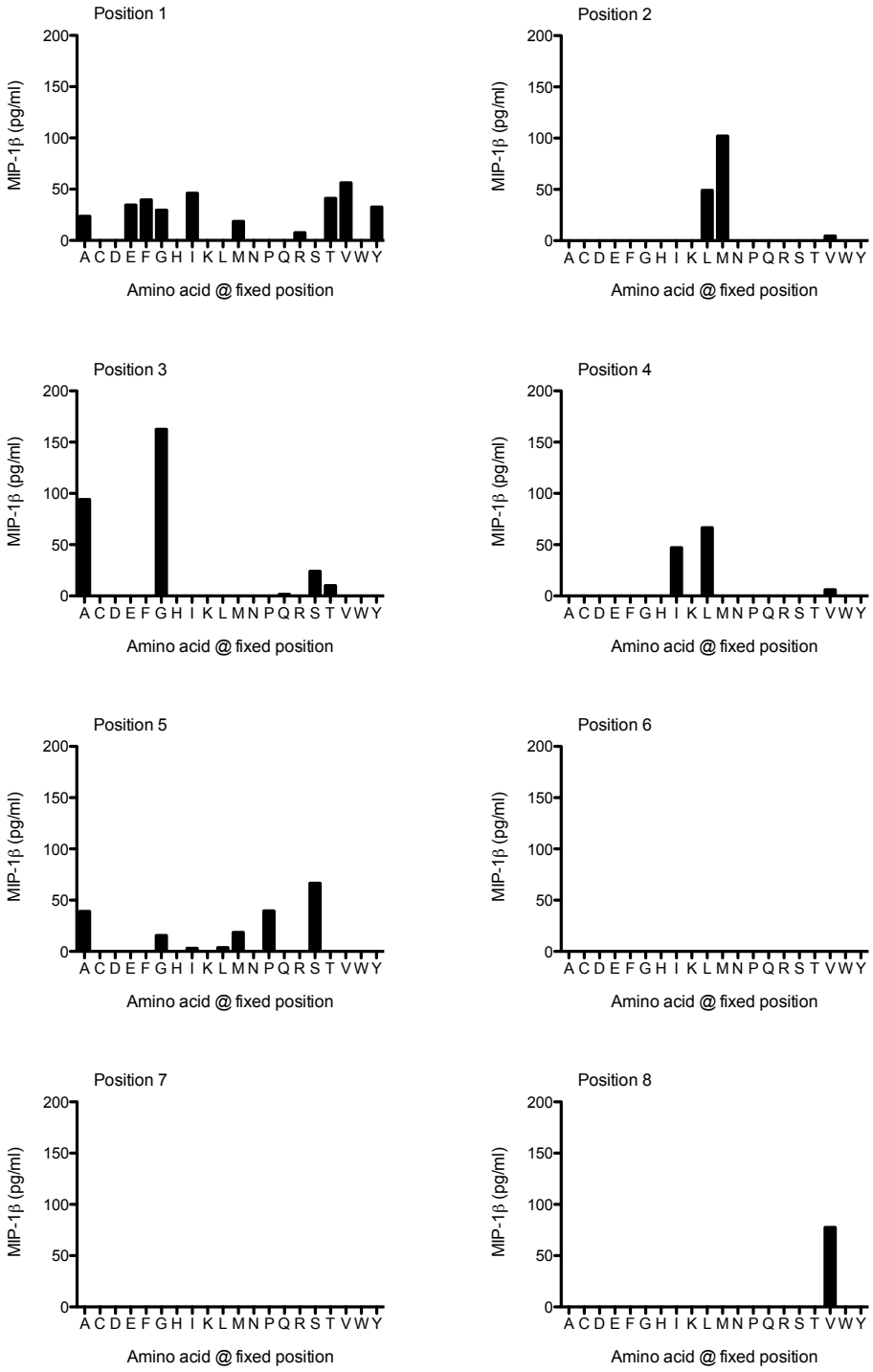


Figure 6.3. 8mer combinatorial peptide library screen for KG19967. On day 1, $CD8^+$ T cells were washed in PSG and put in R2 overnight. On day 2, cell cultures were set up for CPL screens. 5 µl of CPL peptide mix (at a concentration of 1mM) per well was plated in 96 well round bottom plates (in duplicate). For combinatorial peptide library screens (CPL) the 8mer CPL was synthesized in a positional scanning format (Pepscan). 60,000 target cells were added to each well and plates incubated at 37^{o}C for 1-2 hours. 30,000 $CD8^+$ T cells were then added to each well and plates incubated overnight. The following day, supernatant was harvested and MIP1-β ELISA performed.

These results were then inputted into the Warwick Systems Biology Centre webtool for bioinformatics analysis, searching the human viral pathogen and human self-protein databases.[296] Appendix, Section 8.7 details the comprehensive results from this search. Of these peptides, the highest scoring and those derived from human herpesviruses were chosen for peptide titration experiments. Tables 6.5, 6.6 and 6.7 demonstrate those peptides chosen.

Rank	Score	Peptide sequence	Viral ID	Rank	Score	Peptide sequence	Viral ID
1	-12.339322	VMGISSLV*	Human polyomavirus 9	**39**	-14.945093	VMAVSTCV	Macacine herpesvirus 1
2	-12.525908	VMGLAMPV*	Human herpesvirus 1	**40**	-14.945093	VMAVSTCV	Papiine herpesvirus 2
3	-12.886705	VMAISRCV*	Suid Herpesvirus 1	**41**	-14.945093	VMAVSTCV	Herpes simplex virus type 2
4	-12.92213	ILGLSTSV*	Human herpesvirus 6B	**42**	-14.945093	VMAVSTCV	Human herpesvirus 1
5	-13.25906	VLGLASCV*	Human herpesvirus 5	**43**	-14.945093	VMAVSTCV	Human herpesvirus 1
6	-13.269185	ILGISCFV*	Human herpesvirus 6B	**44**	-14.945093	VMAVSTCV	Human herpesvirus 1
7	-13.384254	TLGISHLV*	Human cytomegalovirus	**45**	-14.945093	VMAVSTCV	Human herpesvirus 1
8	-13.455771	ILGLANLV*	GB virus C	**46**	-14.949824	TLSLSLNV	Lake Victoria marburgvirus
9	-13.584583	TLALSQVV*	Encephalomyocarditis virus	**47**	-14.949824	TLSLSLNV	Lake Victoria marburgvirus
10	-13.743453	ELGLAILV*	Hepatitis C virus	**48**	-14.949824	TLSLSLNV	Lake Victoria marburgvirus
11	-13.806443	VLALAPEV*	Human herpesvirus 8	**49**	-14.949824	TLSLSLNV	Marburg marburgvirus
12	-13.891999	EMAIPGQV	Coxsackievirus B5	**50**	-14.949824	TLSLSLNV	Lake Victoria marburgvirus
13	-13.904892	VMSLSGKV	Wesselbron virus	**51**	-15.021849	ALAIAYLV	Yellow fever virus
14	-13.942427	FLGIPESV	Rabies virus	**52**	-15.021849	ALAIAYLV	Yellow fever virus
15	-14.003154	ILALAPAV*	Human herpesvirus 2	**53**	-15.021849	ALAIAYLV	Yellow fever virus
16	-14.105484	TLALPSNV	Banna virus	**54**	-15.021849	ALAIAYLV	Yellow fever virus
17	-14.153498	VLAIALVV*	Human herpesvirus 5	**55**	-15.021849	ALAIAYLV	Yellow fever virus
18	-14.288697	AMAIAKSV	Human parvovirus B19	**56**	-15.038671	GMGVSCTV	Measles virus
19	-14.353902	FLGLMCSV*	Human herpesvirus 4	**57**	-15.038671	GMGVSCTV	Measles virus
20	-14.474466	ALGIASLV	Langat virus	**58**	-15.038671	GMGVSCTV	Measles virus
21	-14.625151	ELAIPEAV	Torgue teno virus 3	**59**	-15.038671	GMGVSCTV	Measles virus
22	-14.666174	ELGLGGRV	Macacine herpesvirus 1	**60**	-15.038671	GMGVSCTV	Measles virus
23	-14.674795	ALALAGGV*	Human herpesvirus 4	**61**	-15.038671	GMGVSCTV	Measles virus
24	-14.729165	VLALGSFV	Suid Herpesvirus 1	**62**	-15.038671	GMGVSCTV	Measles virus
25	-14.736338	EMSISTWV	Human papillomavirus	**63**	-15.038671	GMGVSCTV	Measles virus
26	-14.787583	FMSLAHCV	Yaba-like disease virus	**64**	-15.038671	GMGVSCTV	Measles virus
27	-14.901285	FLALMPTV	Human herpesvirus 3	**65**	-15.038671	GMGVSCTV	Measles virus
28	-14.910185	EMSLPPWV	Thogoto virus	**66**	-15.082923	RLGISSIV	Human herpesvirus 3
29	-14.925875	ILALGLLV	Suid Herpesvirus 1	**67**	-15.090857	VMGKSVLV	Human hepatitis A virus
30	-14.945093	VMAVSTCV*	Human herpesvirus 1	**68**	-15.090857	VMGLVGGV	Papiine herpesvirus 2
31	-14.945093	VMAVSTCV	Human herpesvirus 1	**69**	-15.169798	GLGIGALV	Human immunodeficiency virus type 1
32	-14.945093	VMAVSTCV	Human herpesvirus 1	**70**	-15.182156	YLSLSDPV*	Human herpesvirus 1
33	-14.945093	VMAVSTCV	Human herpesvirus 1	**71**	-15.213557	ELALGFKV	Simian hemorrhagic fever virus
34	-14.945093	VMAVSTCV	Human herpesvirus 1	**72**	-15.243933	VMGLSDDE	Human astrovirus
35	-14.945093	VMAVSTCV	Human herpesvirus 2	**73**	-15.248486	TMGLLSIV	Vaccinia virus
36	-14.945093	VMAVSTCV	Human herpesvirus 2	**74**	-15.248486	TMGLLSIV	Vaccinia virus
37	-14.945093	VMAVSTCV	Human herpesvirus 1	**75**	-15.248486	TMGLLSIV	Variola virus
38	-14.945093	VMAVSTCV	Human herpesvirus 1	**76**	-15.248486	TMGLLSIV	Monkeypox virus

Table 6.5. Results of CPL-driven searching of the human viral pathogen database. *peptide sequences were chosen for peptide screening experiments

Rank	Score	Peptide sequence	Self ID
1	-12.513169	VMGLPWFV*	Sodium bicarbonate cotransporter 3
2	-12.525908	VMGLAAGV*	APC membrane recruitment protein 1
3	-12.539651	VMALSAVV*	Solute carrier family 43
4	-12.709879	IMGLPWFV*	Electroneutral sodium bicarbonate exchanger 1
5	-12.72542	VLGLSAAV*	Multidrug resistance protein 1
6	-12.85143	TMALSVLV	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform d
7	-12.860619	AMGLSLLV	Glutamate [NMDA] receptor subunit epsilon-4 precursor
8	-12.860619	AMGLSRAV	Isoform 2 of ATP-binding cassette sub-family A member 7
9	-12.86222	FMGLPWYV	Electrogenic sodium bicarbonate cotransporter 4 isoform c
10	-12.872962	VMGIALAV	Claudin-4
11	-12.92213	ILGLSAAV	Multidrug resistance protein 3 isoform C
12	-12.980283	GMGISNRV	WNT1-inducible-signaling pathway protein 3 isoform 1 precursor
13	-13.0103	EMGLADVV	Isoform 2 of Uncharacterized protein C1orf167
14	-13.0372	TLGLSCGV	Protein LAS1 homolog isoform 2
15	-13.072474	VLGISAEV	Prostamide/prostaglandin F synthase
16	-13.072474	VLGISLTV	Isoform 2 of Ral guanine nucleotide dissociation stimulator
17	-13.072474	VLGISRDV	Poly [ADP-ribose] polymerase 14
18	-13.072474	VLGISAEV	Prostamide/prostaglandin F synthase isoform a
19	-13.072474	VLGISRDV	Isoform 5 of Poly [ADP-ribose] polymerase 14
20	-13.072474	VLGISAEV	Isoform 3 of Prostamide/prostaglandin F synthase
21	-13.074471	FLGLSPHV	Folliculin isoform 1
22	-13.180612	GMALSVLV	Probable low affinity copper uptake protein 2
23	-13.246321	VLGLPQHV	Uncharacterized protein KIAA1522 isoform 2
24	-13.25906	VLGLAVRV	L-fucose kinase
25	-13.25906	VLGLASIV	Lecithin retinol acyltransferase precursor
26	-13.269185	ILGISGCV	Long-chain fatty acid transport protein 6
27	-13.272803	VLALSTEV	Claudin-5
28	-13.366381	GLGLSGVV	Adiponectin receptor protein 1
29	-13.371097	EMAISKTV	Dual specificity protein phosphatase 6 isoform b
30	-13.38152	AMGLPEAV	Inositol 1,4,5-triphosphate receptor-interacting protein precursor
31	-13.408002	AMALSGHV	Transcription factor SOX-7
32	-13.421525	FLGISIGV	Isoform 2 of Tetraspanin-12
33	-13.422342	FMALANGV	RNA 3'-terminal phosphate cyclase isoform a

Table 6.6. Results of CPL-driven searching of the human self peptide database. *peptide sequences were chosen for peptide screening experiments.

Tube no.	Peptide sequence	Tube no.	Peptide sequence
D1	VMGISSLV	D12	ILALAPAV
D2	VMGLAMPV	E1	VLAIALVV
D3	VMAISRCV	E2	FLGLMCSV
D4	ILGLSTSV	E3	ALALAGGV
D5	VLGLASCV	E4	VMAVSTCV
D6	ILGISCFV	E5	YLSLSDPV
D7	TLGISHLV	E6	VMGLPWFV
D8	ILGLANLV	E7	VMGLAAGV
D9	TLALSQVV	E8	VMALSAVV
D10	ELGLAILV	E9	IMGLPWFV
D11	VLALAPEV	E10	VLGLSAAV

Table 6.7 Tube numbers and corresponding peptides chosen for titration experiments.

Chosen peptides were synthesised by pepscan (Lelystad, The Netherlands) and further MIP-1β assays performed at effector:target (E:T) ratios of 2:1 and 1:1. Although positive responses were observed at high peptide concentrations for E10 (data not shown) and D5 in the 2:1 and 1:1 assays respectively, these are unlikely to be physiologically relevant. Other than for these peptides, both assays at different E:T ratios failed to demonstrate a positive response with any of the other peptides tested. Figure 6.4 demonstrates the results for an E:T ratio of 1:1.

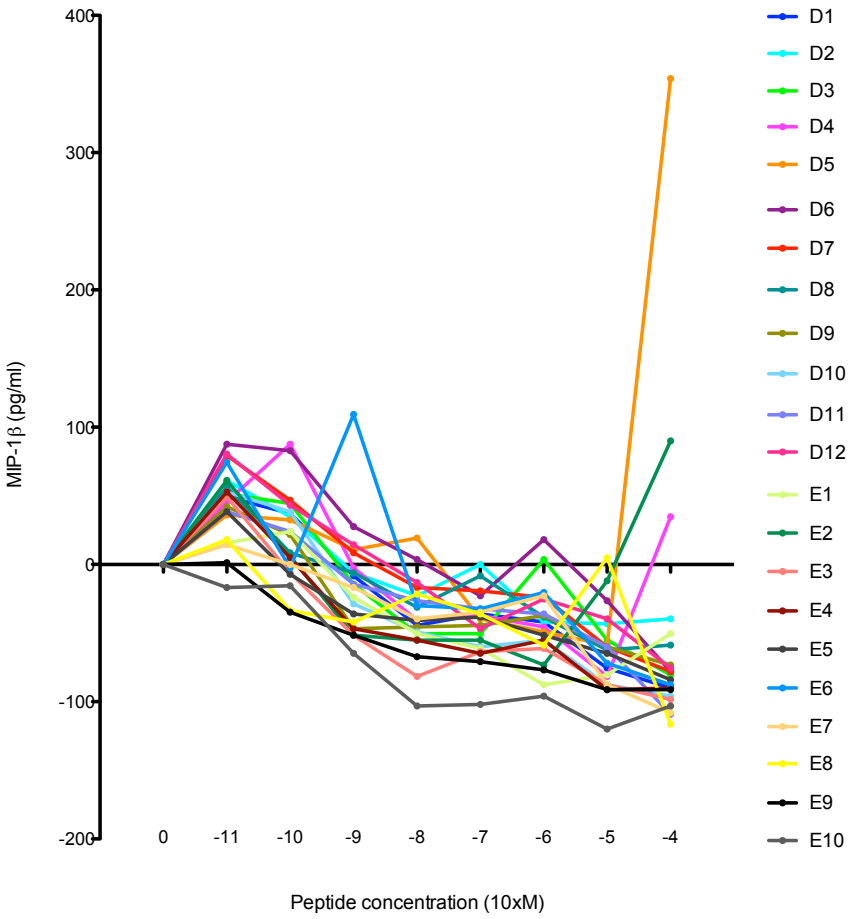


Figure 6.4. Peptide titration experiment with E:T ratio of 1:1. Key for peptides is described in Table 6.7. Peptides were chosen based on scores from the CPL screens and for disease relevance. Peptides were first diluted to an 8mM stock and then further diluted in PSG to a concentration of 1mM (10^{-3}M). Peptides were then diluted to concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}. 5µl of each peptide concentration was then moved to an ELISA plate and made up to final concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} following the addition of 45µl of target cells. MIP-1β ELISA was then performed.

6.3.5 Tumour necrosis factor alpha (TNF-α) capture assay to identify EBV-specific T cells in CSF and peripheral blood

Patients were included for TNF capture if CSF samples had been clonotyped and if there was sufficient frozen PBMCs available. Table 6.8 below details which patient samples were utilised for this assay.

MS		IIH		OND	
Patient ID	**TNF capture**	**Patient ID**	**TNF capture**	**Patient ID**	**TNF capture**
LC20552	No	EC21870	No	SE29703	Yes
LJ20639	No	HS25204	Yes	JG33488	Yes
LS20460	Yes	LH25311	Yes	ND37140	No
MJ19588	No	ML25308	Yes	DL37517	No
EB21510	Yes	SW25353	Yes	CS19395	No
KG19967	No	RY21758	Yes	AG20355	Yes
LH18836	Yes	DC37877	No	JM25229	Yes
NW21326	No	ES37889	No	AB25236	Yes
MW21576	Yes	FC24414	No	CJ26014	Yes
CS21983	No	VE25562	No	JJ37566	No
AL28847	No	CC40712	No	JP24822	No
MK21405	No	RC41200	Yes		
SA23376	Yes	KA38079	Yes		
RM22664	Yes	CC41471	No		
TL22789	No				
CT25364	Yes				
HD21265	Yes				
MH21407	Yes				
RW21309	No				
DD22299	No				
CG41964	No				

Table 6.8. Patients selected for EBV TNF-capture assay.

After the setup of the assays as described, cells were sorted into either $CD4^+$ TNF-α^+ or $CD8^+$ TNF-α^+ cells. Figure 6.5 demonstrates a typical gating strategy that was utilised.

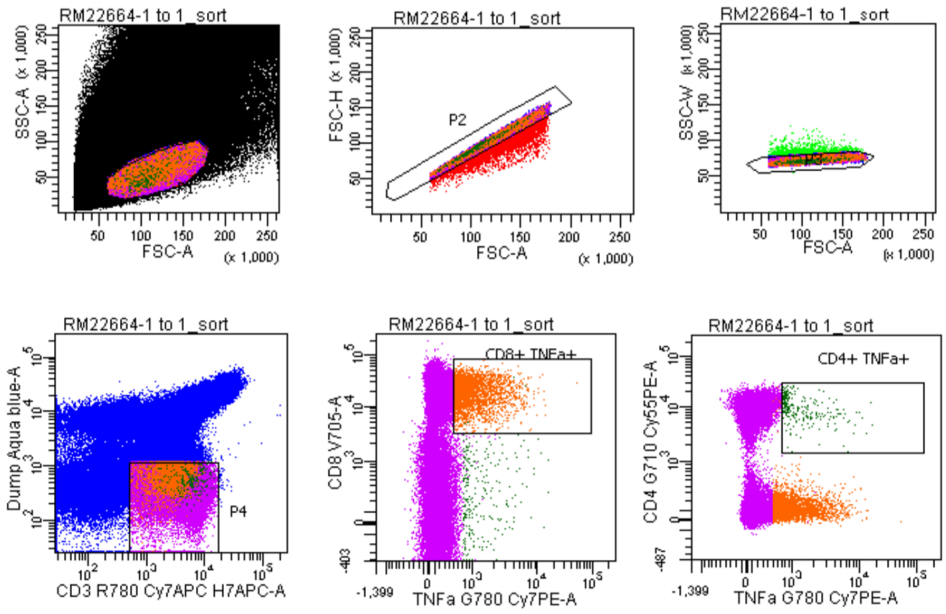


Figure 6.5. Example gating strategy for EBV-TNF capture assay. On the day before the experiment, EBV stimulated T cell lines were restimulated with EBV-LCLs. TAPI-O and anti-TNFα PECy7 was added along with 3µl each of CD28 and CD49d. Samples were then incubated overnight at 37°C. Cells were then stained with aqua and the following antibodies: CD14, CD19, CD3, CD8 and CD4. Flow cytometry and cell sorting was performed of $TNF^+ CD4^+$ and $CD8^+$ T cell populations. Following cell sorting, clonotyping of these cell populations was performed as previously described.

An example result demonstrating the matching of EBV-specific TCRs observed in the $CD8^+$ T cell repertoire of CSF and blood is shown in Figure 6.6. Samples from 9 patients with MS were used in this assay with matching EBV-specific TCRs in the CSF and blood being identified in 3. Matching EBV-specific $CD8^+$ T cells were seen in 3 MS patients (4 different TCRs) and matching EBV-specific $CD4^+$ T cells were seen in 1 MS patient. Within the IIH group, 2 matching EBV-specific $CD8^+$ T cells were identified in 1 patient. In the OND group, 1 EBV-specific $CD4^+$ T cell and 1 EBV-specific $CD8^+$ T cell were identified in two separate patients. The example in the figure below demonstrates 2 matching TCRs in the $CD8^+$ T cell population. Table 6.9 summarises the findings across all patient groups.

MW21576 CSF CD8+ T-cell sort

TRBV	CDR3	TRBJ	Freq (%)	Count	
7-3	CASSPGQGQDEQY	2-7	27.06	23	*match 1
27	CASSGLGRREQY	2-7	21.18	18	
6-2/6-3	CASSLGGTGWTEQF	2-1	10.59	9	
20-1	CSAREAGELF	2-2	8.24	7	
24-1	CATSDLPPTGDTGELF	2-2	4.71	4	*match 2
13	CASSRPFGRPYNEQF	2-1	4.71	4	
2	CASRQLAGGDNEQF	2-1	4.71	4	
7-8	CASSLGQAYEQY	2-7	4.71	4	
6-5	CASGSGYYGYT	1-2	4.71	4	
29-1	CSARLAGDSTDTQY	2-3	3.53	3	
3-1	CASSLLAGGLTDTQY	2-3	2.35	2	
11-2	CASSLDPGWSAGGIAKNIQY	2-4	1.18	1	
7-3	CASSPGQGQGEQY	2-7	1.18	1	
14	CASSQAGIHGYT	1-2	1.18	1	
			100	85	

MW21576 PBMC CD8+ TNF+ T-cell sort

TRBV	CDR3	TRBJ	Freq (%)	Count	
7-3	CASSPGQGQDEQY	2-7	33.8	26	*match 1
7-3	CASSLGTGIYNEQF	2-1	29.9	23	
3-1	CASSPSHRDIWDTQY	2-3	26.0	20	
24-1	CATSDLPPTGDTGELF	2-2	7.8	6	*match 2
5-5	CASSHRTSGSTDKQY	2-3	1.3	1	
5-5	CASSQRTSGSTDTQY	2-3	1.3	1	
			100	77	

Figure 6.6 Example of matching EBV-specific TCRs identified in the CD8$^+$ T cell repertoire of the CSF and blood of an MS patient using an EBV-TNF capture assay. Sorted TNF-α positive CD4$^+$ and CD8$^+$ T cells were clonotyped and analysed as described. Matching TCRs were identified between CSF-resident T cells and those clonotyped in the TNF-α capture assay.

Patient ID	No. of sorted $CD4^+$ T cells	Clonotyped	No. of sorted $CD8^+$ T cells	Clonotyped	Matching TCRs
MS					
LS20460	10,000	Yes	1153	Yes	No
EB21510	304	Yes	4802	Yes	**1 x $CD8^+$**
LH18836	4673	Yes	78	No	No ($CD4^+$ only)
MW21576	491	Yes	1159	Yes	**2 x $CD8^+$**
SA23376	609	Yes	6954	Yes	No
RM22664	466	Yes	5000	Yes	**1 x $CD4^+$ & 1 x $CD8^+$**
CT25364	908	Yes	591	No	No ($CD4^+$ only)
HD21265	3395	Yes	83	Yes	No
MH21407	119	Yes	4338	No	No (CD4 only)
IIH					
HS25204	133	Yes	60	No	No ($CD4^+$ only)
LH25311	1157	Yes	1201	Yes	**2 x $CD8^+$**
ML25308	727	Yes	4097	No	No ($CD4^+$ only)
SW25353	620	Yes	1336	Yes	No
RY21758	962	Yes	2696	No	No ($CD4^+$ only)
RC41200	1530	Yes	101	No	No ($CD4^+$ only)
KA38079	509	Yes	2808	Yes	No
OND					
SE29703	120	No	673	Yes	No ($CD8^+$ only)
JG33488	355	Yes	4122	No	No ($CD4^+$ only)
AG20355	2099	No	5000	Yes	N/A
JM25229	380	Yes	782	Yes	**1 x $CD4^+$**
AB25236	188	Yes	2176	Yes	**1 x $CD8^+$**
CJ26014	148	Yes	90	No	No

Table 6.9. Number of cells sorted and clonotyped from donor PBMCs for EBV-TNF capture assay. Number of matching EBV-specific TCRs also shown. N.B. for sample AG20355, comparison between the clonotyped $CD8^+$ samples was not possible because the CSF $CD8^+$ clonotyping results were discarded due to contamination.

6.4 Discussion

Although more effective therapies have been introduced in the last few years for MS they tend to exert their immunomodulatory effects in a rather indiscriminate manner. For example, in the case of alemtuzumab (discussed in Chapter 3) this acts by depleting all B and T cells through anti-CD52 activity.[364] Depletion and repopulation leads to long-term beneficial effects but secondary autoimmunity and risk of infections remain a concern.[364] In addition, natalizumab, which prevents BBB migration through inhibition of α4-integrin[262] is associated with an increased risk of progressive multifocal leucoencephalopathy (PML) due to lack of CNS surveillance against JC virus.[261] Therefore, it is clearly of interest to be able to identify what the pathogenic triggers and antigenic targets of T cells are so that they can be specifically targeted in more individualised therapy. Analysing the TCR repertoire in individual patients and determining which T cell population is pathogenic could lead to treatment that removes or induces tolerance in pathogenic cells only, leaving the remainder of the immune system intact. This would hopefully reduce the incidence of emergent infections and ameliorate the risk of secondary autoimmune disease.

A recent review by Hohlfeld et al[353] has outlined the methods that should be employed to attempt to identify the target antigens of $CD8^+$ T cells in MS. This includes isolating cells from blood, CSF or brain and establishing T cell lines, which can then be used for antigen screening. In this study, we established the dominant TCR β and α chain populations of CSF-resident T cells from a patient with MS and used cloning and lentiviral technology to establish a $CD8^+$ T cell line expressing this TCR. By utilising combinatorial peptide library screening technology we established an unbiased method for identifying target antigens. To our knowledge, this is the first example of this technique being utilised for CSF-resident $CD8^+$ T cells in MS. Interestingly, our results initially suggested some preference for human herpesvirus 6 (HHV6) and other herpesviruses. However, no preference for myelin or oligodendrocyte proteins was seen. In addition to CPL screening, we also performed TNFα capture assay to attempt to identify EBV-reactive CSF-resident TCRs. This approached revealed EBV-specific TCRs in 3 patients with MS, 1 patient with IIH and 2 different OND patients.

Although the results of the peptide activation assay were negative against the HHV6 peptide (and other peptides), it is interesting to review the possible role of HHV6 in the pathogenesis of MS. Early studies demonstrated elevated anti-HHV6 antibodies in MS patient sera[365, 366] and HHV6 viral DNA in MS plaques.[367] HHV6 was also shown to be present in the brains[368] and CSF[369] of MS patients and controls with higher levels of HHV-6 expression being demonstrated in MS brains compared to controls.[370] In addition, the presence of HHV6 DNA is significantly increased in MS plaques compared with NAWM from normal controls.[371] Previous studies therefore intimate that although HHV6 may be a commensal of normal brain, it's replication and activity is enriched in MS.[372] Interestingly, oligoclonal bands (OCBs) demonstrate HHV6 specificity in approximately 20% of patients with MS.[373] In relation to this, herpesvirus-specific CSF OCBs in MS patients have been shown to be inversely correlated to the presence of viral DNA, whose presence in turn correlates with more contrast enhancing lesions on MRI.[374] In addition to OCBs, HHV6 specific $CD4^+$ T cells show strong intrathecal enrichment across MS patients and those with other inflammatory neurological disease with reactivities more pronounced in patients with MS.[375] Clinically, HHV6 IgG titres have been associated with relapses and disease progression.[376, 377] In addition, an inverse correlation has been shown between the presence of HHV6 in serum and responsiveness to IFN-beta treatment.[378] Molecular mimicry is one suggested mechanism for the involvement of viruses in MS pathology and it is interesting to note that there is an identical stretch of amino acids between HHV6 U24 and human MBP.[372] A higher frequency of T cells reactive to both HHV6 U24 and $(MBP)_{93\text{-}105}$ has also been demonstrated in MS patients compared with controls.[379] It should be recognised that in addition to positive associations with MS, some studies have failed to find any significant correlation with diagnosis [380, 381] or relapses.[382]

Specificity to another member of the human herpesvirus family, EBV (human herpesvirus 4) was also observed in the CPL screen data. Similarly, EBV-specific TCRs were observed across all patient groups in the EBV TNFα capture assays. These data suggest that EBV-specific TCRs are perhaps not pathogenic and may just represent ubiquitous cellular immunity against a common human pathogen. It is also noteworthy that human polyomavirus 9 was identified as the highest match in the CPL screens. JC

virus (polyomavirus 2) is causative of PML and demonstrates that $CD8^+$ viral immunity exists against this virus family and hence is lost when treated with natalizumab.

Although this study was novel and demonstrated some interesting results, several limitations exist when interpreting the data. Firstly, CPL screens were only performed for the dominant $CD8^+$ T cell expansion in one patient. This makes extrapolating the results to a wider MS population difficult in particular without a control population for comparison. For example, although herpesviruses were seen as a 'high-scoring' target in the CPL screens, this may be the case across MS patients and controls but it is not possible to know this without further work.

A second limitation of this study was the limited HLA-restriction of the target cell lines used in the MIP-1β assay of the CPL screens. Our patient's HLA type was HLA-A*02:01/A*30:02, HLA-B*07:02/B*51:01, C*07:02/*16:01 but only an HLA-A2 cell line was chosen as targets. It would be more complete if different target cell lines encompassing all the patient's HLA alleles were used but due to time constraints this was not performed in this initial exploratory study. Ideally, future studies would involve examining TCRs from more MS patients and controls and screening all HLA alleles in the target cells. Indeed, with respect to this, a lack of response was seen in MIP-1β assays when individual chosen peptides were used. This may be explained by the limited use of only one HLA type for the target cells. Perhaps alternative HLA target cells would have yielded more promising results. In addition, the EBV TNFα capture assay was only performed on a limited number of patients determined by the successful outcome of CSF-resident T cell clonotyping and the availability of stored PBMCs. This again makes comparison of EBV-reactive TCRs in CSF across patient groups difficult.

Despite these limitations, this initial study has demonstrated that human TCRs from CSF-derived T cells can be cloned into a lentivirus and used in unbiased screens of peptide libraries. Done on a larger scale, this technique may help to yield important information about potential pathogenic targets and antigenic triggers of MS, knowledge of which could lead to more individualised therapy.

Chapter 7

General Discussion

7.1. Multiple sclerosis, the immune system, CNS immune surveillance and the role of $CD8^+$ T cells

Multiple sclerosis (MS) is a common cause of neurological disability, widely accepted to be autoimmune in origin. Early in the disease, CNS-infiltrating immune cells cause demyelination, reflected clinically as subacute episodes of neurological dysfunction (relapses). As the disease progresses, relapses become less common and progressive neurological disability is the prevalent feature secondary to neurodegeneration. In other forms of the disease, disability progression occurs from onset without overt clinical episodes of subacute neurological dysfunction. The findings from genome wide association studies (GWAS)[2, 45], the histopathological features of MS plaques [47]; and, evidence from animal models of MS[44] support MS being an inflammatory autoimmune disease. In particular, the available evidence suggests that T cells play a central role in the pathogenesis of MS. The current available treatments for MS are aimed at either achieving immunosuppression with subsequent remodelling of the immune system,[9] or immunomodulation.[245, 254, 262, 265, 272, 279]. The observed efficacy of these treatments, particularly against clinical relapses, further supports the accepted notion that the adaptive immune system in general and T cells in particular are key players in disease pathogenesis. Despite the evidence of a central role for T cells in MS disease pathogenesis, the initial antigen that triggers autoreactive T cells is unknown. Similarly, the target antigen within the CNS parenchyma is still to be determined. Within the T cell compartment, focus has largely been on the role of $CD4^+$ T cells in MS, largely because disease in the most commonly used animal model for MS (Experimental Autoimmune Encephalitis (EAE)) is initiated by the $CD4^+$ T cell subset. However, in recent years, there has been increasing evidence for the role of $CD8^+$ T cells in MS pathogenesis and the aim of this thesis was to explore this in more detail. In particular, following a clinical study into the effect of a lymphocyte depleting agent (alemtuzumab) on MS clinical disease, I conducted an in-depth analysis of the phenotype and T cell receptor repertoire of CSF-resident T cells. Furthermore, I

attempted to characterise the antigen specificity of CSF-resident $CD8^+$ T cells in MS patients. An increased understanding of the role of $CD8^+$ T cells in MS is important for defining the disease pathogenesis of MS and ultimately in designing novel therapeutics.

The adaptive immune system consists of antibody-secreting B cells, and T cells that recognise their cognate antigen expressed on the surface of antigen presenting cells. $CD8^+$ and $CD4^+$ T cells recognise peptides presented in association with major histocompatibility complexes class I (HLA-A, -B, -C in humans) and II (HLA-DR, -DQ and –DP in humans) respectively.[35] $CD8^+$ T cells are primary effector cells of the adaptive immune system with the ability to destroy cells infected with intracellular pathogens and cancerous cells.[35] MHC class I is expressed by the majority of cells in the human body, enabling $CD8^+$ T cells to mount a rapid and efficient response to intracellular pathogens.[34] Following pMHCI antigen recognition and activation, $CD8^+$ T cells expand and deliver a range of effector functions.[34] After clearance of target cells, a subpopulation of these expanded cells survive and exist as a memory population in order to mount a further response to recurrent infection.[36]

Before considering how MS pathogenesis may occur, it is important to understand the role of the immune system in normal CNS surveillance. The CNS has long been considered to be an immune privileged site for several reasons:[16]: (1) the expression of MHC molecules within the CNS parenchyma is limited;[17] (2) the entry of adaptive immune cells into the CNS via the blood-cerebrospinal (CSF) fluid barrier, the CSF-brain barrier and the blood-brain-barrier is restricted;[18] and, (3) the antigenic representation in peripheral lymph nodes of CNS antigens may not be an accurate representation of the CNS.[19] However, recent discoveries have led to a revisiting of this immune-privileged status, which are important to the understanding of normal CNS immune surveillance and MS disease pathogenesis. The discovery of CNS lymphatics[21] and understanding the transfer of antigens from parenchymal interstitial fluid to CSF[22] has led to a greater understanding of the lymphatic drainage of the CNS and how immune cells react within it. Indeed, as the CNS is a common target for viral infections and autoimmune disease then T cells must be able to access this supposed immune-privileged compartment. This is further supported by the fact that immunosuppressive

disease (e.g. HIV) and drugs preventing lymphocyte egress into the CNS may lead to CNS infectious disease.[383] It is thought that T cells can either be activated in local CNS-draining lymph nodes or in a distant peripheral site before homing back to the CNS to eliminate the pathogens.[16] It is still to be definitively determined whether autoreactive T cells in MS are activated in the periphery or more centrally. If stimulated in the periphery, it is postulated that autoreactive T cells may become activated through molecular mimicry,[31] bystander activation or the co-expression of T cell receptors (TCRs) with different specificities.[32]

The evidence for $CD8^+$ T cells in MS pathogenesis begins with genome wide association studies. Although the strongest HLA association in GWAS are for HLA class II regions,[42] implicating $CD4^+$ T cells, associations have also been observed for HLA class I.[41] In particular, the HLA-A3 risk allele was identified, with further support for this coming from a landmark study using a mouse model transgenic for HLA-A3. which develops an MS-like disease.[44] In addition to genetic studies, $CD8^+$ T cells have been shown to be the most prominent immune cell present within MS plaques[48], and within the MS plaque, $CD8^+$ T cells have also been shown to interact with antigen presenting cells[64] and directly damage CNS target cells.[67-72] In recent years, there has been a particular focus on $CD8^+$ T cells due to the fact that clonal expansions have been observed in the $CD8^+$ T cell repertoire in the blood, CSF and brain tissue of MS patients. Early studies demonstrated $CD8^+$ T cell clonal expansions in MS patients within the blood and CSF[121-123] with subsequent studies confirming this in CSF and brain lesions.[51-54] Given these observed expansions, it has been suggested that these expanded $CD8^+$ T cell clonotypes are antigen-experienced T cells that may represent a pathogenic T cell population in MS and therefore identifying their target antigens is a research priority for the future. The effect of existing MS therapeutics on $CD8^+$ T cell populations and activity also supports the notion of their central role in disease pathogenesis.

There were four main aims of this thesis. The first aim was to investigate the clinical outcomes of MS patients treated with the anti-CD52, lymphocyte depleting monoclonal antibody, alemtuzumab. The second aim was to perform an in-depth phenotypic analysis of CSF-resident T cell populations in patients with MS. Thirdly I aimed to identify dominant TCRs that reside in the CSF of MS patients. Finally, with

knowledge of these dominant TCRs I aimed to define the pathogenic triggers and antigenic targets of CSF-resident T cells.

7.2 Clinical outcomes of MS patients treated with the anti-CD52, lymphocyte depleting monoclonal antibody, alemtuzumab

The first aim of this thesis was to investigate the clinical outcomes of MS patients treated with alemtuzumab. Alemtuzumab is a monoclonal antibody targeted against the CD52 molecule, present on the cell surface of B- and T- cells, therefore causing immunodepletion with subsequent beneficial reconstitution of the immune system. Clinical trials had demonstrated a significant benefit on clinical and MRI outcomes[9, 240, 241] but this observational, long-term follow up study was performed to examine its use in the 'real-world' setting. Knowledge of this would also be informative as to the role of T cells in disease pathogenesis and the effect of immunodepletion and repopulation on potential side effects and long-term outcomes.

This section of the thesis examined the clinical outcomes of 100 patients treated with alemtuzumab across South Wales and Bristol. The effect of alemtuzumab on clinical relapse rates was profound, with a reduction in the annualised relapse rate of 90% after treatment. In addition, over the 6 years of follow-up data, disability outcomes were also encouraging. In a group of patients deemed to have highly aggressive disease, a very modest mean increase of expanded disability status scale (EDSS)[326] of +0.14 was observed. In addition, only 27% met the definition for 6-month sustained accumulation of disability. Overall, the clinical outcomes were found to be similar to those reported in clinical trials as well as the incidence of the most commonly observed side effect of secondary autoimmune disease. Despite lymphodepletion, immune cells that escaped depletion and subsequent reconstitution meant that only minor infections (commonly urinary, respiratory and herpes zoster-related) were observed.

These data suggest that T cells may play a role in disease pathogenesis and alteration of the immune repertoire following treatment with alemtuzumab is beneficial for clinical outcomes. To further understand the pathogenic role of T cells in MS, a

subsequent detailed investigation of the immunophenotype of CSF-resident T cells was performed.

7.3 Phenotypic analysis of CSF-resident T-cell populations in MS

Several studies have attempted to examine the phenotype of CSF-resident T cells in patients with MS. The findings of these studies have been inconsistent and in some cases limited by the number of cell surface markers studied, the number of patients or a lack of control populations. Both effector memory[93] and central memory T cells[98] have been shown to be the dominant CSF population present in MS patients. To attempt to define these CSF-resident T cell populations further we performed an in-depth phenotypic analysis using multicolour, state-of-the-art flow cytometry. Patients with idiopathic intracranial hypertension (IIH) and other neurological diseases (OND) were included as control populations.

The most significant finding in the chapter of the thesis was the increased number of $CD4^+$ and $CD8^+$ T cells present in the CSF of MS patients compared with controls (IIH and OND). Interestingly, the majority of CSF-resident T cells were of an effector memory phenotype across all patient groups with similar expression of individual phenotypic markers ($CCR7^-CD45RA^-CD27^+CD49^+CD57^-CD95^+$) albeit with more variable expression of CD127 in $CD8^+$ T cells. The only differences observed in the $CD4^+$ T cell compartment were in CD27 and CD49d expression, with significantly higher expression in the MS population compared with IIH patients. Within the $CD8^+$ T cell compartment, the expression of CD27, CD49d and CD57 were all significantly higher in the MS group as compared with IIH. T_{EMRA} cells were significantly higher in the $CD8^+$ T cell compartment compared with $CD4^+$ T cell compartment across all patient groups.

Aside from these minor differences, no other significant differences were observed across the patient groups. Therefore, rather than the phenotype of CSF-resident T cells being a determinant of disease, their presence may be indicative of ubiquitous CNS immune surveillance. Of note however, the increased expression of CD27 in the MS group may be associated with a regulatory phenotype.[348] This would be interesting to explore in future work as Regulatory T cells (Tregs) are thought to play a key role in peripheral immune tolerance and as such, in MS disease pathogenesis.[384] In addition,

CD49d (α-4 integrin) is the main adhesion molecule involved in lymphocyte trafficking to the CNS[342] and the significantly higher expression of CD49d suggests greater homing activity of CSF-resident T cells in MS. In order to further investigate the pathological relevance of these CSF-resident T cells I performed TCR repertoire analysis.

7.4 T cell receptor repertoire (TCR) analysis of CSF-resident T cells and identification of dominant TCRs

The current literature suggests that the $CD8^+$ T cell repertoire present in the central nervous system in patients with MS is characterised by clonal expansions suggesting that they are antigen experienced and therefore likely pathogenic.[51-54, 128, 130] However, small numbers of study patients and either limited or no controls hamper interpretation. In addition, when controls have been present, there has been limited comparison between CSF and CNS compartments. Therefore, to attempt to understand the TCR repertoire further and answer whether these observed clonal expansions in the $CD8^+$ T cell repertoire are a pathological finding, I performed in depth clonotyping of CSF-resident T cells from MS patients and controls.

In MS patients, within the $CD4^+$ T cell compartment, the TCR repertoire was significantly more diverse (higher Simpson's diversity index) and had more unique clonotypes than both control groups individually and when combined. In addition, there was a significantly higher number of TCRs representing the top 10%, which contributed significantly less to the overall TCR repertoire. Within the $CD8^+$ compartment, the TCR repertoire was significantly more diverse with more unique clonotypes in the MS group compared to controls, when both the IIH and OND groups were combined. As in the $CD4^+$ T cell compartment, there was a significantly higher number of clonotypes within the top 10% of the repertoire, which contributed less to the overall repertoire compared to both control groups combined. Within the MS group a significantly higher number of unique clonotypes and more diversity was observed in the $CD4^+$ compared to the $CD8^+$ compartment. Conversely, no significant differences were observed in the control groups.

My results demonstrate that clonal expansions can be observed in both the $CD4^+$ and $CD8^+$ T cell repertoires of CSF in MS patients, which is consistent with the current

literature. However, this is not a unique feature of MS and more skewing is actually observed in the $CD4^+$ and $CD8^+$ T cell repertoires of CSF from control patients. Set against the concept that $CD8^+$ T cell clonal expansions in MS are proof of pathogenesis, these results offer a different interpretation. The control groups in this study were largely considered not to be inflammatory in aetiology. As such, the finding that TCR repertoires were more skewed in both the $CD4^+$ and $CD8^+$ T cell repertoires in the control groups suggests activation and expansion of CSF-resident T cells is an important part of CNS immune surveillance. The repertoires may be more 'evened out' to some extent in the MS groups because of non-specific T cell infiltration into the CSF and CNS parenchyma. However, it is more likely that the observed increase in TCR diversity in MS patients is due to the fact that the T cells in MS patients have a lower activation threshold and as a result, a more diverse response to antigenic stimulus is observed. Despite this perhaps somewhat surprising discovery, there is evidence for the role of $CD8^+$ T cells in MS pathogenesis. Therefore although there is an 'evening out' of the TCR repertoire there must still be a population of cells that are pathogenic. It is of obvious importance to be able to recognise this population for the understanding of MS disease pathogenesis and for targeted therapeutics. As such, I aimed to investigate the antigenic specificity of these TCRs using a novel experimental approach utilising lentiviral and combinatorial peptide screening technology in addition to TNFα capture assays.

7.5 Identifying the pathogenic triggers and antigenic targets of CSF-resident $CD8^+$ T cells in Multiple sclerosis

Although a pathogenic role for $CD8^+$ T cells in MS patients has been suggested, the antigen-specificity of these cells remains unknown. Studies to date have tried to define antigen specificity using a candidate antigen approach. These have included studying the reactivity of $CD8^+$ T cells to myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), glial fibrillary acidic protein and transaldolase.[63, 142, 143, 145, 146, 148] The results of these studies have been inconclusive – increased $CD8^+$ T cell responses have been observed in MS patients in some studies [142, 145, 148] but not in others[104, 143, 149, 288]. $CD8^+$ T cells specific to MOG,[179, 180, 357] MBP,[181], GFAP,[144] in addition to haemagglutinin[182] and ovalbumin expressed by oligodendrocytes have been shown to be pathogenic in

animal models.[183] In addition to antigenic targets, the initial triggering antigen in MS is also unknown. Considerable attention has been focused on Epstein-Barr virus (EBV). Based on epidemiological,[153] serological[154, 155], CSF[156-159] and EBV-reactive T cell frequencies[163-167] there is some evidence that this virus may play a role in disease pathogenesis.

Within this section of the thesis, I aimed to identify the pathogenic triggers of dominant CSF-resident TCRs in MS patients. Two strategies were utilised; the first was to perform an EBV-antigen focused approached utilising a TNFα capture assay.[292] The second approach was a novel, unbiased investigation using lentiviral and combinatorial peptide library screening technology. The results of the EBV TNFα capture assay demonstrated CSF-resident EBV-specific T cells across all patient groups. Results were obtained for 9 patients in the MS group with EBV-specific TCRs observed in 3 patients; 1x $CD8^+$ TCR in 1 patient; 2x $CD8^+$ T CRS in 1 patient; 1x $CD4^+$ and 1x $CD8^+$ TCR in a final patient sample. In the IIH group, results were obtained for 7 patients. Two EBV-specific $CD8^+$ TCRs were observed in 1 patient. In the OND group, results were obtained for 6 patients with 1 $CD4^+$ EBV-specific TCR observed in 1 patient and 1 $CD8^+$ EBV-specific TCR observed in another. Utilising a novel, unbiased combinatorial peptide screening approach for 1 patient I identified the antigen specificities for the most dominant CSF-resident MHCI restricted TCR. These results demonstrate a strong affiliation with several herpes viruses although not including EBV (human herpesvirus 4). Unfortunately, the patient's TCR did not show a response against the selected peptides so more work is required.

7.6 Overall results and limitations

The clinical chapter of this thesis helped to confirm the impressive outcomes of patients treated with the lymphocyte-depleting agent alemtuzumab. This confirmed the findings of previous studies and adds to the weight of evidence implicating T cells in MS disease pathogenesis. Although there is evidence for both $CD4^+$ and $CD8^+$ T cells in MS aetiology, this thesis focused on the potential pathogenic role of $CD8^+$ T cells. With recent evidence suggesting a clonal expansion of $CD8^+$ T cells indicative of antigen-stimulation I focused on this population with the aim to ultimately identify the

pathogenic triggers and antigenic targets of these cells. However, although $CD4^+$ T cells were not the main focus of this thesis, data for this population was also collected.

The detailed immunophenotyping of CSF-resident T cells did not establish many differences between MS and control patients. In addition, the clonotyping analysis of these T cell populations demonstrated more unique clonotypes, less diversity and more contribution to the overall TCR frequencies of the top TCRs in the control patients compared to MS. This finding was present for both $CD4^+$ and $CD8^+$ T cell compartments. These results in combination offer an intriguing insight into normal CNS immune surveillance. It is thought that T cells must regularly travel around the CNS through blood, CSF and newly discovered lymphatic channels.[16, 21] This is essential in removing pathogenic antigens including infectious organisms. From these results it would seem reasonable to postulate that effector memory T cells are a common finding in CSF along with clonal expansions in the $CD8^+$ T cell repertoire, which is 'evened out' in MS. In further support of this, TNFα capture assay identified EBV-specific TCRs across all patient groups. This suggests that T cells are constantly surveying the CNS and are activated against common antigens such as EBV. However, these results do not exclude a role for $CD8^+$ T cells in the pathogenesis of MS. There is clearly enough evidence to support their role and although an 'evening out' of the repertoire is observed, the dominant populations present may still be pathogenic. Therefore it is still of importance to try and identify the pathogenic triggers and antigenic targets of these cells. As such, we performed a novel, unbiased approach to try and answer this question.

There were several aspects of this thesis that contributes to the quality of the results but also several limitations, which should be considered when reflecting on the overall results obtained. In the alemtuzumab clinical chapter, the relatively large number of patients and detailed long-term follow-up data aided the results obtained. However, although the results suggest a central role for T cells in general, they are not specific to $CD8^+$ T cells and therefore the results should not be over interpreted with respect to these cells. However, clinical trials of $CD4^+$ T cell targeted monoclonal antibodies alone have not proven to be successful.[236-238] With respect to the immunophenotyping chapter, the number of cell surface markers used was extensive and it was important

to be able to compare with control data. However, although we had control data and they were as close to normal as our ethics would allow, they were still not entirely representative of a 'normal' population. This may explain differences between the control immunophenotype in our study and those in others.[50, 103] Of note, these earlier studies demonstrating central memory T cells as the most dominant population used different cell markers to determine differentiation status (CD45RA, CD27) rather than the more accepted CCR7 and CD45RA markers that are used now.[96] Furthermore, it should be noted that I did not attempt to identify regulatory T cells in this study, which would be interesting to examine in future studies especially due to the significant differences observed with the expression of CD27.

Similar positive attributes and limitations can be noted with regards to the TCR clonotyping studies performed. The presence of control populations was important for comparisons but again these may not entirely reflect the 'normal' population. In addition, the relatively low cell numbers in these groups made clonotyping technically difficult with samples of less than 100 cells not being clonotyped. These factors resulted in a lower success rate of clonotyping and hence less sequencing results for the samples. However, the difference in sorted cell numbers was investigated by Dr Venturi who concluded that the results of the MS patient samples were likely an underestimate i.e. they were likely to be more diverse than that observed. Currently we are aiming to perform further control experiments on different numbers of sorted cells in order to understand the effect of the number of sorted cells on TCR diversity analysis in order to confirm this finding.

With respect to antigen specificity, the approach used to examine this was novel and to our knowledge has not been attempted in CSF-resident T cells from MS patients before. The lentiviral construction and peptide library screening utilised was time-consuming and therefore this approach was only used in one MS patient. Because of this it is difficult to draw definitive conclusions from the results obtained. It was disappointing that the transduced patient TCR did not demonstrate a response to the selected peptides but this may have been because only one of the patient's HLA types was expressed by the target cell line used. In the future, it would be interesting to perform combinatorial peptide library screens for more patients and more TCRs across

a wider range of HLA types. This would be an unbiased way of examining T-cell antigen specificity across a wide range of patients and controls.

Aside from this, it is extremely encouraging that this approach was ultimately successful and could be harnessed again in future studies. Other overall limitations in this thesis included the lack of comparison with peripheral blood and the lack of access to CNS tissue. Obviously, comparison with brain or spinal cord tissue would be extremely intuitive but this is difficult to obtain from living patients. In addition, post-mortem tissue has the obvious limitation that it may not be reflective of the situation in life. Access to CSF from normal people would also allow a greater understanding of normal immune surveillance and MS pathogenesis but our existing ethics did not allow this. This could be a consideration for future ethics applications but may be difficult to justify an invasive procedure with potential complications in normal individuals.

7.7 Concluding remarks and future directions

This thesis set out to better characterize the role of $CD8^+$ T cells in MS disease pathogenesis but the results perhaps led in a slightly different direction. They have offered an insight into normal CNS immune surveillance and led to further questions about how $CD8^+$ T cells contribute to the aetiology of MS and how best to identify pathogenic subsets. This will have particular importance for designing future therapeutics. With the existing evidence for the role of $CD8^+$ T cells, it is clear that they are contributing to MS disease pathogenesis, but there must be additional mechanisms rather than just having a clonally expanded population. Our data demonstrate that there is an increased number of $CD4^+$ and $CD8^+$ T cells in the CSF of MS patients as may be expected and perhaps these T cells have other attributes that influences their pathogenicity. For example, they may be more cross-reactive, have different activation thresholds[25] or may have different CNS migration markers.[350] In addition, they may have more reactivity against multiple CNS epitopes. Recent approaches may also offer a novel approach to identifying antigen specificity.[385, 386] Understanding this and identifying pathogenic subsets will be important for future therapeutics. Current therapies are aimed at modulating the immune system in the periphery. Although they are effective at preventing relapses, they are often associated with other features of immunosuppression and immune reconstitution such

as secondary autoimmune disease, opportunistic infections and malignancies. Understanding pathogenesis so that more individualised, targeted therapeutics can be developed will therefore be an important area of future enquiry.

Appendix

8.1 Immunophenotyping antibodies used per patient group

8.1.1 Multiple sclerosis

Patient ID	CD45RA	CCR7	CD27	CD49d	CD57	CD95	CD127	Pan γδ	PD-1
LC20552	✓	✓	✓	✗	✓	✓	✓	✗	✗
LJ20639	✓	✓	✓	✗	✓	✓	✓	✗	✗
LS20460*									
MJ19588	✓	✓	✓	✓	✓	✓	✓	✗	✓
EB21510	✓	✓	✓	✓	✓	✓	✓	✗	✓
KG19967	✓	✓	✓	✓	✓	✓	✓	✗	✓
LH18836	✓	✓	✓	✓	✓	✓	✓	✗	✓
NW21326	✓	✓	✓	✓	✓	✓	✓	✗	✓
MW21576	✓	✓	✓	✓	✓	✓	✓	✗	✓
CS21983	✓	✓	✓	✓	✓	✓	✓	✗	✓
AL28847	✓	✓	✓	✓	✓	✓	✓	✓	✗
MK21405	✓	✓	✓	✓	✓	✓	✓	✗	✓
SA23376	✓	✓	✓	✓	✓	✓	✓	✓	✗
RM22664	✓	✓	✓	✓	✓	✓	✓	✗	✓
TL22789	✓	✓	✓	✓	✓	✓	✓	✓	✗
CT25364	✓	✓	✓	✓	✓	✓	✓	✓	✗
HD21265	✓	✓	✓	✓	✓	✓	✓	✗	✓
MH21407	✓	✓	✓	✓	✓	✓	✓	✗	✓
RW21309	✓	✓	✓	✓	✓	✓	✓	✗	✓
DD22299*									
CG41964	✓	✓	✓	✓	✓	✓	✓	✓	✗

8.1.2 Idiopathic intracranial hypertension

Patient ID	CD45RA	CCR7	CD27	CD49d	CD57	CD95	CD127	Pan γδ	PD-1
EC21870	✓	✓	✓	✓	✓	✓	✓	✓	✗
HS25204	✓	✓	✓	✓	✓	✓	✓	✓	✗
LH25311	✓	✓	✓	✓	✓	✓	✓	✓	✗
ML25308	✓	✓	✓	✓	✓	✓	✓	✓	✗
SW25353	✓	✓	✓	✓	✓	✓	✓	✓	✗
RY21758	✓	✓	✓	✓	✓	✓	✓	✓	✗
DC37877	✓	✓	✓	✓	✓	✓	✓	✓	✗
ES37889	✓	✓	✓	✓	✓	✓	✓	✓	✗
FC24414	✓	✓	✓	✓	✓	✓	✓	✓	✗
VE25562*									
CC40712	✓	✓	✓	✓	✓	✓	✓	✓	✗
RC41200	✓	✓	✓	✓	✓	✓	✓	✓	✗
KA38079	✓	✓	✓	✓	✓	✓	✓	✓	✗
CC41471	✓	✓	✓	✓	✓	✓	✓	✓	✗

8.1.3 Other neurological diseases

Patient ID	CD45RA	CCR7	CD27	CD49d	CD57	CD95	CD127	Pan γδ	PD-1
SE29703*									
JG33488	✓	✓	✓	✓	✓	✓	✓	✓	✗
ND37140	✓	✓	✓	✓	✓	✓	✓	✓	✗
DL37517*									
CS19395	✓	✓	✓	✓	✓	✓	✓	✗	✓
AG20355	✓	✓	✓	✓	✓	✓	✓	✗	✓
JM25229	✓	✓	✓	✓	✓	✓	✓	✓	✗
AB25236	✓	✓	✓	✓	✓	✓	✓	✓	✗
CJ26014	✓	✓	✓	✓	✓	✓	✓	✓	✗
JJ37566*									
JP24822*									

8.2 T cell receptor clonotyping of CSF-resident T Cells – Raw data

8.2.1 Multiple sclerosis

1. LC20552

LC20552 CSF CD4⁺ T cell sort (5591 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSAGLAGHNEQF	2-1	11.27	8
29-1	CSVGRLAGGSYNEQF	2-1	8.45	6
4-1	CASSQDLGPYNEQF	2-1	8.45	6
12-3/12-4	CASSLTGGYQPQH	1-5	8.45	6
6-5	CASKKDRADTEAF	1-1	8.45	6
28	CASSGRGAPSTDTQY	2-3	4.23	3
25-1	CASSERTTGGAKLF	1-4	2.82	2
6-2/6-3	CASSYGQENYGYT	1-2	2.82	2
20-1	CSAPHPGANVLT	2-6	2.82	2
18	CASSPRVLRTESPLH	1-6	1.41	1
15	CATSKASGSWADTQY	2-3	1.41	1
7-2	CASSKPGLAEQETQY	2-5	1.41	1
5-6	CASSLNTGRISYEQY	2-7	1.41	1
11-1	CASSQKPGQVGEQY	2-7	1.41	1
7-9	CASSLRGLGGNEQF	2-1	1.41	1
7-2	CASSSHWGGRNEQY	2-7	1.41	1
7-2	CASSPSGGANYGYT	1-2	1.41	1
6-5	CASSYNGRSQETQY	2-5	1.41	1
6-5	CASSSRQGDTDTQY	2-3	1.41	1
5-1	CASSENSGANEKLF	2-2	1.41	1
5-1	CASSLADGGNSPLH	1-6	1.41	1
5-1	CASSLPDSGNSPLH	1-6	1.41	1
2	CASSELGQINYGYT	1-2	1.41	1
29-1	CSVGRGGSYNEQF	2-1	1.41	1
20-1	CSARSGSVTGEQY	2-7	1.41	1
20-1	CSARDSQRTYEQY	2-7	1.41	1
9	CASSPWTGPYEQY	2-7	1.41	1
7-2	CASSQRGPYNEQF	2-1	1.41	1
28	CASTGTLGNEQF	2-1	1.41	1
27	CASSRKPDRPQH	1-5	1.41	1
19	CASKDRVFTEAF	1-1	1.41	1
18	CASSPDWNYEQY	2-7	1.41	1
11-3	CASSFEENNEQF	2-1	1.41	1
10-3	CAIRREGGTEAF	1-1	1.41	1
10-1	CASSMGRTYEQY	2-7	1.41	1
4-2	CASSQGPFDEQY	2-7	1.41	1
20-1	CSAGVVGTSAF	1-1	1.41	1
13	CPSSHEQF	2-1	1.41	1
13	CASSHEQF	2-1	1.41	1
			100	71

LC20552 CSF CD8⁺ T cell sort (798 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSLGWGDTEAF	1-1	20.31	13
5-1	CASSFGAPAYNSPLH	1-6	15.63	10
13	CASSTGTSGSNEQF	2-1	9.38	6
27	CASRTSGVKNIQY	2-4	7.81	5
2	CASSDAGGHTEAF	1-1	7.81	5
4-1	CASSQETGLWDEQF	2-1	6.25	4
28	CASSLWAPSASSYEQY	2-7	4.69	3
7-9	CASSSHQGALNTEAF	1-1	4.69	3
2	CASRDSPGLTNTEAF	1-1	4.69	3
27	CASSNDRANQPQH	1-5	4.69	3
4-1	CASSPWTMDTQY	2-3	3.13	2
9	CASSPDRGVSGANVLT	2-6	1.56	1
5-1	CVSSFGAPAYNSPLH	1-6	1.56	1
13	CASSTGTSGGNEQF	1-1	1.56	1
10-3	CVISESGRGLAEAF	1-1	1.56	1
6-6	CASSEEDIRYTEAF	1-1	1.56	1
6-6	CATGTSGDSYEQY	2-7	1.56	1
5-4	CASSPLGGDEQF	2-1	1.56	1
			100	64

2. LJ20639

LJ20639 CSF CD4⁺ T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASRNSGSRDEQF	2-1	12.50	5
5-1	CASSLFQGPDTEAF	1-1	10.00	4
5-1	CASSVGDTDTQY	2-3	7.50	3
15	CATSSPFAGGQWEQF	2-1	5.00	2
5-1	CASSFPWTGGDTEAF	1-1	5.00	2
7-8	CASSLAGPMNTEAF	1-1	5.00	2
6-5	CASSYSIRQNQPQH	1-5	5.00	2
7-9	CASSLRSGGSPLH	1-6	5.00	2
5-1	CASSRQNEQF	2-1	5.00	2
3-1	CASSQRLAGDGTDTQY	2-3	2.50	1
6-1	CASSEPRTGKNTEAF	1-1	2.50	1
5-5	CASSLAWSSYNSPLH	1-6	2.50	1
5-1	CASSLARHPQDTEAF	1-1	2.50	1
20-1	CSARDMGKGNEKLF	2-2	2.50	1
4-1	CASSQAELRSYEQY	2-7	2.50	1
29-1	CSVGDLGLRGELF	2-2	2.50	1
6-6	CASSYSLFNEKLF	2-2	2.50	1
4-2	CASSQDGTGGEQY	2-7	2.50	1
29-1	CSVTGATTDTQY	2-3	2.50	1
29-1	CSVGTAFDNEQF	2-1	2.50	1
4-2	CASSQTRTDTQY	2-3	2.50	1
6-2/6-3	CASSYDRGEQY	2-7	2.50	1
6-1	CASIYRATEAF	1-1	2.50	1
7-8	CASSGGGEQY	2-7	2.50	1
3-1	CASSRDYEQY	2-7	2.50	1
			100	40

LJ20639 CSF CD4+ T cell sort (repeat)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSVGSVGTEAF	1-1	6.8	5
18	CASSRAGQAAGELF	2-2	5.5	4
6-5	CASSYSTPRGVNTEAF	1-1	4.1	3
6-1	CASSEPRTGKNTEAF	1-1	4.1	3
27	CASSLLGLAADTQY	2-3	4.1	3
6-5	CASSYGTSGSYEQY	2-7	4.1	3
5-1	CASSVGDTDTQY	2-3	4.1	3
12-4	CASSLGQRAAF	1-1	4.1	3
28	CASSPSRGLSGANVLT	2-6	2.7	2
5-1	CASSFPWTGGDTEAF	1-1	2.7	2
20-1	CSARDMGKGNEKLF	1-4	2.7	2
12-3	CASGRDNQGTGELF	2-2	2.7	2
9	CASSPRSDSGNTIY	1-3	2.7	2
11-2	CASSLYGGTNEKLF	1-4	2.7	2
29-1	CSVVGTGYNEQF	2-1	2.7	2
20-1	CSETSGSGNEQY	2-7	2.7	2
24-1	CASRGADTEAF	1-1	2.7	2
28	CASSYGNEQF	2-1	2.7	2
5-1	CASSRQNEQF	2-1	2.7	2
3-1	CASSHTSEAF	1-1	2.7	2
15	CATSSPFAGGQWEQF	2-1	1.4	1
5-1	CASSLARHPQDTEAF	1-1	1.4	1
2	CASRWSRGSYNEQF	2-1	1.4	1
6-5	CASSYSIRQNQPQH	1-5	1.4	1
2	CASSEGSHGANVLT	2-6	1.4	1
29-1	CSASRGQNTGELF	2-2	1.4	1
28	CASSFRRTGNTEAF	1-1	1.4	1
20-1	CSAVLAGGRNEQF	2-1	1.4	1
12-3	CASRNSGSRDEQF	2-1	1.4	1
7-6	CASSLEVRKDSPLH	1-6	1.4	1
5-1	CASSLFQGPDTEAF	1-1	1.4	1
3-1	CASSQVSARETQY	2-5	1.4	1
29-1	CSVGDLGLRGELF	2-2	1.4	1
7-9	CASSLRSGGSPLH	1-6	1.4	1
20-1	CSAVSANTGELF	2-2	1.4	1
6-6	CASSYSLFNEKLF	1-4	1.4	1
5-1	CASSSGQYYGYT	1-2	1.4	1
4-2	CASSQTRTDTQY	2-3	1.4	1
10-3	CAISPRGLDEQY	2-7	1.4	1
29-1	CSVDPGTIVQF	2-1	1.4	1
12-3	CASSFAGETQY	2-5	1.4	1
7-8	CASSGGGEQY	2-7	1.4	1
			100	73

LJ20639 CSF CD8+ T cell sort (673 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
6-6	CASSFGGNEQF	2-1	17.65	12
6-1	CASSELFSKGNQPQH	1-5	13.24	9
4-2	CASSLDGLARSPQY	2-5	8.82	6
4-1	CASSQDRLAGVEQF	2-1	7.35	5
27	CASSFGSGANVLT	2-6	7.35	5
27	CASSSPEWGYGYT	1-2	7.35	5
28	CASSIDRVETQY	2-5	7.35	5
9	CASSVAQGGEQY	2-7	7.35	5
5-1	CASSFGSYTGELF	2-2	5.88	4
28	CASSLASGNYNEQF	2-1	4.41	3
7-2	CASSLGSVYTEAF	1-1	4.41	3
5-1	CASSYALSYEQY	2-7	2.94	2
7-9	CASSRVSYEQY	2-7	2.94	2
5-4	CASSPGTSGNWNEQF	2-1	1.47	1
12-3/12-4	CASSLAFVGYEQY	2-7	1.47	1
			100	68

3. LS20460

LS20460 CSF CD4+ T cell sort (1176 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASIPLPAVGRDYGYT	1-2	21.43	15
20-1	CSAPEGARLNTEAF	1-1	12.86	9
7-2	CASSLGLADNEQF	2-1	11.43	8
28	CASTRPGGRSNQPQH	1-5	10.00	7
11-1	CASSNHMGQGRGYT	1-2	5.71	4
5-1	CASRTGPISGNTIY	1-3	5.71	4
15	CATSPTSGSKGNTGELF	2-2	4.29	3
7-8	CASRYRGENSPLH	1-6	2.86	2
7-2	CASSVRDSSYEQY	2-7	2.86	2
28	CASSRDRGETQY	2-5	2.86	2
5-1	CASSLGSNQPQH	1-6	2.86	2
3-1	CASRGLGNSPLH	1-6	2.86	2
12-3/12-4	CASPTTEPSSGANVLT	2-6	1.43	1
30	CAWGAGQGFYNEQF	2-1	1.43	1
6-2/6-3	CASWETYSSGNTIY	1-3	1.43	1
5-1	CASSFGSGGGETQY	2-5	1.43	1
5-1	CASSLRQGDTGELF	2-2	1.43	1
7-2	CASNLGLADNEQF	2-1	1.43	1
5-1	CASSLGGSRDEQY	2-7	1.43	1
5-1	CASSLAAANSPPH	1-6	1.43	1
20-1	CSAPGSNRNEQF	2-1	1.43	1
5-1	CASRMGSNQPQH	1-5	1.43	1
			100	70

LS20460 CSF CD8^{+} T cell sort (393 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CATGKARSGTGELF	2-2	48.86	43
5-5	CASSPWTGLDNEQF	2-1	35.23	31
20-1	CSARDRQGLIGYNEQF	2-1	13.64	12
7-9	CATGKVRSGTGELF	2-2	1.14	1
5-5	CASSPGTGLDNEQF	2-1	1.14	1
			100	88

4. MJ19588

MJ19588 CSF CD4^{+} T cell sort (5984 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSPQGLAGDQETQY	2-5	5.41	4
5-1	CASSSSGSSGTGELF	2-2	5.41	4
27	CASSINGVQETQY	2-5	5.41	4
20-1	CSARSLLSYEQY	2-7	5.41	4
28	CASSGHRRTGFSYEQY	2-7	4.05	3
7-8	CASSLGAGAGPIGELF	2-2	2.70	2
5-1	CASSLETGKGETQY	2-5	2.70	2
5-4	CASSFISGGEETQY	2-5	2.70	2
29-1	CSARGTSGSINEQF	2-1	2.70	2
20-1	CSARDAGGPNEQF	2-1	2.70	2
5-1	CASSPGNGPYEQY	2-7	2.70	2
12-3/12-4	CASTRQMNTEAF	1-1	2.70	2
7-9	CASILGGNTEAF	1-1	2.70	2
5-1	CASRSDTGNEQF	2-1	2.70	2
12-3/12-4	CASSAGDTEAF	1-1	2.70	2
28	CAIKGNTEAF	1-1	2.70	2
7-8	CASSPPGELF	2-2	2.70	2
15	CATSTQPQH	1-5	2.70	2
28	CASRPTRGAGYTGELF	2-2	1.35	1
20-1	CSASGTGGGSTDTQY	2-3	1.35	1
20-1	CSARGQRDRPAGELF	2-2	1.35	1
6-5	CASRDLAGDSYNEQF	2-1	1.35	1
6-5	CASKGQGFEDNSPLH	1-6	1.35	1
6-5	CASSSLTGAGSTEAF	1-1	1.35	1
4-3	CASSQGTSGDTGELF	2-2	1.35	1
29-1	CSVEGTSGATDTQY	2-3	1.35	1
29-1	CSVPLANRVGTEAF	1-1	1.35	1
27	CASSPDRANSYEQY	2-7	1.35	1
12-3/12-4	CASSLVRSAYNEQF	2-1	1.35	1
11-3	CASSLNTGPYNEQF	2-1	1.35	1
7-2	CASSSLASGSSEQY	2-7	1.35	1
7-2	CASSLIIGLNTEAF	1-1	1.35	1
5-6	CASSSPGTGISGYT	1-2	1.35	1
5-1	CASSIITGETETQY	2-5	1.35	1
5-1	CASSIETGGSTEAF	1-1	1.35	1
5-1	CASSDERTANTEAF	1-1	1.35	1
2	CASSEALGGDQPQH	1-5	1.35	1
27	CASSLWGQAYGYT	1-2	1.35	1
20-1	CSAPSGSINNEQF	2-1	1.35	1
20-1	CSARDPTGPDGYT	1-2	1.35	1
12-3/12-4	CASSLGPGKETQY	2-5	1.35	1
6-5	CASSRPSGTDTQY	2-3	1.35	1
5-1	CASSLGRGVNEQY	2-7	1.35	1
5-1	CASSLGLAGIEQF	2-1	1.35	1
30	CAWRTGFNTEAF	1-1	1.35	1
7-8	CASSPWANTEAF	1-1	1.35	1
6-2/6-3	CATSQGNNQPQH	1-5	1.35	1
5-1	CASRSDTGKEQF	2-1	1.35	1
7-8	CASSEQTGELF	2-2	1.35	1
5-1	CASSLRTNEQF	2-1	1.35	1
12-3/12-4	CASSSQYEQY	2-7	1.35	1
			100	78

MJ19588 CSF CD8+ T cell sort (887 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSVDLYGDDYGYT	1-2	23.86	21
20-1	CSAKGLAGRNEQF	2-1	23.86	21
7-9	CASSLGGTTAHTNTGELF	2-2	17.05	15
28	CASTPWANQETQY	2-5	12.50	11
12-5	CASGLLPRDRGDYGYT	1-2	4.55	4
7-3	RASSLRREGNQPQH	1-5	4.55	4
27	CASSLRTERAGELF	2-2	3.41	3
12-3/12-4	CASSRGQNIDEQF	2-1	3.41	3
5-1	CASSLGVANQPQH	1-5	2.27	2
7-9	CASSLGGTTAHRNTGELF	2-2	1.14	1
29-1	CSVAGLAGKTAETQY	2-5	1.14	1
7-3	RASSLRGEGNQPQH	1-5	1.14	1
29-1	CSAYDSSYEQY	2-1	1.14	1
			100	88

5. EB21510

EB21510 CSF CD4^{+} T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSARHSTGKIYYEQY	2-7	5.41	4
20-1	CSVPRGVPYNEQF	2-1	5.41	4
5-6	CASSQNWNEAF	1-1	5.41	4
20-1	CSASLGLAVMTSTDTQY	2-3	4.05	3
12-3/12-4	CASSFLPGRGLDGYT	1-2	4.05	3
29-1	CSVEWGRGDGYT	1-2	4.05	3
28	CASSWMTGLSSGNTIY	1-3	2.70	2
7-2	CASSLMPQGASYGYT	1-2	2.70	2
12-3/12-4	CASTLGLRGYGYT	1-2	2.70	2
28	CASSLKAGGTEQY	2-7	2.70	2
28	CAGTSGRGETQY	2-5	2.70	2
6-2/6-3	CASRRGGNTEAF	1-1	2.70	2
7-9	CASSFPGNTIY	1-3	2.70	2
5-1	CASSQNWNEAF	1-1	2.70	2
9	CASSPSSSSLSYNEQF	2-1	1.35	1
9	CASLRVGKGLSGNTIY	1-3	1.35	1
28	CASSLRAGRGTYEQY	2-7	1.35	1
28	CASRETDRAGANVLT	2-6	1.35	1
6-6	CASSYGDFSYNSPLH	1-6	1.35	1
5-1	CASSLGARSSYNEQF	2-1	1.35	1
3-1	CASSPTQLGAKNIQY	2-4	1.35	1
2	CASSKSRDFSYNEQF	2-1	1.35	1
29-1	CSVEDLGRGDTEAF	1-1	1.35	1
28	CASSTTGGLSYEQY	2-7	1.35	1
28	CASSLFPGMGYEQY	2-7	1.35	1
28	CASSSGGADSNEQF	2-1	1.35	1
27	CASSLRRLPGETQY	2-5	1.35	1
20-1	CSATEPKRGHEKLF	1-4	1.35	1
12-3/12-4	CASSSPAGSTDTQY	2-3	1.35	1
11-3	CASSLGTSGYNEQF	2-1	1.35	1
10-3	CAISGQLSGANVLT	2-6	1.35	1
9	CASSVASGTYNEQF	2-1	1.35	1
9	CASSGGQTVNSPLH	1-6	1.35	1
7-2	CASRTGTGLTGELF	2-2	1.35	1
7-2	CASSLGSSYNSPLH	1-6	1.35	1
5-1	CASSLNPSGRDEQY	2-7	1.35	1
29-1	CSVEEQGRGSPLH	1-6	1.35	1
28	CASSFYRGGYEQY	2-7	1.35	1
11-3	CASSLHGRPDTQY	2-3	1.35	1
7-2	CASSVGGVNYEQY	2-7	1.35	1
6-2/6-3	CASWGQTFTGELF	2-2	1.35	1
5-1	CASSFVTSTDTQY	2-3	1.35	1
5-1	CASSLFGQDTEAF	1-1	1.35	1
12-3/12-4	CASSLGRLGEQY	2-7	1.35	1
7-2	CASSLDYRGGYT	1-2	1.35	1
5-1	CASSLGYQETQY	2-5	1.35	1
2	CASRGQGGTEAF	1-1	1.35	1
25-1	CASAAPGTEAF	1-1	1.35	1
10-3	CAISEEVYEQY	2-7	1.35	1
10-3	CAISEAVYGYT	1-2	1.35	1
10-3	CAISEPPEAF	1-1	1.35	1
			100	74

EB21510 CSF CD8+ T cell sort (911 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASTPSGANVLT	2-6	36.99	27
27	CASSFGGLEKLF	1-4	8.22	6
7-2	CASSLGGGQGLDWTEAF	1-1	6.85	5
11-2	CASSPYPSGRDVEQF	2-1	6.85	5
28	CASSLRLYEQY	2-7	6.85	5
5-5	CASSVVGALNQY	2-4	5.48	4
7-9	CASSLVERAEAF	1-1	4.11	3
7-3	CASSLTTNTEAF	1-1	4.11	3
5-5	CASSLTETGFNQPQH	1-5	2.74	2
28	CASTPRGGGYQPQH	1-5	2.74	2
6-2/6-3	CASSYVGLAEETQY	2-5	2.74	2
7-9	CASRGGRDAEKLF	1-4	2.74	2
6-6	CASLDGSTNEKLF	1-4	2.74	2
20-1	CSATDLASHQETQY	2-5	1.37	1
7-9	CASSDQDKGTDTQY	2-3	1.37	1
9	CASSFGTGNTEAF	1-1	1.37	1
20-1	CSARGRGVQPQH	1-5	1.37	1
29-1	CSVRGLAGVQY	2-7	1.37	1
			100	73

6. KG19967

KG19967 CSF CD4+ T cell sort (1582 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASSLAGTDTQY	2-3	18.29	15
27	CASSFYKTSYEQY	2-7	15.85	13
20-1	CSAPPRVPNTEAF	1-1	13.41	11
3-1	CASSRDLAGGQETQY	2-5	12.20	10
12-3/12-4	CASSRQGDSSPLH	1-6	9.76	8
27	CASSRTKQGNTEAF	1-1	6.10	5
7-2	CASSSGAAWGGEQF	2-1	4.88	4
29-1	CSVAEGNEQY	2-7	4.88	4
5-1	CASSLEFGETQY	2-5	3.66	3
29-1	CSVEDPGTVYT	1-2	3.66	3
11-3	CASSPVGRADNEQF	2-1	2.44	2
20-1	CSARDFGNTEAF	1-1	2.44	2
18	CASSPSGTGGTNEKLF	1-4	1.22	1
6-6	CASTRLGAGNTIY	1-3	1.22	1
			100	82

KG19967 CSF CD8^{+} T cell sort (256 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASSYGAYNEQF	2-1	59.30	51
29-1	CSVTGQGTTEQY	2-7	17.44	15
11-2	CASSLTAGGYEQY	2-7	12.79	11
29-1	CSVSMVGTSGRYEQF	2-1	6.98	6
20-1	CSAPQGVNTGELF	2-2	2.33	2
3-1	CASSPATGNTEAF	1-1	1.16	1
			100	86

7. LH18836

LH18836 CSF CD4^{+} T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSLLSGSTDTQY	2-3	7.58	5
6-6	CASSYSDRLSGRYEQY	2-7	4.55	3
28	CASSPPDSSNQPQH	1-5	4.55	3
20-1	CSARTPRRADSEKLF	1-4	3.03	2
29-1	CSAETRADSGNTIY	1-3	3.03	2
20-1	CSARDPARSNEKLF	1-4	3.03	2
11-2	CASSPRLAGGYEQY	2-7	3.03	2
30	CAWSPGYKGQPQH	1-5	3.03	2
12-3/12-4	CASSPSGYTYEQY	2-7	3.03	2
5-1	CASSLLRTEEGYT	1-2	3.03	2
29-1	CSVTTGNTEAF	1-1	3.03	2
29-1	CSVEDKARGF	2-1	3.03	2
7-3	CANSPQPPGLAGSDTDTQY	2-3	1.52	1
5-1	CASSLPLAGEGPTDTQY	2-3	1.52	1
28	CASSLVRTGGLPYEQY	2-7	1.52	1
7-8	CASSLGVLAGVTGELF	2-2	1.52	1
5-6	CASSLGFMGQGQDTQY	2-3	1.52	1
5-1	CASSSGTSGHTRNTQY	2-3	1.52	1
4-1	CASSQATAGTSAGELF	2-2	1.52	1
25-1	CASSAALAGLTDTQY	2-3	1.52	1
6-5	CASSYQSPGVGTEAF	1-1	1.52	1
5-1	CASSPAASGNTGELF	2-2	1.52	1
5-1	CASSSSFGGRSGEQF	2-1	1.52	1
18	CASSRRQGNDSPLH	1-6	1.52	1
11-3	CASSLVQAGPDTQY	2-3	1.52	1
11-2	CASSPPGRNLETQY	2-5	1.52	1
9	CASSVDGRVLGGYT	1-2	1.52	1
7-8	CASSLRQGGPYEQY	2-7	1.52	1
29-1	CSVGTWSGTDTQY	2-3	1.52	1
19	CASSSDRWNYGYT	1-2	1.52	1
12-3/12-4	CASTRASGTYEQY	2-7	1.52	1
12-3/12-4	CASSRTGTGNTIY	1-3	1.52	1
7-9	CASSLTPVTDTQY	2-3	1.52	1
7-2	CASSLVSFTDTQY	2-3	1.52	1
6-5	CASSYRGQTYGYT	1-2	1.52	1
6-1	CASSPLGGRAEQF	2-1	1.52	1
5-1	CASSLLQTEEGYT	1-2	1.52	1
2	CASRKSGSLDEQY	2-7	1.52	1
20-1	CSARGPRLNEQF	2-1	1.52	1
12-3/12-4	CASSTRTLYEQY	2-7	1.52	1
9	CASSVSPRNEQF	2-1	1.52	1
5-1	CASSAEGTQPQH	1-5	1.52	1
5-1	CASSFGTDATAF	1-1	1.52	1
2	CASGRDSPYEQY	2-7	1.52	1
28	CASLGQNYGYT	1-2	1.52	1
12-3/12-4	CASMLSYNEQF	2-1	1.52	1
9	CASSVGFGELF	2-2	1.52	1
29-1	CSVDGAHGYT	1-2	1.52	1
29-1	CSGIGRNEAF	1-1	1.52	1
			100	66

LH18836 CSF CD8+ T cell sort (1795 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLVKAGGNEEQY	2-7	8.57	6
29-1	CSVEATGLAGAQEQF	2-1	7.14	5
12-3/12-4	CASSFPTGYYNEQF	2-1	7.14	5
7-8	CASSFGGRASNEQF	2-1	7.14	5
4-1	CASSGRTGTNYGYT	1-2	5.71	4
27	CASSLQGANYGYT	1-2	5.71	4
13	CASSRQGIPEAF	1-1	5.71	4
9	CASSEKGLAGADEQY	2-7	4.29	3
5-1	CASSLSNTGELF	2-2	4.29	3
20-1	CSAIIMLAGGPWHTDTQY	2-3	2.86	2
6-6	CASSPEKTGLNYGYT	1-2	2.86	2
20-1	CSAVSQQGGSYEQY	2-7	2.86	2
7-2	CASSLTSGPYNEQF	2-1	2.86	2
27	CASSLAAGGETQY	2-5	2.86	2
7-2	CASSSTLDNQPQH	1-5	2.86	2
29-1	CSVPGTVNNEQF	2-1	2.86	2
29-1	CSVPGTVNTEAF	1-1	2.86	2
7-9	CASSLDPAPEAF	1-1	2.86	2
27	CASHRKWLAGITNTGELF	2-2	1.43	1
20-1	CSAIIILAGGPWHTDTQY	2-3	1.43	1
11-3	CASTRGGHLKYSNQPQH	1-5	1.43	1
7-9	CASGGTLAGDYNEQF	2-1	1.43	1
7-2	CASSESQGPNTGELF	2-2	1.43	1
5-1	CASSLVKAGGSEEQY	2-7	1.43	1
27	CASSLRRQGEETQY	2-5	1.43	1
7-2	CASSLRGTTLDEQF	2-1	1.43	1
4-1	CASSEGLAGAYEQY	2-7	1.43	1
3-1	CASSHSVGARHEQF	2-1	1.43	1
7-3	CASSSTLDNQPQH	1-5	1.43	1
27	CASSSPTWDTQY	2-3	1.43	1
28	CASSLGYEQY	2-7	1.43	1
			100	70

8. NW21326

NW21326 CSF CD4+ T cell sort (534 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLGLGRNTEAF	1-1	44.44	40
5-1	CASSLGGQETQY	2-5	27.78	25
28	CASRASGRGPGELF	2-2	10.00	9
7-2	CASSLSFSSSGHEQY	2-7	7.78	7
28	CASSRQETQY	2-5	5.56	5
28	CASSLPGQGFPGELF	2-2	2.22	2
5-1	CASSLGLGRSTEAF	1-1	1.11	1
29-1	CSVEESTYTEAF	1-1	1.11	1
			100	90

NW21326 CSF CD8+ T cell sort (116 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CASSLGGTEAF	1-1	69.12	47
19	CASSYGRAVGELF	2-2	7.35	5
5-5	CASSFWEANEQF	2-1	7.35	5
4-1	CASSQDSTPTHSNQPQH	1-5	5.88	4
7-9	CASSLPGSSYEQY	2-7	5.88	4
7-9	CAGSLGGTEAF	1-1	2.94	2
20-1	CSVQDRTYEQY	2-7	1.47	1
			100	68

9. MW21576

MW21576 CSF CD4+ T cell sort (1956 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLIPGDGYT	1-2	10.26	8
9	CASSVDISGNTIY	1-3	6.41	5
5-1	CASSLGPDLNTEAF	1-1	6.41	5
5-1	CASSLGQGLF	2-1	6.41	5
7-8	CASSLGSRGQSTDTQY	2-3	5.13	4
6-2/6-3	CASSQGVLLSGYT	1-2	5.13	4
11-2	CASSLALLTSGKGQF	2-1	3.85	3
7-9	CASSQEYGGNTDTQY	2-3	3.85	3
7-6	CASSVQVGHSYEQY	2-7	3.85	3
18	CASSTGTDNTEAF	1-1	3.85	3
11-2	CASSFKQGDHSGNTIY	1-3	2.56	2
7-2	CASSFFTSGDRTDTQY	2-3	2.56	2
3-1	CASSQGAVAGFAETQY	2-5	2.56	2
27	CASSLLGSGGQPQH	1-5	2.56	2
3-1	CASSSGTGVGNEQF	2-1	2.56	2
30	CAWSPGTPLGYT	1-2	2.56	2
6-5	CASRSPRYNEQF	2-1	2.56	2
24-1	CATSVGIQPQH	1-5	2.56	2
28	CATSLSSGGRPDTQY	2-3	1.28	1
5-5	CASSLRTGRVNTEAF	1-1	1.28	1
29-1	CSVEYSGGGTGELF	2-2	1.28	1
24-1	CATSVLDTAGNTQY	2-3	1.28	1
12-3/12-4	CASSAAYRGGETQY	2-5	1.28	1
6-5	CASSYSAVFTDTQY	2-3	1.28	1
2	CASSQDGGSNQPQH	1-5	1.28	1
29-1	CSVEVMTSTDTQY	2-3	1.28	1
7-2	CASSLAGGLLEQY	2-7	1.28	1
6-6	CASYGQGRNSPLH	1-6	1.28	1
6-5	CASSLRQGNEKLF	1-4	1.28	1
6-5	CASSYGTGSLRAF	1-1	1.28	1
6-2/6-3	CASRRGGAGNEQF	2-1	1.28	1
6-1	CASSPGTGGQPQH	1-5	1.28	1
5-1	CASSLDKNNSPLH	1-6	1.28	1
20-1	CSANRQGGTEAF	1-1	1.28	1
2	CASKRQLNTEAF	1-1	1.28	1
29-1	CSVGQGHGELF	2-2	1.28	1
11-1	CASSSRQGEAF	1-1	1.28	1
			100	78

MW21576 CSF CD8^{+} T cell sort (313 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-3	CASSPGQGQDEQY	2-7	27.06	23
27	CASSGLGRREQY	2-7	21.18	18
6-2/6-3	CASSLGGTGWTEQF	2-1	10.59	9
20-1	CSAREAGELF	2-2	8.24	7
24-1	CATSDLPPTGDTGELF	2-2	4.71	4
13	CASSRPFGRPYNEQF	2-1	4.71	4
2	CASRQLAGGDNEQF	2-1	4.71	4
7-8	CASSLGQAYEQY	2-7	4.71	4
6-5	CASGSGYYGYT	1-2	4.71	4
29-1	CSARLAGDSTDTQY	2-3	3.53	3
3-1	CASSLLAGGLTDTQY	2-3	2.35	2
11-2	CASSLDPGWSAGGIAKNIQY	2-4	1.18	1
7-3	CASSPGQGQGEQY	2-7	1.18	1
14	CASSQAGIHGYT	1-2	1.18	1
			100	85

10. CS21983

CS21983 CSF CD4^{+} T cell sort (2125 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSTGLAGETQY	2-5	10.26	8
5-1	CASSLTSGSLSSYEQY	2-7	6.41	5
12-3/12-4	CASSLTNNQPQH	1-5	6.41	5
6-2/6-3	CASSRIGQEQF	2-1	6.41	5
7-8	CASSTVGGGTNTEAF	1-1	5.13	4
4-1	CASSQEFGGRNQPQH	1-5	5.13	4
29-1	CSVRTGGDGGYT	1-2	5.13	4
12-3/12-4	CASSPRRGQGPYGYT	1-2	3.85	3
11-2	CASSLVGTGSYNEQF	2-1	3.85	3
24-1	CATSPGGNMNTEAF	1-1	3.85	3
5-1	CASSTSRDRGYQETQY	2-5	2.56	2
12-3/12-4	CASSLITGWFQPQH	1-5	2.56	2
5-1	CASSFASGRTDTQY	2-3	2.56	2
2	CARRGARGNTGELF	2-2	2.56	2
5-1	CASSLRGDSYEQY	2-7	2.56	2
5-5	CASSKPETVWGT	1-1	2.56	2
5-1	CASRRTGSNEQF	2-1	2.56	2
5-1	CASRDLRGNEQF	2-1	2.56	2
7-8	CASSLQGEQY	2-7	2.56	2
6-1	CASRASGTSGRESTDTQY	2-3	1.28	1
5-1	CASSLTSGSLGSYEQY	2-7	1.28	1
28	CASSSPGTGLLYEQY	2-7	1.28	1
11-2	RASSLVGTGSYNEQF	2-1	1.28	1
7-8	CASSPSRLTASYEQY	2-7	1.28	1
5-1	CASSFGGVRTNEKLF	1-4	1.28	1
24-1	CAASPGGNMNTEAF	1-1	1.28	1
6-2/6-3	CASSYSDASLYEQY	2-7	1.28	1
29-1	CSVSSAYGANVLT	2-6	1.28	1
7-9	CASSDPGYSYEQY	2-7	1.28	1
7-3	CASSRTSGQETQY	2-5	1.28	1
7-2	CASSRALSGNTIY	1-3	1.28	1
7-2	CASSRAFSGNTIY	1-3	1.28	1
4-1	CASRPLAGGNEQF	2-1	1.28	1
12-3/12-4	CASSMSLSSPLH	1-6	1.28	1
29-1	CSGGQGVHEQF	2-1	1.28	1
			100	78

CS21983 CSF CD8^{+} T cell sort (590 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
9	CASSKGEGSMNTEAF	1-1	12.99	10
24-1	CATSEDTGFGSYNEQF	2-1	11.69	9
7-6	CASSLSGYSSYEQY	2-7	10.39	8
7-8	CASSWNTGGWEQY	2-7	10.39	8
10-3	CAIGDSPENTIY	1-3	10.39	8
20-1	CSAREGDRVNYGYT	1-2	7.79	6
12-3/12-4	CASRPENTGELF	2-2	7.79	6
6-5	CASGGTGPYNEQF	2-1	5.19	4
7-9	CASSRGLREQF	2-1	5.19	4
6-6	CASGHGDEQY	2-7	5.19	4
28	CASSFRQGYQETQY	2-5	2.60	2
6-2/6-3	CASSPPTGGNQPQH	1-5	2.60	2
9	CASSELTGTGRETQY	2-5	1.30	1
28	CVSSFRQGYQETQY	2-5	1.30	1
19	CASSIQETTNEKLF	2-2	1.30	1
6-2/6-3	CASRPPGRTYEQY	2-7	1.30	1
7-9	CASSLVRVNTEAF	1-1	1.30	1
6-6	CASSPRENIQY	2-4	1.30	1
			100	77

11. AL28847

AL28847 CSF CD4^{+} T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
6-5	CASSYVRLSERGYEQY	2-7	6.58	5
28	CASSLIHTGELF	2-2	6.58	5
5-1	CASSEDRAQF	2-1	6.58	5
20-1	CSAGNTGTGVNEQF	2-1	5.26	4
5-1	CASSLDSVQETQY	2-5	5.26	4
5-1	CASSLGSAFSYEQY	2-7	3.95	3
12-3/12-4	CASTRDRVEETQY	2-5	3.95	3
6-5	CASSPQGRDSRQY	2-7	3.95	3
5-1	CASSLDTGNTIY	1-3	3.95	3
2	CASSSSRNYEQY	2-7	2.63	2
5-1	CASSLWDEQF	2-1	2.63	2
18	CASSPLPGLAGPKNTGELF	2-2	1.32	1
25-1	CASSESPGQGADGDIQY	2-4	1.32	1
5-1	CASSSRTSGRAHGGEQF	2-1	1.32	1
28	CASSIGQGFSGANVLT	2-6	1.32	1
6-5	CAGSYVRLSERGYEQY	2-7	1.32	1
5-1	CASSLGPLRDIQETQY	2-5	1.32	1
30	CAWSVGGGAGANVLT	2-6	1.32	1
20-1	CSASRVQGAENYGYT	1-2	1.32	1
6-2/6-3	CASTWRQGARNTEAF	1-1	1.32	1
28	CASSPQGLAGGEQY	2-7	1.32	1
28	CASRLGQGGNQPQH	1-5	1.32	1
9	CASSVGAGAGNEQF	2-1	1.32	1
7-8	CASSLRTSRANEQY	2-7	1.32	1
7-8	CASNRGGVGGTEAF	1-1	1.32	1
6-2/6-3	CASSTGWTQTYEQY	2-7	1.32	1
5-1	CASRPRTGGLGEQY	2-7	1.32	1
5-1	CASSLGPAFSYEQY	2-7	1.32	1
5-1	CASSLVMGKNTEAF	1-1	1.32	1
12-3/12-4	CASSSTGTLNEQF	2-1	1.32	1
12-3/12-4	CASRQGMSNQPQH	1-5	1.32	1
7-6	CASSQSGSTDTQY	2-3	1.32	1
7-3	CASSLSGKSYEQY	2-7	1.32	1
7-3	CASRLGGRTGELF	2-2	1.32	1
6-2/6-3	CASSHVQGVETQY	2-5	1.32	1
5-1	CASSPRTKGNEQY	2-7	1.32	1
5-1	CASSPIAGVDTQY	2-3	1.32	1
5-1	CASSPNTIANEQF	2-1	1.32	1
30	CAWDRQGGTEAF	1-1	1.32	1
29-1	CSVSLGGSETQY	2-5	1.32	1
12-3/12-4	CASSSGIGTPKH	1-5	1.32	1
10-3	CAISEGVTYEQY	2-7	1.32	1
5-1	CASSFDRTYEQY	2-7	1.32	1
28	CASSLLYEKLF	1-4	1.32	1
20-1	CSARQGAYTLH	1-6	1.32	1
7-9	CASSLTEGRN	1-2	1.32	1
7-3	CASSSDTGELF	2-2	1.32	1
5-1	CASSLRYNEQF	2-1	1.32	1
			100	76

AL28847 CSF CD8+ T cell sort (2537 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
2	CASSEGGAYEQY	2-7	16.67	13
7-2	CASSPPGRAGYEQY	2-7	8.97	7
6-2/6-3	CASSYWGSYDEQY	2-7	8.97	7
28	CASSTQGAVLGYT	1-2	7.69	6
20-1	CSAGGTGGSYEQY	2-7	6.41	5
12-3/12-4	CASSLYRGEKLF	1-4	6.41	5
4-1	CASSQAGQETQY	2-5	5.13	4
7-9	CASSPGGISNTEAF	1-1	3.85	3
7-9	CASSLGQGFNEQY	2-7	3.85	3
7-2	CASSAAGGAHEQY	2-7	3.85	3
5-5	CASSLGSDQPQH	1-5	3.85	3
3-1	CASSQDFSGSAKNIQY	2-4	2.56	2
20-1	CSARDTGGYSGNTIY	1-3	2.56	2
7-9	CASSPEVRGAYEQY	2-7	2.56	2
15	CATSTRDGEETQY	2-5	2.56	2
20-1	CSARVRGLPSSGANVLT	2-6	1.28	1
10-1	CASSESRAAGPTGELF	2-2	1.28	1
6-2/6-3	CASSYKMTRGFRNEQF	2-1	1.28	1
28	CASSLPGQGVTGELF	2-2	1.28	1
9	CASRETSGSLGEQF	2-1	1.28	1
7-9	CASSPEARGAYEQY	2-7	1.28	1
6-2/6-3	CASSYSGARLDTQY	2-3	1.28	1
5-6	CASSRGFSSYNEQF	2-1	1.28	1
7-9	CASSLALGRDEQY	2-7	1.28	1
7-8	CASSSGTGNTEAF	1-1	1.28	1
6-1	CASVAGDYQETQY	2-5	1.28	1
			100	78

12. MK21405

MK21405 CSF CD4^{+} T cell sort (3887 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSGPLSNEQF	2-2	8.97	7
5-5	CASSFNQGETEAF	1-1	7.69	6
7-3	CASSFSGGAPPDTQY	2-3	5.13	4
6-1	CASSEAPGLGRFHEQY	2-7	3.85	3
5-6	CASSSTSGGDYNEQF	2-1	3.85	3
7-2	CASSLATGVGEEQF	2-1	3.85	3
5-1	CASSLGLAKNNEQF	2-1	3.85	3
28	CASSITGSQETQY	2-5	3.85	3
5-1	CASSLAPGAGTQY	2-3	3.85	3
7-2	CASSSSPVPEQF	2-1	3.85	3
6-2/6-3	CASSYGEGYT	1-2	3.85	3
20-1	CSAETGTSGGTEQY	2-7	2.56	2
6-1	CASTLFPGMSYGYT	1-2	2.56	2
5-1	CASSSGLAEGNEQF	2-1	2.56	2
28	CASSSGPMGQRAF	1-1	2.56	2
5-1	CASSRAEGQETQY	2-5	2.56	2
5-1	CASRVYAQGTEAF	1-1	2.56	2
10-3	CAISVGHEQY	2-7	2.56	2
27	CASSLQAGAYHLRLAGAYEQY	2-7	1.28	1
6-1	CANSEAPGLGRFHEQY	2-7	1.28	1
25-1	CASSVTSGGTLGEQF	2-1	1.28	1
7-2	CASSLVATQSSYEQY	2-7	1.28	1
28	CASSSDRGLDNEQF	2-1	1.28	1
20-1	CSARNPTSGEGEQY	2-7	1.28	1
7-2	CASSLFGSSNQPQH	1-5	1.28	1
7-2	CASSLGQGVNYGYT	1-2	1.28	1
6-1	CASSFTDSTNYGYT	1-2	1.28	1
6-1	CASTFSPGMSYGYT	1-2	1.28	1
5-1	CVSSLDTGSLETQY	2-5	1.28	1
4-3	CASSQRDRDSGGYT	1-2	1.28	1
30	CAWSRPAGGNEQF	2-1	1.28	1
12-3/12-4	CASSPGQGLYGYT	1-2	1.28	1
7-3	CASSPPGGLIEQF	2-1	1.28	1
6-1	CASSNSGSYNEQF	2-1	1.28	1
28	CASSLAGANVLT	2-6	1.28	1
27	CASSMTGSDEQF	2-1	1.28	1
9	CASSVASNTEAF	1-1	1.28	1
29-1	CSYRRDSDTQY	2-3	1.28	1
28	CATRAADDGYT	1-2	1.28	1
19	CASSRGQYGYT	1-2	1.28	1
29-1	CSVRQQETQY	2-5	1.28	1
			100	78

MK21405 CSF CD8+ T cell sort (717 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSVEGDGRSYEQY	2-7	22.58	14
12-5	CASGSTGPGDSPLH	1-6	19.35	12
27	CASSLLQGDTGELF	2-2	17.74	11
19	CASSITGTLGQPQH	1-5	16.13	10
28	CASSPTTATNEKLF	1-4	6.45	4
2	CASSGREVSPGELF	2-2	6.45	4
20-1	CSARVPGGVNNEQF	2-1	3.23	2
9	CASSVALGDYGYT	1-2	3.23	2
7-8	CASSESAGILAGGRDEQF	2-1	1.61	1
27	CASSLLQGVSGELF	2-2	1.61	1
15	CATSRDSAGAEPQH	1-5	1.61	1
			100	62

13. SA23376

SA23376 CSF CD4^{+} T cell sort (4463 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLAGPNSPLH	1-6	10.13	8
12-3/12-4	CASSFGGQGLSQFNQPQH	1-5	7.59	6
29-1	CSVGQTNTGELF	2-2	6.33	5
5-1	CASSAGRSGNSDTQY	2-3	3.80	3
5-1	CASSSGGQASGGYT	1-2	3.80	3
19	CASSSPTSYQPQH	1-5	3.80	3
5-5	CASSGGFSDNEQF	2-1	3.80	3
12-3/12-4	CASSLNSGTEQF	2-1	3.80	3
7-2	CASRPGLAGTDTQY	2-3	2.53	2
6-1	CASRRGTTSTDTQY	2-3	2.53	2
5-1	CASSSNRGTNEKLF	1-4	2.53	2
2	CASRWLAGVTDTQY	2-3	2.53	2
10-2	CASLGGRSTDTQY	2-3	2.53	2
6-5	CASNRGGRNYGYT	1-2	2.53	2
2	CASSGTVANYGYT	1-2	2.53	2
18	CASSPTDGQPQH	1-5	2.53	2
12-3/12-4	CASSPQATGELF	2-2	2.53	2
7-9	CASSLGDSNEQY	2-7	2.53	2
5-1	CASSRSGAELF	2-2	2.53	2
24-1	CATSDLSQGSRENNQPQH	1-5	1.27	1
7-8	CASRLLASGAFYNEQF	2-1	1.27	1
12-3/12-4	CASSYGGSAGANVLT	2-6	1.27	1
5-1	CASSIRTGALTDTQY	2-3	1.27	1
20-1	CSAREPRGDTGELF	2-2	1.27	1
6-1	CASNPLQGGSYGYT	1-2	1.27	1
4-1	CASSQEVGFSYEQY	2-7	1.27	1
4-1	CASSRNRDYNQPQH	1-5	1.27	1
3-1	CASSQWTTNTGELF	2-2	1.27	1
28	CASTPGGVTDTQY	2-3	1.27	1
25-1	CASSRGGVHSPLH	2-6	1.27	1
11-2	CASGRLAGGNEQF	2-1	1.27	1
7-2	CASSSPGGFWGYT	1-2	1.27	1
5-1	CASGLAGRNSPLH	1-6	1.27	1
4-1	CASSYMVLGNTIY	1-3	1.27	1
29-1	CSVGQTSTGELF	2-2	1.27	1
28	CASSSSTSNEQF	2-1	1.27	1
7-9	CASTRGDTGELF	2-2	1.27	1
7-9	CASSRDSNQPQH	1-5	1.27	1
5-6	CASSLGLADEQF	2-1	1.27	1
5-1	CASSFIASETQY	2-5	1.27	1
5-1	CASSSIASETQY	2-5	1.27	1
20-1	CSASSPGTQY	2-3	1.27	1
			100	79

SA23376 CSF CD8^{+} T cell sort (961 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
11-2	CASSLDRDSPSSYNEQF	2-1	28.99	20
10-3	CAISSGTVSPYGYT	1-2	28.99	20
25-1	CASRKGTAYEQY	2-7	7.25	5
7-6	CASSLGQGTNLMNTEAF	1-1	5.80	4
27	CASSPSAGRREKLF	1-4	5.80	4
3-1	CASSQDRQGGQPQH	1-5	5.80	4
10-3	CAISESSRGQGGRTGELF	2-2	4.35	3
9	CASSWTSSYNEQF	2-1	4.35	3
5-1	CASSLRDSLSGNTIY	1-3	1.45	1
9	CASSVMTGDNYGYT	1-2	1.45	1
27	CASSSGGPYEQF	2-1	1.45	1
6-1	CASSEAASYEQY	2-7	1.45	1
24-1	CATSEGNSYT	1-2	1.45	1
6-4	CASSDGYGYT	1-2	1.45	1
			100	69

14. RM22664

RM22664 CSF CD4+ T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSPLAASYNEQF	2-1	5.80	4
20-1	CSATGQGGGYGYT	1-2	4.35	3
7-7	CASSWGLAEETQY	2-5	4.35	3
7-2	CASSWGQGANGYT	1-2	4.35	3
24-1	CAAPGTGWYEQY	2-7	4.35	3
6-1	CASGKAPGEQY	2-7	4.35	3
16	CASSQQAGPSSGTQY	2-3	2.90	2
27	CASSLSISGRAEQY	2-7	2.90	2
7-2	CASSLTVLSTDTQY	2-3	2.90	2
6-2/6-3	CASSSARGNNSPLH	1-6	2.90	2
27	CASSKLAGRDTQY	2-3	2.90	2
12-3/12-4	CASSFSSSGNTIY	1-3	2.90	2
10-3	CAITRQGARNEQF	2-1	2.90	2
12-3/12-4	CASSLGSVYEQY	2-7	2.90	2
28	CASSFTNTIY	1-3	2.90	2
27	CASSSGIGQLPANYGYT	1-2	1.45	1
4-3	CASSQAPIGGAGQETQY	2-5	1.45	1
7-6	CASSQGGLAGATDTQY	2-3	1.45	1
29-1	CSVPGTGEKFNYGYT	1-2	1.45	1
28	CASSPPGSPYQETQY	2-5	1.45	1
15	CATSRNPHRGQETQY	2-5	1.45	1
11-2	CASSSRAATGVYEQF	2-1	1.45	1
9	CASSLTSGGVQETQY	2-5	1.45	1
27	CASSLRGVVQDTQY	2-3	1.45	1
7-6	CASSPGAGSADTQY	2-3	1.45	1
7-2	CASNPLAASYNEQF	2-1	1.45	1
6-2/6-3	CASRLGTGRGNEQF	2-1	1.45	1
6-1	CASKPGASYFEKLF	1-4	1.45	1
5-1	CASSSSTGRQETQY	2-5	1.45	1
29-1	CSVVQRGIGTEAF	1-1	1.45	1
28	CASSFWAAQETQY	2-5	1.45	1
20-1	CSAREPGRSTEAF	1-1	1.45	1
12-3/12-4	CASSLSGTGNTIY	1-3	1.45	1
12-3/12-4	CASRKGRRNTEAF	1-1	1.45	1
10-3	CAVTRQGARNEQF	2-1	1.45	1
29-1	CSVDGTGGVEAF	1-1	1.45	1
20-1	CSVSGTNTDTQY	2-3	1.45	1
20-1	CSSPGDTAYGYT	1-2	1.45	1
12-3/12-4	CASSWDRTYEQY	2-7	1.45	1
12-3/12-4	CASGGNQVNTQY	2-3	1.45	1
5-6	CASSLAGRYEQY	2-7	1.45	1
5-1	CASSDRGAHEQY	2-7	1.45	1
12-3/12-4	CASSPDRYEQY	2-7	1.45	1
12-3/12-4	CASSFRNQPQH	1-5	1.45	1
2	CASRWNQGMQY	2-5	1.45	1
29-1	CSARGNTEAF	1-1	1.45	1
18	CASQTNTEAF	1-1	1.45	1
			100	69

RM22664_CSF CD8+ T cell sort (4881 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSPGQAYEQY	2-7	8.20	5
9	CASSAMTSGGADTQY	2-3	4.92	3
7-7	CASSNEQGLSTDTQY	2-3	4.92	3
4-1	CASTRGTSSYNSPLH	1-6	4.92	3
27	CASSSRDSSGNTIY	1-3	4.92	3
7-9	CASSLSALGNEQF	2-1	4.92	3
27	CASTPGQGYEQY	2-7	4.92	3
28	CASSFEGTSGGTDTQY	2-3	3.28	2
7-2	CASSLGGTGPFNSPLH	1-6	3.28	2
29-1	CSVGTGGTNEKLF	1-4	3.28	2
28	CASSFSTDVGGYT	1-2	3.28	2
7-2	CASSVGTEYNEQF	2-1	3.28	2
6-5	CASRASGSSYEQY	2-7	3.28	2
6-2/6-3	CASSPGIYTYEQY	2-7	3.28	2
4-1	CASSLPGDPYEQY	2-7	3.28	2
29-1	CSVETGVVEAF	1-1	3.28	2
6-2/6-3	CASSSILQGLDTGELF	2-2	1.64	1
4-3	CASSHPTPAGSTDTQY	2-3	1.64	1
4-3	CASSHDTPGGRTDTQY	2-3	1.64	1
28	CASSLTDGRLNQPQH	1-5	1.64	1
27	CASSLDGRALHQPQH	1-5	1.64	1
5-1	CASSLGQGRFTDTQY	2-3	1.64	1
20-1	CSARGLSVRNTEAF	1-1	1.64	1
2	CASSEALRTPYGHT	1-2	1.64	1
30	CAWSLGQPTGELF	2-2	1.64	1
27	CASRTHRASDEQY	2-7	1.64	1
12-3/12-4	CASSPGTGGHEQF	2-1	1.64	1
9	CASSPSGVQETQY	2-5	1.64	1
4-1	CASSQGSEGFEQY	2-7	1.64	1
11-2	CASTWGAHNEQF	2-1	1.64	1
11-2	CASTLGAHNEQF	2-1	1.64	1
7-2	CASSAGRGTTF	1-1	1.64	1
6-6	CASSYRRAEAF	1-1	1.64	1
5-6	CASSLRGNEQF	2-1	1.64	1
6-2/6-3	CASSLSYEQY	2-7	1.64	1
6-5	CASTADTQY	2-3	1.64	1
			100	61

15. TL22789

TL22789 CSF CD4+ T cell sort (4994 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSSPSGGNEKLF	1-4	24.4	19
6-1	CASRTGTSGNEQF	2-1	7.7	6
7-2*02 or 7-2*03	CASSLQVLDTQY	2-3	7.7	6
29-1	CSAAGASNQPQH	1-5	6.4	5
12-3	CASTPPPTGNAEAF	1-1	5.1	4
29-1	CSVETGGGNTEAF	1-1	5.1	4
7-9	CASSLEVTQY	2-5	6.4	5
25-1	CASSPFGAGGSDEQY	2-7	3.8	3
9	CASRASGSSSYNEQF	2-1	3.8	3
9	CASSGGSLGNTEAF	1-1	3.8	3
5-1	CASSLARLAGAGNIQY	2-4	2.6	2
7-7	CASSPLVGRPDTQY	2-3	2.6	2
5-6	CASSLGTGVGGTEAF	1-1	2.6	2
20-1	CSARGLAGEVTQY	2-3	2.6	2
11-2	CASSAGK	1-2	2.6	2
9	CASSVEFGTGTDTQY	2-3	1.3	1
5-1	CASSLAWDTSYNEQF	2-1	1.3	1
20-1	CSASMGGMGANVLT	2-6	1.3	1
5-1	CASSLAWGTSYNEQF	2-1	1.3	1
20-1	CSARGLAGEVAQY	2-3	1.3	1
5-1	CASSLAISNSYEQY	2-7	1.3	1
27	CASRPITGIASPLH	1-6	1.3	1
10-3	CAISGGTDNSPLH	1-6	1.3	1
20-1	CSAQPDSAYNEQF	2-1	1.3	1
29-1	CSVIRAAETQY	2-5	1.3	1
			100	78

TL22789 CSF CD8+ T cell sort (837 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
4-1	CASSQDGGVSMNTEAF	1-1	98.7	77
5-1	CASSNRGQGLNTEAF	1-1	1.3	1
			100	78

16. CT25364 (CD4+ only)

CT25364 CSF CD4+ T cell sort (1649 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
18	CASSPGGDTQY	2-3	92.9	65
5-4	CASSSGSFQETQY	2-5	7.1	5
			100	70

17. HD21265 (CD4+ only)

HD21265 CSF CD4+ T cell sort (1485 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
24-1	CATSDGNLQETQY	2-5	19.5	16
7-6	CASSSYSGGHNEQF	2-1	14.6	12
7-2	CASSFTGGSGNTIY	1-3	11.0	9
29-1	CSVPDTGRLGNTIY	1-3	8.5	7
7-3	CASSSGRHNEQF	2-1	7.3	6
12-3	CASSFSPAGQETQY	2-5	4.9	4
7-2	CASSLGAGGETQY	2-5	4.9	4
18	CASSTVQETQY	2-5	4.9	4
27	CASSLRGAGDNSPLH	1-6	3.7	3
7-7	CASSGPGGSQETQY	2-5	2.4	2
12-4	CASSGNRGMNTEAF	1-1	2.4	2
24-1	CATTSGLSTDTQY	2-3	2.4	2
20-1	CSGGQGDYNEQF	2-1	2.4	2
12-4	CASTLTGGYGYT	1-2	2.4	2
19	CASTNRGRVLDTEAF	1-1	1.2	1
18	CASSPPETGGRGYT	1-2	1.2	1
12-5	CASEPRTGTYNEQF	2-1	1.2	1
20-1	CSARGPLAAGELF	2-2	1.2	1
7-3	CASSLGGLNTEAF	1-1	1.2	1
20-1	CSATGQFYEQY	2-7	1.2	1
7-9	CASSSSYEQYF	2-7	1.2	1
			100	82

18. MH21470

MH21470 CSF CD4+ T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSFLGQGASETQY	2-5	19.4	12
19	CASSIKGSGNTIY	1-3	9.7	6
12-4	CAIRGDTQY	2-3	9.7	6
7-2	CASSQISGTGSSYEQY	2-7	8.1	5
28	CASSSPAAGGGTDTQY	2-3	6.5	4
9	CASSVAPAGANTGELF	2-2	6.5	4
6-1	CASRNTGTGRTDTQY	2-3	4.8	3
27	CASRRTTGGIGEKLF	1-4	4.8	3
6-5	CASSYGGNYGYT	1-2	4.8	3
7-3	CASSPLAGGPASYNEQF	2-1	3.2	2
27	CASRLTGTVSHYGYT	1-2	3.2	2
7-2	CASTSRSGSSGELF	2-2	3.2	2
7-2	CASSLRPYEQY	2-7	3.2	2
2	CASRSTDYGYT	1-2	3.2	2
19	CASSIGTGWYPDTQY	2-3	1.6	1
7-3	CASSLMEGTENEQF	2-1	1.6	1
7-2	CASSFDDRLNEQF	2-1	1.6	1
6-5	CASSCGGNYGYT	1-2	1.6	1
12-3	CASNIRGGGGYT	1-2	1.6	1
4-3	CASSLTVYNEQF	2-1	1.6	1
			100	62

MH21470 CSF CD8+ T cell sort (836 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSVDLGDPEQF	2-1	56.1	46
2	CASSEASGGYYNEQF	2-1	26.8	22
13	CASSLEGKGGPQETQY	2-5	11.0	9
7-6	CASSLVLAGTSYNEQF	2-1	4.9	4
12-3	CASSLGVEKLFF	1-4	1.2	1
			100	82

19. RW21309

RW21309 CSF CD4^{+} T cell sort (548 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
30	CAWQLQGSHQPQH	1-5	28.6	24
5-1	CASSQGGNNQPQH	1-5	25.0	21
13	CASSARAHNEQF	2-1	14.3	12
3-1	CASSLAGSSYNEQF	2-1	9.5	8
6-5	CASRLGQGGGYT	1-2	6.0	5
7-2	CASSPFTGELF	2-2	6.0	5
9	CASSLNPRPGNTIY	1-3	2.4	2
11-1	CASSQDRYGYT	1-2	2.4	2
24-1	CATSDTDRGYGFVQETQY	2-5	1.2	1
19	CASLRESNRGNGYT	1-2	1.2	1
30	CAWQLPGSHQPQH	1-5	1.2	1
28	CASGLGGGIYGYT	1-2	1.2	1
6-1	CASGTGGWTDTQY	2-3	1.2	1
			100	84

RW21309 CSF CD8^{+} T cell sort (234 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CASTQTGDSYGYT	1-2	61.8	47
7-9	CASRGFTQY	2-3	28.9	22
27	CASSPKGPRWQPQH	1-5	7.9	6
19	CASSTLDYNEQF	2-1	1.3	1
			100	76

20. CG41964

CG41964 CSF CD4+ T cell sort (5000 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSAKGALAGTISYNEQF	2-1	27.6	8
7-9	CASSYRGADTGELF	2-2	20.7	6
25-1	CASSEWSSYNSPLH	1-6	17.2	5
4-1	CASSQGGTSGVSGDTQY	2-3	10.3	3
5-5	CASSLVHVPNSNQPQH	1-5	6.9	2
18	CASSPLAGGHNEQF	2-1	6.9	2
5-1	CASSWKASGVFDEQF	2-1	3.4	1
27	CASRVGAGATGEGF	2-2	3.4	1
5-6	CASSFKALSYNEQF	2-1	3.4	1
			100	29

CG41964 CSF CD4+ T cell sort (repeat)

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSAKGALAGTISYNEQF	2-1	31.8	21
25-1	CASSEWSSYNSPLH	1-6	19.7	13
4-1	CASSQGGTSGVSGDTQY	2-3	13.6	9
18	CASSPLAGGHNEQF	2-1	10.6	7
7-9	CASSYRGADTGELF	2-2	6.1	4
7-9	CASSYRGADAGELF	2-2	4.5	3
7-2	CASSSSNRGQWVETQY	2-5	3.0	2
5-5	CASSLVHVPNSNQPQH	1-5	3.0	2
5-1	CASSWKASGVFDEQF	2-1	3.0	2
7-9	CASSYRGTDTGELF	2-2	1.5	1
5-5	CASSWDINTGELF	2-2	1.5	1
5-1	CASSPGRE*GYT	1-2	1.5	1
			100	66

CG41964 CSF CD8+ T cell sort (514 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
4-1	CASSQEDRGYGYT	1-2	17.1	6
5-6	CASSLSYGSWGLNTGELF	2-2	14.3	5
7-9	CASSSQIMDLNYGYT	1-2	8.6	3
5-6	CASSLFYGSWGIDTGELF	2-2	5.7	2
20-1	CSASVRGFDGPYNEQF	2-1	5.7	2
27	CASSLAEGGSTEAF	1-1	5.7	2
6-4	CASSDSSTDTQY	2-3	5.7	2
27	CASSPLRGESSTEAF	1-1	2.9	1
7-9	CASSPTSGGGKNEQF	2-1	2.9	1
7-8	CASSPGVGGMNTEAF	1-1	2.9	1
5-1	CASSARGESYNSPLH	1-6	2.9	1
7-9	CASSRVGGPGDEQY	2-7	2.9	1
27	CASSPTGSQGKLF	1-4	2.9	1
18	CASSPRIREYEQY	2-7	2.9	1
28	CASSLGTAGEQF	2-1	2.9	1
28	CASSLGTPGEQF	2-1	2.9	1
20-1	CSALAGGPGEQF	2-1	2.9	1
7-3	CASSSELRSPLH	1-6	2.9	1
12-4	CASSLNIYEQY	2-7	2.9	1
11-2	CASSSLTREQF	2-1	2.9	1
			100	35

CG41964 CSF CD8+ T cell sort (repeat)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-6	CASSLSYGSWGLNTGELF	2-2	14.3	8
20-1	CSASVRGFDGPYNEQF	2-1	14.3	8
4-1	CASSQEDRGYGYT	1-2	10.7	6
7-9	CASSSQRRDLNYGYT	1-2	7.1	4
7-2	CASSLNRGGNEQF	2-1	7.1	4
28	CASSLGTAGEQF	2-1	7.1	4
28	CASSLSYMTSGSPDTQY	2-3	5.4	3
28	CASSFGGGTSGGDTQY	2-3	5.4	3
27	CASSPTGSQGKLF	1-4	3.6	2
20-1	CSALAGGPGEQF	2-1	3.6	2
6-4	CASSDSSTDTQY	2-3	3.6	2
23-1	CASSQSWDRDEGGNQPQH	1-5	1.8	1
28	CASRPPTTRREVGEQF	2-1	1.8	1
18	CASSPGQGPTSDYGYT	1-2	1.8	1
7-9	CVSSPTSGGGKNEQF	2-1	1.8	1
28	CASSTAGDYYNEQF	2-1	1.8	1
27	CASSLAEGGSTEAF	1-1	1.8	1
12-4	CASSFFGKADTQY	2-3	1.8	1
11-3	CASSLGTEDIYGYT	1-2	1.8	1
11-2	CASSSLTREQF	2-1	1.8	1
6-5	CASSYRLGEQF	2-1	1.8	1
			100	56

8.2.2 Idiopathic Intracranial Hypertension

1. EC21870 (CD4+ only)

EC21870 CSF CD4⁺ T cell sort (555 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
6-1	CASGASGRGLYEQY	2-7	53.4	47
12-3	CASSWDRGQSYNEQF	2-1	17.0	15
27	CASSLSAGTPNTEAF	1-1	10.2	9
7-3	CASSEAGTDTQY	2-3	10.2	9
29-1	CSVVSGGNQPQH	1-5	8.0	7
4-2	CASSQDLSGTRVTDTQY	2-3	1.1	1
			100	88

2. HS25204 (CD4⁺ only)

HS25204 CSF CD4⁺ T cell sort (1073 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CASSLPAPQGRYEQY	2-7	40.5	17
5-1	CASSLGGLYNEQF	2-1	23.8	10
5-1	CASSLSQPGANVLT	2-6	21.4	9
5-1	CASSARGESYNSPLH	1-6	7.1	3
7-9	CASSLPTPQGRYEQY	2-7	4.8	2
7-7	CASSLATAPWTSKETQY	2-5	2.4	1
			100	42

HS25204 CSF CD4⁺ T cell sort (repeat)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLSQPGANVLT	2-6	41.4	29
7-9	CASSLPAPQGRYEQY	2-7	24.3	17
5-1	CASSLGGLYNEQF	2-1	22.9	16
5-1	CASSARGESYNSPLH	1-6	5.7	4
7-7	CASSLATAPWTSKETQY	2-5	2.9	2
18	CASSIGVAGGRDTQY	2-3	2.9	2
			100	70

3. LH25311

LH25311 CSF CD4+ T cell sort (1096 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLGQGQYPLH	1-6	43.8	32
6-2/6-3	CASSYISAGNQPQH	1-5	20.5	15
29-1	CSVSGTSITDTQY	2-3	16.4	12
11-3	CASRRGGTGKLYTGELF	2-2	6.8	5
24-1	CATSDLPPGLAESTDTQY	2-3	4.1	3
11-2	CASSLEYSVNTGELF	2-2	2.7	2
23-1	CASSLDRLSSYEQY	2-7	2.7	2
4-2	CASSQDLSGTIPREQF	2-1	1.4	1
7-9	CASSLGSLLGQPQH	1-5	1.4	1
			100	73

LH25311 CSF CD8+ T cell sort (312 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-6	CASSHMTGDEREQY	2-7	74.7	62
20-1	CASSHMTGDEREQY	2-4	25.3	21
			100	83

4. ML25308 (CD4+ only)

ML25308 CSF CD4+ T cell sort (1014 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
4-2	CASSPSTSGGAAYNEQ	2-1	92.9	79
12-4	CASSISGGAQGDTQY	2-3	3.5	3
29-1	CSVHRDGNTIY	1-3	2.4	2
7-2	CASSSATGAGNTIY	1-3	1.2	1
			100	85

5. SW25353

SW25353 CSF CD4+ T cell sort (4219 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3	CASSPTGSTSGYT	1-2	65.2	45
5-1	CASSLSFSGSSHPY	2-3	11.6	8
7-9	CASSPPGQGNTIY	1-3	7.2	5
5-1	CASSRTGGARVTQY	2-3	5.8	4
12-4	CASSSGTQNQPQH	1-5	4.3	3
5-1	CASTLSFRGSSHPY	2-3	1.4	1
5-1	CASSHDPKDYGYT	1-2	1.4	1
5-1	CASSTGQNQPQH	1-5	1.4	1
12-4	CASSLVLGGKAF	1-1	1.4	1
			100	69

SW25353 CSF CD8+ T cell sort (302 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
2	CASSENNEQF	2-1	29.6	24
6-5	CASSLSSGGLYNEQF	2-1	16.0	13
3-1	CASSQETEGPNQPQH	1-5	14.8	12
10-1	CASSEEDPNSPLH	1-6	12.3	10
30	CAWSPQGGMRQPQH	1-5	7.4	6
6-1	CASNTGGQPQH	1-5	7.4	6
7-9	CASRNRGDRGIEETQY	2-5	3.7	3
4-1	CASSQDPPPHSPLH	1-6	2.5	2
20-1	CSAPGQGTDTQY	2-3	2.5	2
4-1	CASSQPLAGGIGELF	2-2	1.2	1
27	CASSFQGEGNEQF	2-1	1.2	1
3-1	CASSSTGDIQY	2-4	1.2	1
			100	81

6. RY21758 (CD4+ only)

RY21758 CSF CD4+ T cell sort (987 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-8	CASRVTGVNTEAF	1-1	39.2	29
2	CASRTGGGEKLF	1-4	24.3	18
7-8	CASSLRQPGQHSNQPQH	1-5	16.2	12
25-1	CASRFLCGSSSYNEQF	2-1	4.1	3
5-1	CASSATGMRGSNTEAF	1-1	2.7	2
29-1	CSVGTSGLGNEQF	2-1	1.4	1
28	CASSLFSRGQGSYNEQF	2-1	1.4	1
28	CASSLGQRAGFSEKLF	1-4	1.4	1
19	CASSSQREGEQF	2-1	1.4	1
11-2	CASSFDYEQY	2-7	1.4	1
7-9	CASSPVPETGNTEAF	1-1	1.4	1
5-4	CASSLRLAGLRPDTQC	2-3	1.4	1
4-2	CASSQERTSYEQY	2-7	1.4	1
4-1	CASSQDLNRGANIQY	2-4	1.4	1
2	CASSPGTSGAGELF	2-2	1.4	1
			100	74

7. ES37889 (CD4+ only)

ES37889 CSF CD4+ T cell sort (411 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSHRRGGDNTGELF	2-2	100	56
			100	56

8. CC40712

CC40712 CSF CD4+ T cell sort (2140 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
24-1	CATSDSGTGFQETQY	2-5	23.2	16
20-1	CSAPKGGGEQY	2-7	15.9	11
5-5	CASSLGGTSNTEAF	1-1	10.1	7
20-1	CSARTSGRASYNEQF	2-1	7.2	5
18	CASSPGQTEKLF	1-4	7.2	5
20-1	CSAREAGRVNTEAF	1-1	5.8	4
11-2	CASSPYNTETT	1-2	4.3	3
5-5	CASSFGLGTGGNYEQY	2-7	2.9	2
5-5	CASSLASGRGNQPQH	1-5	2.9	2
7-2	CASSLVGGGLAGSVGQF	2-1	1.4	1
7-2	CASSTRGGPNSYNEQF	2-1	1.4	1
7-9	CASSLRTGGAGTEAF	1-1	1.4	1
28	CASSPPRGQGDGYT	1-2	1.4	1
24-1	CAFSQSRLETGELF	2-2	1.4	1
7-6	CASSLLEGANEKLF	1-4	1.4	1
5-4	CASSLGGTSNTEDF	1-1	1.4	1
18	CASSPGLAEETQY	2-5	1.4	1
6-1	CATWLGGSSYEQY	2-7	1.4	1
29-1	CSVWDRGHTEAF	1-1	1.4	1
12-4	CASSSGEALGLF	1-4	1.4	1
20-1	CSSDRLPYEQY	2-7	1.4	1
18	CASSSYGDTQY	2-3	1.4	1
12-3	CASRRTVGEQF	2-1	1.4	1
			100	69

CC40712 CSF CD8+ T cell sort (181 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSSEYSYEQY	2-2	39.7	27
24-1	CATRGRGETYNEQF	2-1	16.2	11
5-1	CASRVGGGYEQY	2-7	14.7	10
28	CASSSEYSYEQY	2-7	11.8	8
6-4	CASSALGKTTTDTQY	2-3	7.4	5
5-6	CSSSLFSGQGREKLF	1-4	1.5	1
28	CASSFRALNSYEQY	2-7	1.5	1
27	CASSFSAGSPYEQY	2-7	1.5	1
24-1	CATSEGQAAGETQY	2-5	1.5	1
5-1	CASSLEGVGNQPQH	1-5	1.5	1
9	CASGGTTSSEQY	2-7	2.9	2
			100	68

9. KA38079 (CD4+ excluded from analysis as frequency of clonotypes <50)

KA38079 CSF CD4+ T cell sort (3505 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSSRDSGANVLT	2-6	8.6	3
6-5	CASSYSQPWDRGFEQY	2-7	5.7	2
7-2	CASNTVGHPGNEQF	2-1	5.7	2
6-6	CASSQGGKNTEAF	1-1	5.7	2
5-6	CASSPGTARYGYT	1-2	5.7	2
5-1	CASSLAGSPNEQF	2-1	5.7	2
29-1	CSGVVDGGTEAF	1-1	5.7	2
10-3	CAISPGGSETQY	2-5	5.7	2
20-1	CSPQRNTEAF	1-1	5.7	2
6-2	CVSRPPPGQKREDTEAF	1-1	2.9	1
5-1	CASSKPTGTNQGYTEAF	1-1	2.9	1
10-2	VQSCNIVGTGSTDTQY	2-3	2.9	1
7-9	CASSSGTVGNSGNTIY	1-3	2.9	1
7-2	CASSRVTSAGSYNEQF	2-1	2.9	1
20-1	CSAPGLAGVQETQY	2-5	2.9	1
12-4	CASSLGKAWGQAQH	1-5	2.9	1
10-2	CATRDRQVINSPLH	1-6	2.9	1
5-1	CASSPDAWNPYEQY	2-7	2.9	1
5-1	CASSLEGAPNYGYT	1-2	2.9	1
12-5	CASGTDRLNEKLF	1-4	2.9	1
12-3	CASSLNSGTDTQY	2-3	2.9	1
5-6	CASSLGGGSETQY	2-5	2.9	1
5-6	CASSLERINTEAF	1-1	2.9	1
27	CASSFRRITEAF	1-1	2.9	1
5-1	CAIRDREREQY	2-7	2.9	1
			100	35

KA38079 CSF CD4+ T cell sort (repeat)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3	CASSLRGQNTGELF	2-2	16.7	3
5-1	CASGLPPTGAPNTEAF	1-1	11.1	2
20-1	CSAVKLGGAFKGYT	1-2	11.1	2
5-1	CASSLGSADYNEQF	2-1	11.1	2
5-1	CAIRDREREQY	2-7	11.1	2
20-1	CSPQRNTEAF	1-1	11.1	2
7-9	CASSLSGTGASEQY	2-7	5.6	1
5-1	CASSGGRVQETQY	2-5	5.6	1
2	CASRSTGIDQPQH	1-5	5.6	1
12-3	CASSSPLGYGYT	1-2	5.6	1
10-3	CAISPGGSETQY	2-5	5.6	1
			100	18

KA38079 CSF CD8⁺ T cell sort (212 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-9	CASSPTSGGGNEQF	1-4	85.2	46
7-9	CASRSGLSGEKLF	1-4	13.0	7
9	CASSEPLDSDSGNTIY	1-3	1.9	1
			100	54

10. CC41471 (CD4⁺ only)

CC41471 CSF CD4⁺ T cell sort (660 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSVAGLAGVDTQY	2-3	25.0	16
7-3	RASSLWVREVPESYNEQF	2-1	20.3	13
18	CASSPRIREYEQY	2-7	14.1	9
5-1	CASSPTTGTSSHEQY	2-7	10.9	7
7-2	CASSLGLGPTGELF	2-2	9.4	6
10-2	CASKRADSYNEQF	2-1	6.3	4
10-3	CAIRPGTGAYEQY	2-7	4.7	3
5-6	CASYRRTSGITYNEQF	2-1	3.1	2
19	CASKGLQGASEQF	2-1	3.1	2
29-1	CSVEPRGGDGYT	1-2	1.6	1
12-4	CASMRSYNEQF	2-1	1.6	1
			100	64

11. RC41200 (CD4⁺ only)

RC41200 CSF CD4⁺ T cell sort (747 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
2	CASSTLYEQY	2-7	100	89
			100	89

8.2.3 Other Neurological Diseases

1. SE29703

SE29703 CSF CD4⁺ T cell sort (2505 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
11-2	CASSLGTGALNTGELF	2-2	98.8	83
11-2	CASSLGTEALNTGELF	2-2	1.2	1
			100	84

SE29703 CSF CD8⁺ T cell sort (305 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSFESGTHNEQF	2-1	65.8	50
10-3	CATRPDREDQPQH	1-5	28.9	22
24-1	CATREGQTNAEAF	1-1	2.6	2
7-7	CASSPGLAGAFRDTQY	2-3	1.3	1
5-1	CASSWESGTHNEQF	2-1	1.3	1
			100	76

2. JG22488 (CD4⁺ only)

JG22488 SF CD4⁺ T cell sort (793 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSRRGQGGTEAF	1-1	95.2	80
25-1	CASPGTGGRNGYT	1-2	1.2	1
12-3	CASRPRGEGFGYT	1-2	1.2	1
12-4	CASSGGSYNEQF	2-1	1.2	1
28	CASSTRRAEAF	1-1	1.2	1
			100	84

3. ND37140

ND37140 CSF CD4$^+$ T cell sort (643 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
29-1	CSAGRVAEAF	1-1	63.0	34
7-2	CASSSEIYNEQF	2-1	25.9	14
5-5	CASSLDPGSSNQPQH	1-5	9.3	5
5-1	CASSLGQSRATEAF	1-1	1.9	1
			100	54

ND37140 CSF CD8$^+$ T cell sort (129 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
6-1	CASGRKGTIQPQH	1-5	52.3	34
4-1	CASSQGAGGGGTEAF	1-1	20.0	13
7-9	CASSYSEAGNNEQF	2-1	7.7	5
5-1	CASSLEGQASSYEQY	2-7	7.7	5
5-5	CASSLNMLAVTYNEQF	2-1	4.6	3
7-9	CASSSTGGAGDEQF	2-1	4.6	3
7-8	CASSLGQTQAQY	2-7	3.1	2
			100	65

4. CS19395 (CD4$^+$ only)

CS19395 CSF CD4$^+$ T cell sort (356 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
28	CASRTGGTRSYNEQF	2-1	18.52	15
20-1	CSARDFGSLYNEQF	2-1	18.52	15
20-1	CSAMGGAGSTDTQY	2-3	16.05	13
28	CASSLQGRSSYEQY	2-7	9.88	8
6-5	CASSKSLVWNEQF	2-1	8.64	7
5-1	CASRAGTGTDTQY	2-3	6.17	5
28	CASSSHTGELF	2-2	6.17	5
28	CASRANSGGELF	2-2	4.94	4
28	CASSPRPPPGELF	2-2	2.47	2
20-1	CSARGGAGSTDTQY	2-3	1.23	1
7-2	CASSFGTASGNTIY	1-3	1.23	1
29-1	CSVDLGQGSYEQY	2-7	1.23	1
2	CASKVRGQNNEQF	2-1	1.23	1
28	CAGRANSGGELF	2-2	1.23	1
6-6	CASSYARSDEQF	2-1	1.23	1
28	CASSSRTGELF	2-2	1.23	1
			100	81

5. AG20355 (CD4+ only)

AG20355 CSF CD4+ T cell sort (432 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSPGQGAGTEAF	1-1	31.6	25
7-8	CASSPSMREQF	2-1	25.3	20
28	CASRIQTGKRGTDTQY	2-3	20.3	16
7-9	CASSSPAEAF	1-1	8.9	7
28	CGGQGRGQPQH	1-5	5.1	4
27	CASLPLRGVYNEQF	2-1	3.8	3
2	CASSERRSF	1-1	2.5	2
4-3	CASSQVSGDNEAF	1-1	1.3	1
4-3	CASSQVSGDSEAF	1-1	1.3	1
			100	79

6. JM25229

JM25229 CSF CD4+ T cell sort (2810 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSAPASRGTGELF	2-2	41.3	33
12-3	CASSLGLYEQY	2-7	21.3	17
11-3	CASSGRAPRTQY	2-3	12.5	10
5-6	CASSLADQPQH	1-5	8.8	7
9	CASSVVGLSDTQY	2-3	5.0	4
7-8	CASSLDMQGINEKLF	1-4	3.8	3
3-1	CASSQDGASRDGTDTQY	2-3	2.5	2
12-3	CASSLGTGKADTQY	2-3	1.3	1
20-1	CSAPASRGAGELF	2-2	1.3	1
5-1	CASSLEGDYTEAF	1-1	1.3	1
5-6	CASSLMGVYEQY	2-7	1.3	1
			100	80

JM25229 CSF CD8+ T cell sort (650 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSLVGGTGGTQY	2-5	80.7	46
7-9	CASSLVGQESPDEQF	2-1	14.0	8
7-9	CASSLVGRESPDEQF	2-1	3.5	2
7-2	CASSLVGGTGRTQY	2-5	1.8	1
			100	57

7. AB25236

AB25236 CSF CD4^{+} T cell sort (4005 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3	CASSIPSGRAEEQF	2-1	98.8	79
12-3	CASSIPSGRAEDKF	2-1	1.3	1
			100	80

AB25236 CSF CD8^{+} T cell sort (545 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
15	CATSRSRGASYEQY	2-7	26.6	17
6-2	CASSSWTGLGNTEAF	1-1	17.2	11
12-3	CASSFDVRGETQY	2-5	15.6	10
2	CASSEEAAKNQETQY	2-5	12.5	8
4-2	CASSLETGTAPEQY	2-7	7.8	5
6-2	CASIQGPETYEQY	2-7	7.8	5
5-1	CASSLELAGYGYT	1-2	6.3	4
27	CASSLHSGQGFYEQY	2-7	3.1	2
10-3	CAISARDGREDTEAF	1-1	1.6	1
7-9	CASSEGVRGYT	1-2	1.6	1
			100	64

7. CJ26014

CJ26014 CSF CD4+ T cell sort (844 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSARPLRDLGEAF	1-1	19.4	13
7-9	CASSLIGESETQY	2-5	17.9	12
12-4	CASSRGQGFFGNTEAF	1-1	13.4	9
12-4	CASSPRNSAEAF	1-1	11.9	8
12-3	CASSSGQGNRYSNQPQH	1-5	7.5	5
6-2/6-3	CASSYSSSGDTDTQY	2-3	7.5	5
12-4	CAGGRGGMNTEAF	1-1	7.5	5
7-2	CASEGRSGANVLT	2-6	3.0	2
5-1	CASSFRLGQDYYEQY	2-7	1.5	1
5-1	CASSRPPGRQPYEQY	2-7	1.5	1
20-1	CSARPLRDLSEAF	1-1	1.5	1
7-9	CASRVIGESETQY	2-5	1.5	1
9	CASSVGDNKKAF	1-1	1.5	1
9	CASSVGDNTEAF	1-1	1.5	1
4-2	CASSQDDNYGYT	1-2	1.5	1
30	CAWTDRKAF	1-1	1.5	1
			100	67

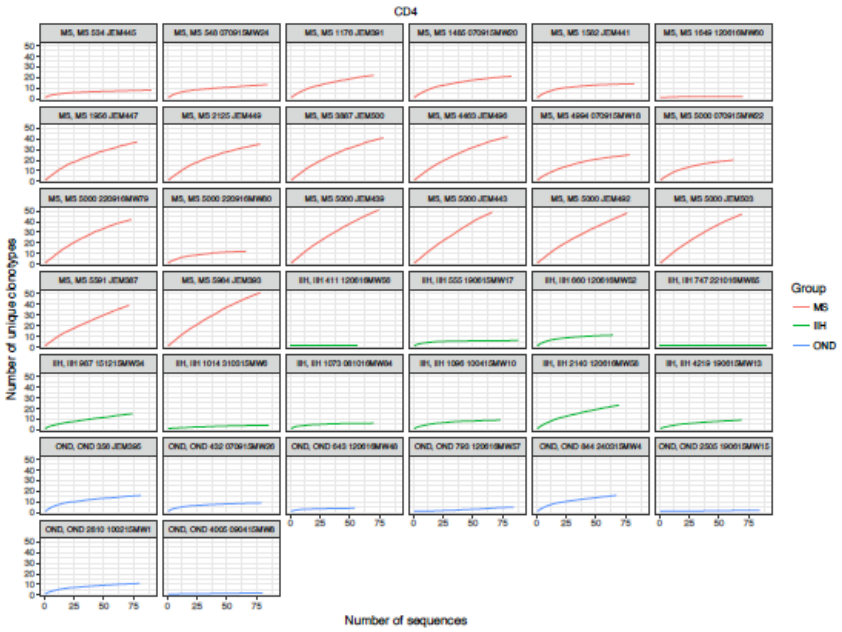
CJ26014 CSF CD8+ T cell sort (122 sorted cells)

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSLGRVRDEQY	2-7	87.7	57
7-9	CASSGRGSLYGYT	1-2	7.7	5
4-1	CASSQDVWYEQY	2-7	3.1	2
5-1	CASSLGRVRGEQY	2-7	1.5	1
			100	65

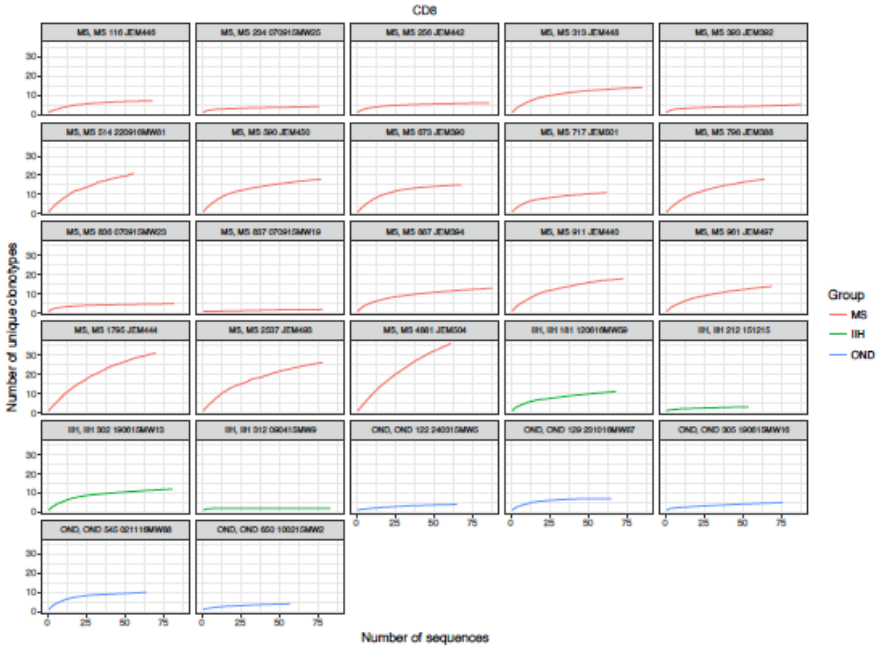
8.3 Accumulation of unique TCR clonotypes with increasing number of sequences for the CD4⁺ TCR repertoire of individual patients

To better understand the correlation between sample TCR diversity and number of sorted cells for both CD4+ and CD8+ T cell populations across MS patients but not for control patients, the depth of clonotype sequencing for the individual TCR repertoires was examined. Species accumulation curves were used to plot the accumulation of unique TCR clonotypes for increasing-sized subsamples of sequences from the original data. A plateau in the number of unique clonotypes at higher numbers of sequences (i.e. no new clonotypes are identified as more sequences are considered) demonstrates that the diversity estimate is approaching the population diversity.

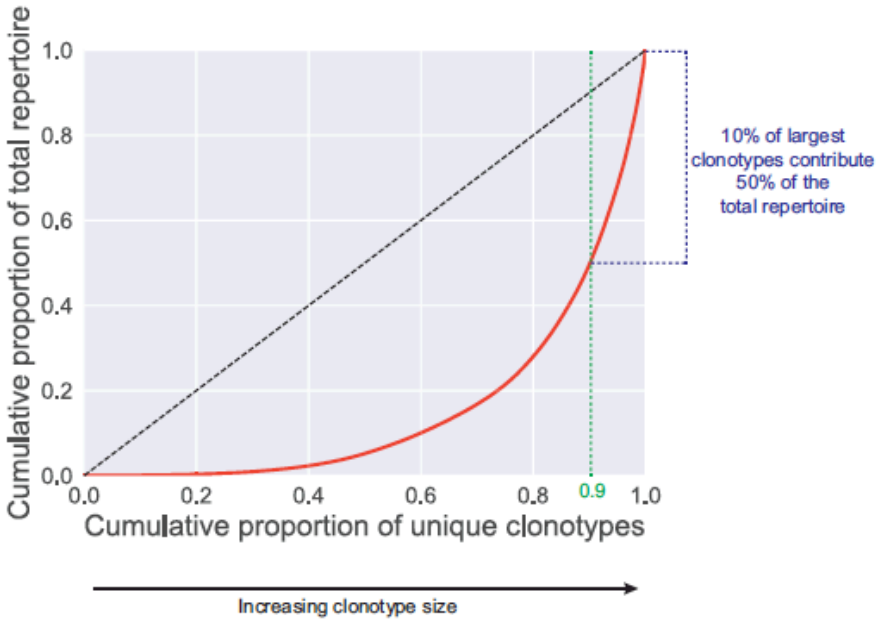
CD4⁺



CD8⁺

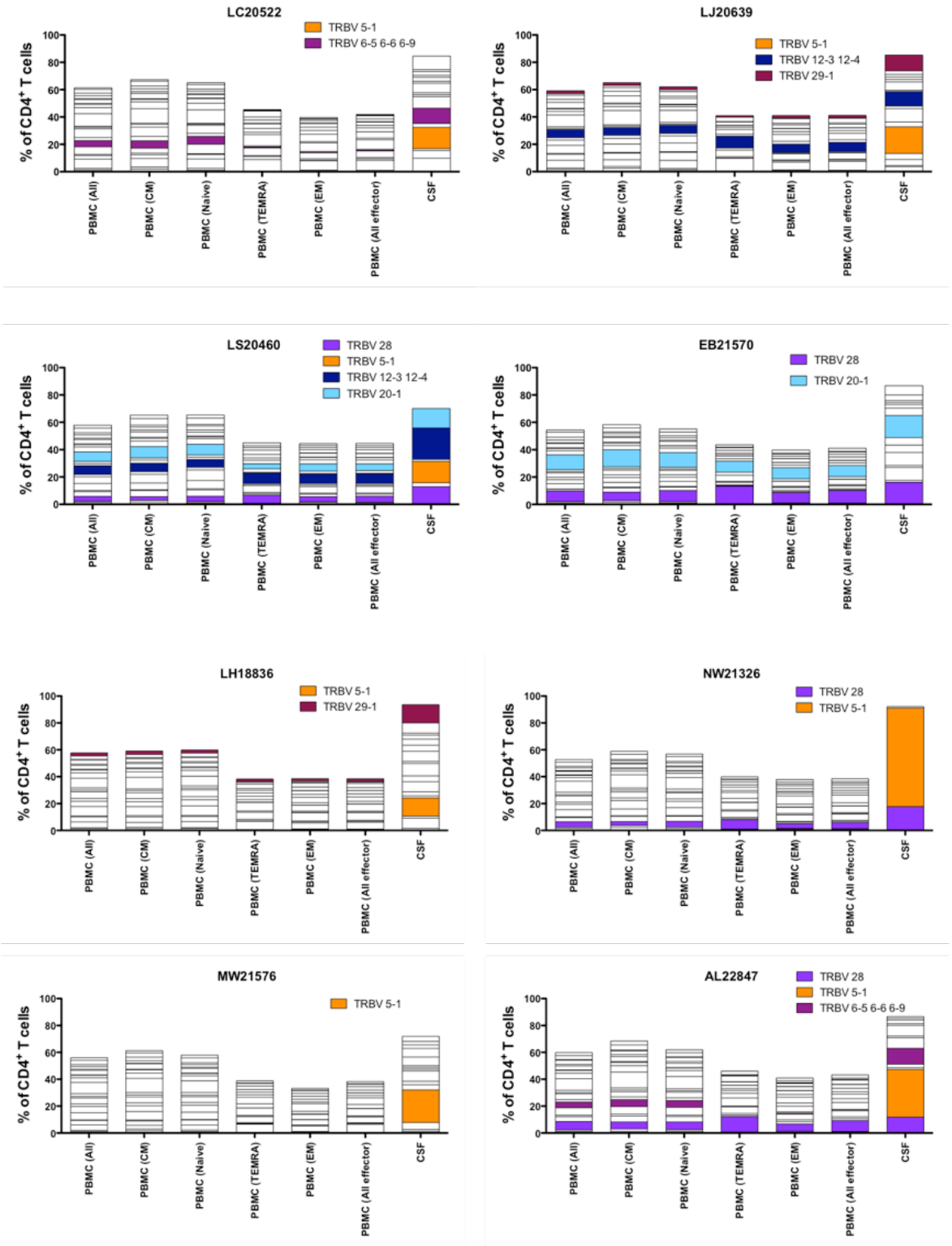


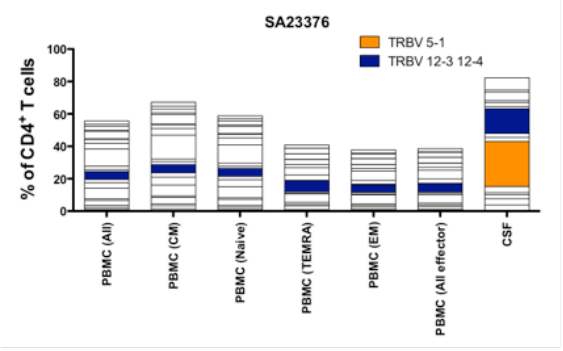
8.4 Example showing interpretation of the cumulative frequency distributions

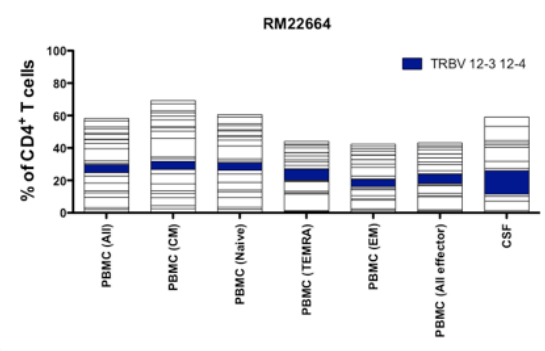


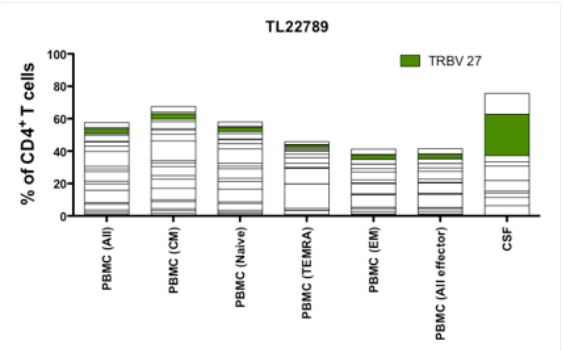
8.5 TCR Vβ comparisons between CSF and peripheral blood

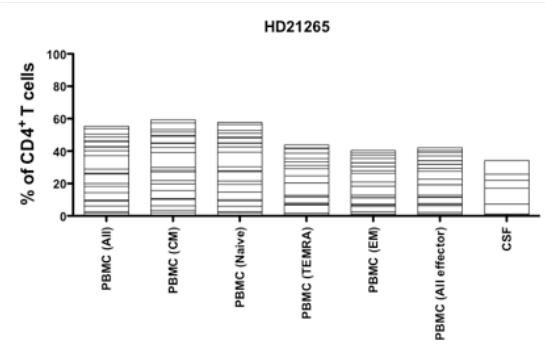
8.5.1 Multiple sclerosis CD4⁺ T cells

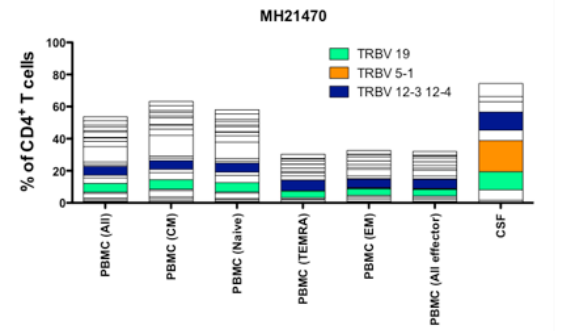


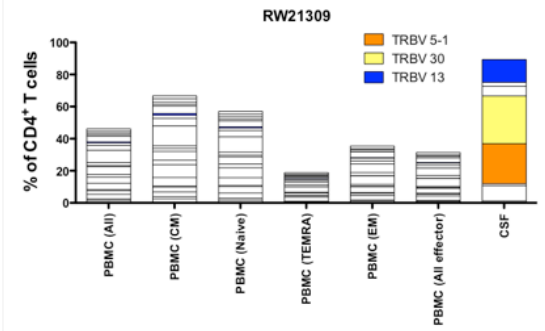

SA23376
TRBV 5-1
TRBV 12-3 12-4
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF


RM22664
TRBV 12-3 12-4
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

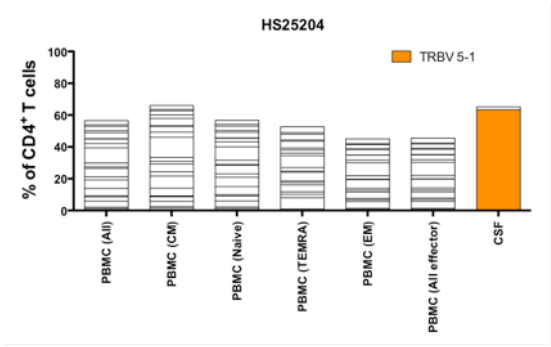

TL22789
TRBV 27
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

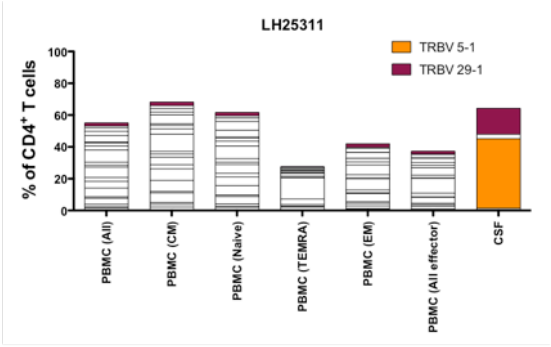

HD21265
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

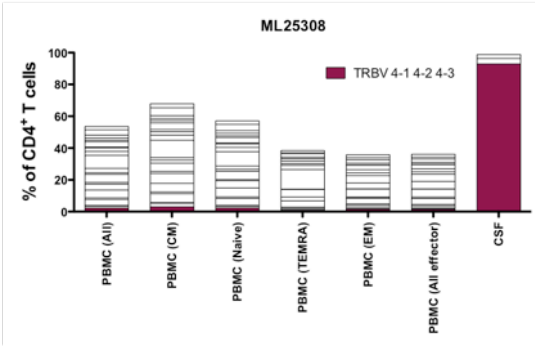

MH21470
TRBV 19
TRBV 5-1
TRBV 12-3 12-4
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

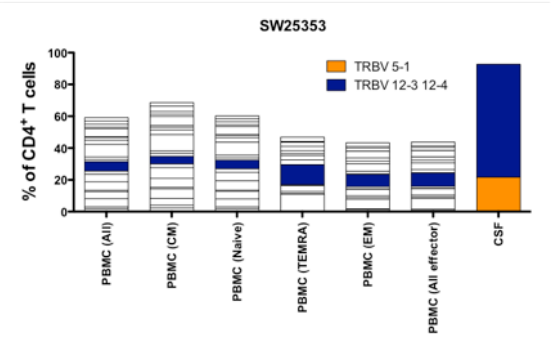

RW21309
TRBV 5-1
TRBV 30
TRBV 13
% of CD4+ T cells
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

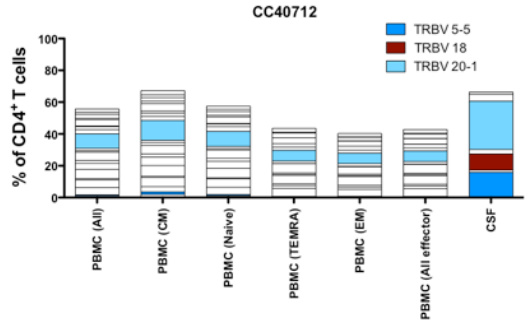
8.5.2 Idiopathic intracranial hypertension CD4+ T cells



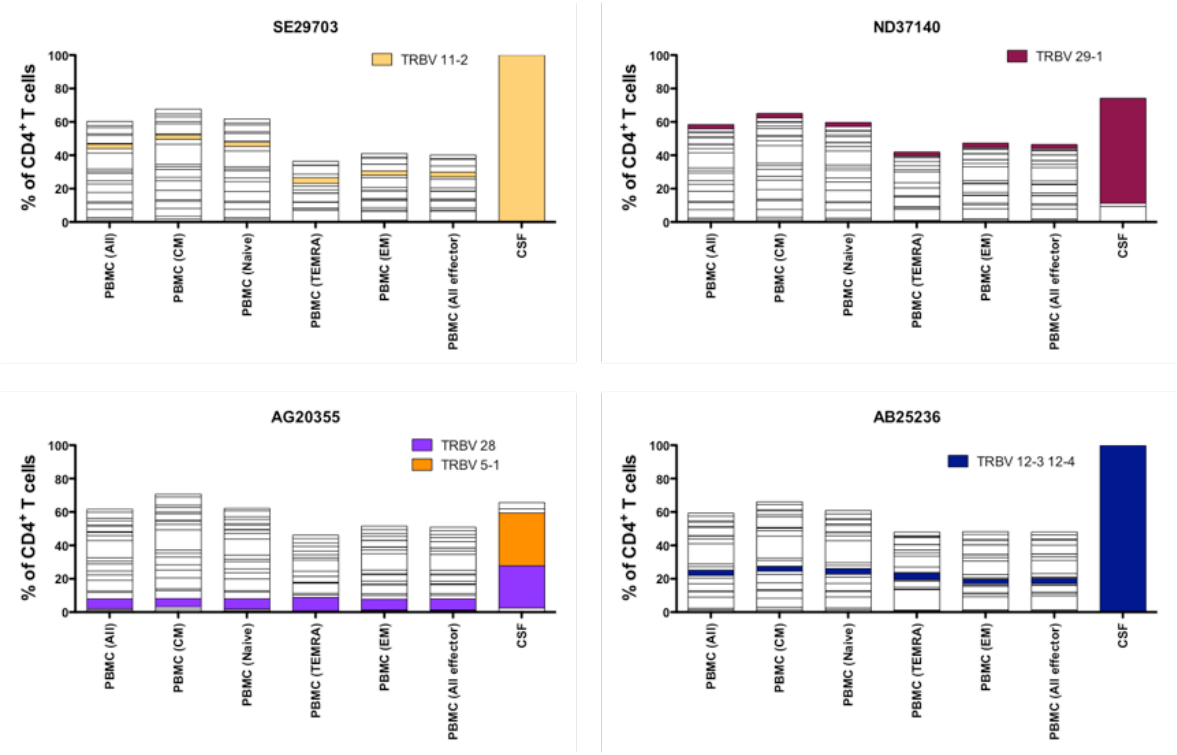




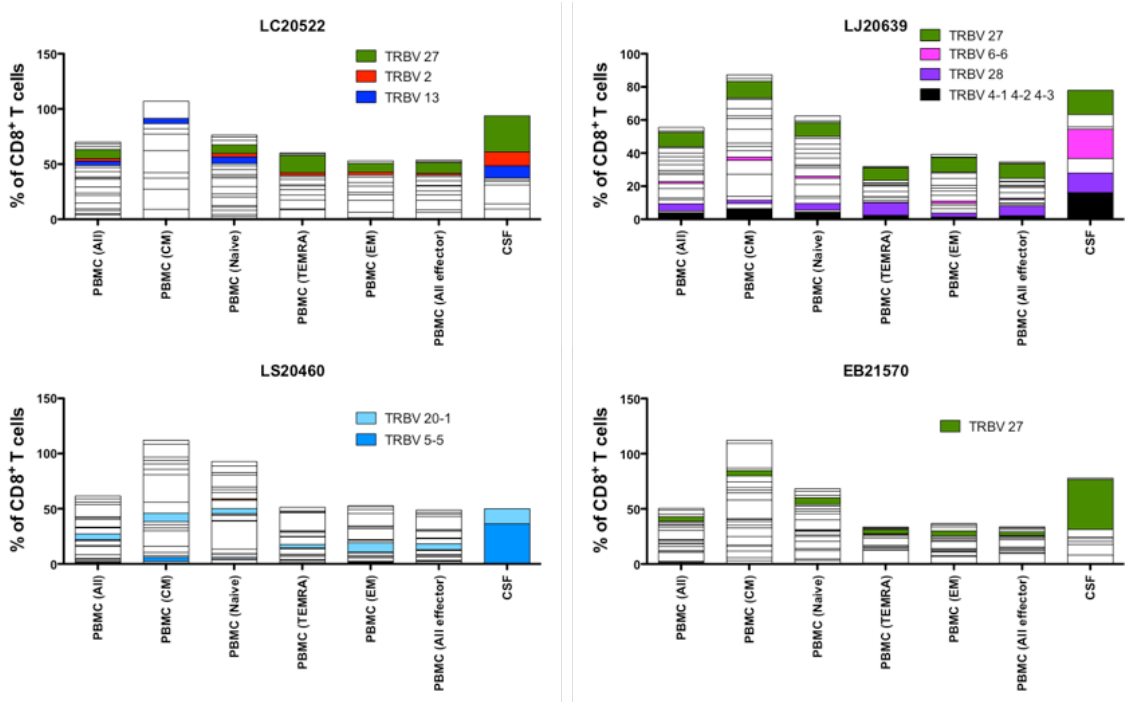


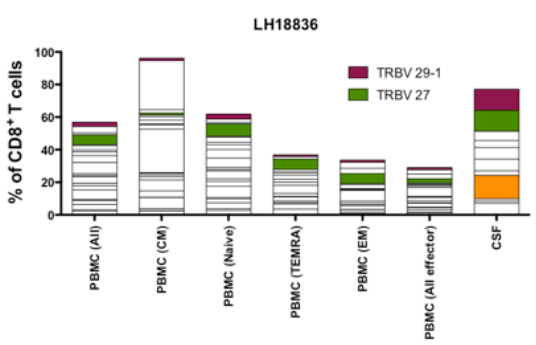


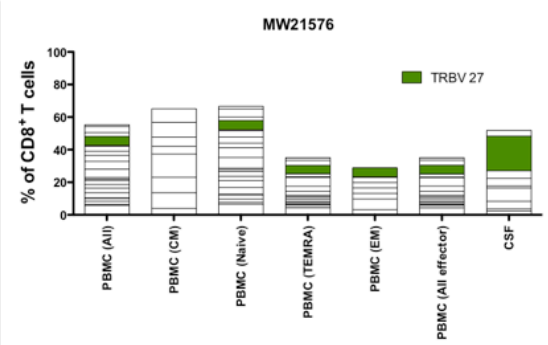
8.5.3 Other neurological diseases CD4⁺ T cells

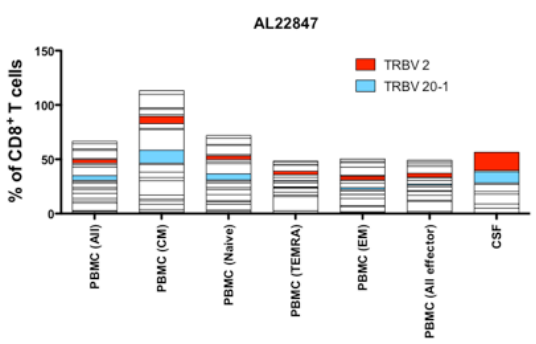


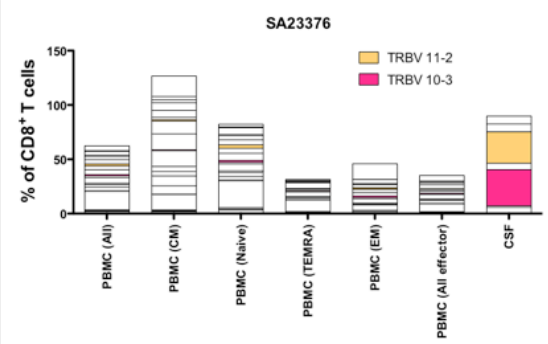
8.5.4 Multiple sclerosis CD8⁺ T cells

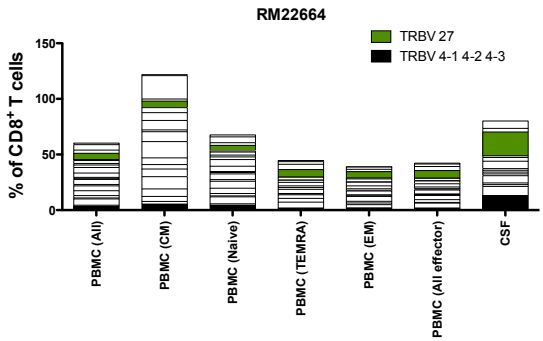


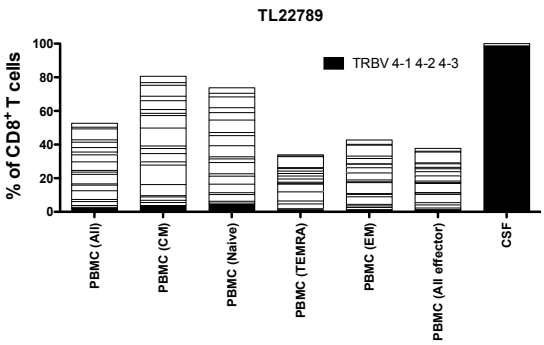

LH18836
% of CD8+ T cells
TRBV 29-1
TRBV 27
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

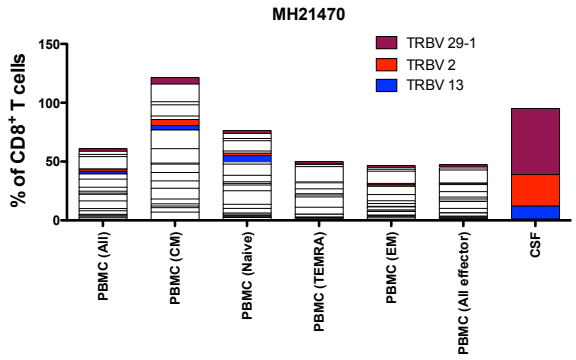

MW21576
% of CD8+ T cells
TRBV 27
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF


AL22847
% of CD8+ T cells
TRBV 2
TRBV 20-1
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

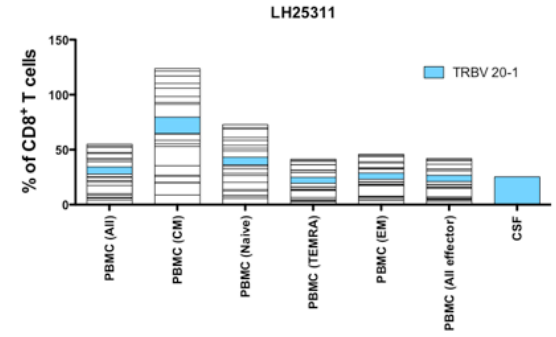

SA23376
% of CD8+ T cells
TRBV 11-2
TRBV 10-3
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

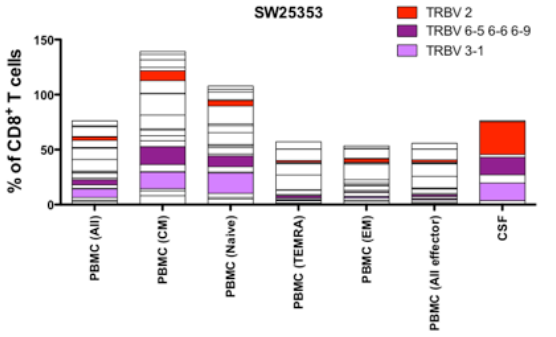

RM22664
% of CD8+ T cells
TRBV 27
TRBV 4-1 4-2 4-3
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

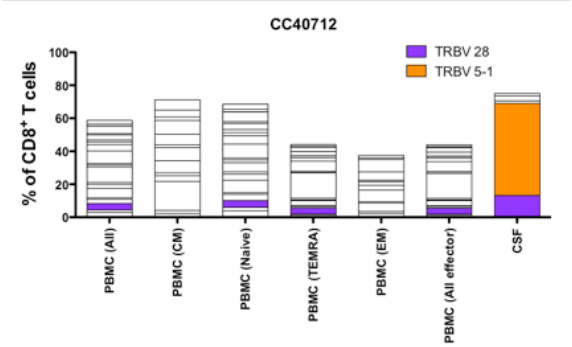

TL22789
% of CD8+ T cells
TRBV 4-1 4-2 4-3
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF


MH21470
% of CD8+ T cells
TRBV 29-1
TRBV 2
TRBV 13
PBMC (All)
PBMC (CM)
PBMC (Naive)
PBMC (TEMRA)
PBMC (EM)
PBMC (All effector)
CSF

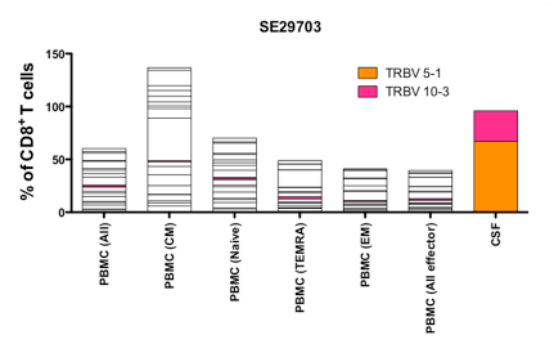
8.5.5 Idiopathic intracranial hypertension CD8⁺ T cells

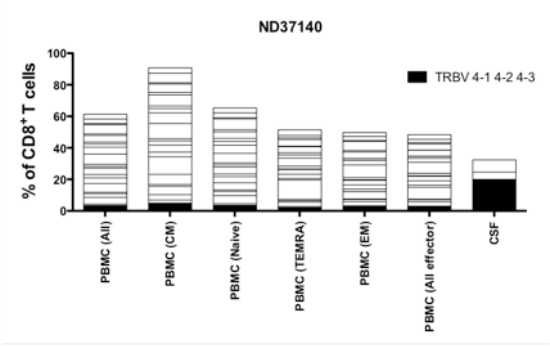


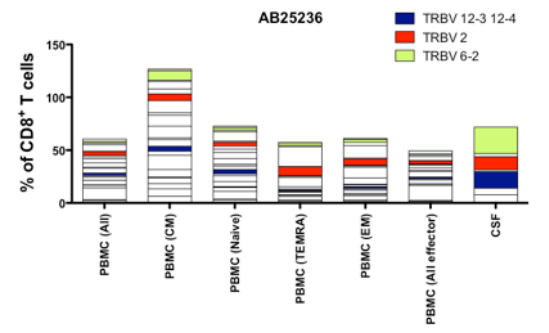




8.5.6 .Other neurological diseases CD8⁺ T cells







8.6 CSF T cell Vβ usage – Raw data from clonotyping (Grey boxes = TCRs >10% frequency; Orange = not covered by PBMC Vβ screen; Green = PBMC stains excluded)

8.6.1 Multiple sclerosis CSF CD4⁺ T cell Vβ

	EB21570	LC20552	LH18836	LJ20639	LS20460	MW21576	NW21326	AL22847	SA23376	RM22664	TL22789	HD21265	MH21407	RW21309
TRBV 4-1 4-2 4-3		9.9	1.5	3.6					3.8	1.4			1.6	
TRBV 5-5				0.9		1.3			3.8					
TRBV 28	16.2	5.6	7.6	4.4	12.9	1.3	17.8	11.8	2.5	5.8			6.5	1.2
TRBV 3-1	1.4			4.4	2.9	5.1			1.3					9.5
TRBV 19		1.4	1.5						3.8			1.2	11.3	1.2
TRBV 14														
TRBV 5-1	9.5	15.5	13.6	19.5	15.7	24.4	73.3	35.5	27.8	2.9	6.4		19.4	25.0
TRBV 18		2.8	1.5	3.5		3.8		1.3	2.5	1.4		6.1		
TRBV 30			3		1.4	2.6		2.6						29.8
TRBV 6-5 6-6 6-9		11.3	7.5	9.8		7.7		11.8	2.5				6.5	6.0
TRBV 6-6	1.4		4.5	1.8		1.3								
TRBV 12-3 12-4	9.5	8.5	9.1	10.7	22.9	1.3		7.9	15.2	14.5	5.1	9.8	11.3	
TRBV 5-6	5.4	1.4	1.5						1.3	1.4	2.6			
TRBV 10-3	5.4	1.4		0.9				1.3		4.3	1.3			
TRBV 20-1	16.2	7	7.6	6.2	14.3	1.3		7.9	2.5	8.7	6.4	4.9		
TRBV 9	5.4	1.4	4.5	1.8		6.4		1.3		1.4	9		6.5	2.4
TRBV 11-2			4.5	1.8		6.4			1.3	1.4	2.6			
TRBV 13		2.8												14.3
TRBV 2	2.7	1.4	3	1.8		2.6		2.6	5.1	1.4			3.2	
TRBV 25-1	1.4	2.8	1.5					1.3	1.3		3.8			
TRBV 27	1.4	1.4	7.6	2.7		2.6				8.7	25.6	3.7	8.1	
TRBV 4-3														
TRBV 6-2	4.1													
TRBV 29-1	6.8	9.9	13.6	11.5		3.8	1.1	1.3	7.6	5.8	12.8	8.5		
TRBV 24-1				1.8		3.8			1.3	4.3		22		1.2
TRBV 7-2	8.1	5.6	1.5		15.7	3.8	7.8		3.8	14.5	7.7	15.9	16.1	6.0
TRBV 7-3			1.5					3.9				8.5	4.8	
TRBV 12-5												1.2		
TRBV 7-6				0.9		3.8		1.3		2.9		14.6		
TRBV 7-7										4.3	2.6	2.4		
TRBV 7-9	2.7	1.4	1.5	2.7		3.8		1.3	5.1		6.4	1.2		
TRBV 5-4														
TRBV 11-3	2.7	1.4	1.5											
TRBV 7-8			3	3.5	2.9	5.1		2.6	1.3					
TRBV 6-1			1.5	4.4		1.3			3.8	5.8	7.7		4.8	1.2
TRBV 5-1														
TRBV 7-3														
TRBV 6-2/6-3		2.8		0.9	1.4	6.4		3.9		4.3				
TRBV 15		1.4		2.7	4.3					1.4				
TRBV 10-1		1.4												
TRBV 6-4														
TRBV 11-1		1.4			5.7	1.3								2.4
TRBV 10-2		2.5							2.5					
TRBV 16										2.9				
TRBV 23-1														

8.6.2 Multiple sclerosis CSF CD8^{+} T cell Vβ

	EB21570	LC20552	LH18836	LJ20639	LS20460	MW21576	AL22847	SA23376	RM22664	TL22789	MH21407
TRBV 4-1 4-2 4-3		9.38	7.1	16.17			5.1		13.1	98.7	
TRBV 5-5	8.2				36.4		3.8				
TRBV 28	9.6	4.69	1.4	11.76			9		8.2		
TRBV 3-1			1.4			2.4	2.6	5.8			
TRBV 19											
TRBV 14						1.2					
TRBV 5-1		17.19	14.3	8.82				1.4	1.6	1.3	
TRBV 18											
TRBV 30									1.6		
TRBV 6-5 6-6 6-9						4.7			6.5		
TRBV 6-6	2.7	3.13	2.9	17.65					1.6		
TRBV 12-3 12-4			7.1	1.47			6.4		1.6		1.2
TRBV 5-6							1.3		1.6		
TRBV 10-3		1.56						33.3			
TRBV 20-1	2.7		7.1		13.6	8.2	10.3		1.6		
TRBV 9	1.4	1.56	4.3	7.35			1.3	5.8	6.6		
TRBV 11-2	6.8					1.2		29	3.3		
TRBV 13		10.94	5.7			4.7					11
TRBV 2		12.5				4.7	16.7		1.6		26.8
TRBV 25-1								7.2			
TRBV 27	45.2	32.81	12.9	14.71		21.2		7.2	21.3		
TRBV 4-3									3.3		
TRBV 6-2											
TRBV 29-1	1.4		12.9			3.5			6.6		56.1
TRBV 24-1						4.7		1.4			
TRBV 7-2	6.8		8.6	4.41			12.8		8.2		
TRBV 7-3	4.1		1.4								
TRBV 12-5											
TRBV 7-6								5.8			4.9
TRBV 7-7									4.9		
TRBV 7-9	8.2	4.69	4.3	2.94	50		12.8		4.9		
TRBV 5-4		1.56		1.47							
TRBV 11-3			1.4								
TRBV 7-8			7.1			4.7	1.3				
TRBV 6-1				13.24			1.3	1.4			
TRBV 7-3						28.2					
TRBV 6-2/6-3	2.7					10.6	11.5		6.6		
TRBV 15							2.6				
TRBV 10-1							1.3				
TRBV 6-4								1.4			
TRBV 11.1											
TRBV 10-2											
TRBV 16											
TRBV 23-1											

8.6.3 Idiopathic intracranial hypertension CSF $CD4^+$ T cell Vβ

	HS25204	LH25311	ML25308	SW25353	CC40712
TRBV 4-1 4-2 4-3		1.4	92.9		
TRBV 5-5					15.9
TRBV 28					1.4
TRBV 3-1					
TRBV 19					
TRBV 14					
TRBV 5-1	63.4	43.8		21.7	
TRBV 18	1.8				10.1
TRBV 30					
TRBV 6-5 6-6 6-9					
TRBV 6-6					
TRBV 12-3 12-4			3.5	71	2.8
TRBV 5-6					
TRBV 10-3					
TRBV 20-1					30.4
TRBV 9					
TRBV 11-2		2.7			4.3
TRBV 13					
TRBV 2					
TRBV 25-1					
TRBV 27					
TRBV 4-3					
TRBV 6-2					
TRBV 29-1		16.4	2.4		1.4
TRBV 24-1		4.1			24.6
TRBV 7-2			1.2		2.9
TRBV 7-3					
TRBV 12-5					
TRBV 7-6					1.4
TRBV 7-7	2.7				
TRBV 7-9	32.1	1.4		7.2	1.4
TRBV 5-4					1.4
TRBV 11-3		6.8			
TRBV 7-8					
TRBV 6-1					1.4
TRBV 5-1					
TRBV 7-3					
TRBV 6-2/6-3		20.5			
TRBV 15					
TRBV 10-1					
TRBV 6-4					
TRBV 11-1					
TRBV 10-2					
TRBV 16					
TRBV 23-1		2.7			

8.6.4 Idiopathic intracranial hypertension CSF $CD8^+$ T cell Vβ

	LH25311	SW25353	CC40712
TRBV 4-1 4-2 4-3		3.7	
TRBV 5-5			
TRBV 28			13.2
TRBV 3-1		16	
TRBV 19			
TRBV 14			
TRBV 5-1			55.9
TRBV 18			
TRBV 30		7.4	
TRBV 6-5 6-6 6-9		16	
TRBV 6-6			
TRBV 12-3 12-4			
TRBV 5-6			1.5
TRBV 10-3			
TRBV 20-1	25.3	2.5	
TRBV 9			2.9
TRBV 11-2			
TRBV 13			
TRBV 2		29.6	
TRBV 25-1			
TRBV 27		1.2	1.5
TRBV 4-3			
TRBV 6-2			
TRBV 29-1			
TRBV 24-1			17.6
TRBV 7-2			
TRBV 7-3			
TRBV 12-5			
TRBV 7-6	74.7		
TRBV 7-7			
TRBV 7-9		3.7	
TRBV 5-4			
TRBV 11-3			
TRBV 7-8			
TRBV 6-1		7.4	
TRBV 7-3			
TRBV 6-2/6-3			
TRBV 15			
TRBV 10-1		12.3	
TRBV 6-4			7.4
TRBV 11.1			
TRBV 10-2			
TRBV 16			
TRBV 23-1			

8.6.5 Other neurological diseases CSF CD4$^+$ T cell Vβ

	SE29703	ND37140	AG20355	AB25236
TRBV 4-1 4-2 4-3			2.5	
TRBV 5-5		9.3		
TRBV 28			25.3	
TRBV 3-1				
TRBV 19				
TRBV 14				
TRBV 5-1		1.9	31.6	
TRBV 18				
TRBV 30				
TRBV 6-5 6-6 6-9				
TRBV 6-6				
TRBV 12-3 12-4				100
TRBV 5-6				
TRBV 10-3				
TRBV 20-1				
TRBV 9				
TRBV 11-2	100			
TRBV 13				
TRBV 2			2.5	
TRBV 25-1				
TRBV 27			3.8	
TRBV 4-3				
TRBV 6-2				
TRBV 29-1		63		
TRBV 24-1				
TRBV 7-2		25.9		
TRBV 7-3				
TRBV 12-5				
TRBV 7-6				
TRBV 7-7				
TRBV 7-9			8.9	
TRBV 5-4				
TRBV 11-3				
TRBV 7-8			25.3	
TRBV 6-1				
TRBV 5-1				
TRBV 7-3				
TRBV 6-2/6-3				
TRBV 15				
TRBV 10-1				
TRBV 6-4				
TRBV 11-1				
TRBV 10-2				
TRBV 16				
TRBV 23-1				

8.6.6 Other neurological diseases CSF CD8$^+$ T cell Vβ

	SE29703	ND37140	AB25236
TRBV 4-1 4-2 4-3		20	7.8
TRBV 5-5		4.6	
TRBV 28			
TRBV 3-1			
TRBV 19			
TRBV 14			
TRBV 5-1	67.1	7.7	6.3
TRBV 18			
TRBV 30			
TRBV 6-5 6-6 6-9			
TRBV 6-6			
TRBV 12-3 12-4			15.6
TRBV 5-6			
TRBV 10-3	28.9		1.6
TRBV 20-1			
TRBV 9			
TRBV 11-2			
TRBV 13			
TRBV 2			12.5
TRBV 25-1			
TRBV 27			3.1
TRBV 4-3			
TRBV 6-2			25
TRBV 29-1			
TRBV 24-1	2.6		
TRBV 7-2			
TRBV 7-3			
TRBV 12-5			
TRBV 7-6			
TRBV 7-7	1.3		
TRBV 7-9		12.3	1.6
TRBV 5-4			
TRBV 11-3			
TRBV 7-8		3.1	
TRBV 6-1		52.3	
TRBV 7-3			
TRBV 6-2/6-3			
TRBV 15			26.6
TRBV 10-1			
TRBV 6-4			
TRBV 11.1			
TRBV 10-2			
TRBV 16			
TRBV 23-1			

8.7 Peptide library screen results for KG19967 $CD8^+$ TCR

8.7.1 Viral database results

Score	Peptide	Protein
-12.339322	VMGISSLV	4POQ A[HUMAN POLYOMAVIRUS 9]
-12.339322	VMGISSLV	>GI|326910937|REF|YP_004243705.1| VP1 [HUMAN POLYOMAVIRUS 9]
-12.525908	VMGLAMPV	>GI|9628341|REF|NP_042932.1| ENVELOPE GLYCOPROTEIN B [HUMAN HERPESVIRUS 6A]
-12.525908	VMGLAMPV	>GI|9633110|REF|NP_050220.1| GLYCOPROTEIN B [HUMAN HERPESVIRUS 6B]
-12.525908	VMGLAMPV	SP|P36319|GB_HHV6G ENVELOPE GLYCOPROTEIN B OS=HUMAN HERPESVIRUS 6A (STRAIN GS) GN=GB PE=3 SV=1
-12.886705	VMAISRCV	>GI|51557496|REF|YP_068330.1| ENVELOPE GLYCOPROTEIN B [SUID HERPESVIRUS 1]
-12.92213	ILGLSTSV	>GI|9633101|REF|NP_050211.1| CAPSID ASSEMBLY PROTEIN [HUMAN HERPESVIRUS 6B]
-12.92213	ILGLSTSV	SP|P52437|UL37_HHV6U CAPSID ASSEMBLY PROTEIN UL37 HOMOLOG OS=HUMAN HERPESVIRUS 6A (STRAIN UGANDA-1102) GN=U30 PE=3 SV=1
-13.25906	VLGLASCV	>GI|52139241|REF|YP_081514.1| ENVELOPE GLYCOPROTEIN B [HUMAN HERPESVIRUS 5]
-13.25906	VLGLASCV	SP|P06473|GB_HCMVA ENVELOPE GLYCOPROTEIN B OS=HUMAN CYTOMEGALOVIRUS (STRAIN AD169) GN=GB PE=1 SV=1
-13.25906	VLGLASCV	SP|P13201|GB_HCMVT ENVELOPE GLYCOPROTEIN B OS=HUMAN CYTOMEGALOVIRUS (STRAIN TOWNE) GN=GB PE=1 SV=1
-13.269185	ILGISCFV	SP|Q9QJ45|U21_HHV6Z U21 GLYCOPROTEIN OS=HUMAN HERPESVIRUS 6B (STRAIN Z29) GN=U21 PE=3 SV=2
-13.269185	ILGISCFV	>GI|9628323|REF|NP_042914.1| MEMBRANE PROTEIN U21 [HUMAN HERPESVIRUS 6A]
-13.269185	ILGISCFV	>GI|9633090|REF|NP_050201.1| PUTATIVE MEMBRANE GLYCOPROTEIN [HUMAN HERPESVIRUS 6B]
-13.384254	TLGISHLV	SP|P07387|TEGU_HCMV TEGUMENT PROTEIN OS=HUMAN CYTOMEGALOVIRUS PE=4 SV=1
-13.455771	ILGLANLV	>GI|9628706|REF|NP_043570.1| POLYPROTEIN PRECURSOR [GB VIRUS C]
-13.455771	ILGLANLV	>GI|28971391|REF|NP_803203.1| PUTATIVE E2 PROTEIN [GB VIRUS C]
-13.584583	TLALSQVV	SP|P17594|POLG_EMCVD GENOME POLYPROTEIN OS=ENCEPHALOMYOCARDITIS VIRUS (STRAIN EMC-D DIABETOGENIC) PE=1 SV=2
-13.584583	TLALSQVV	SP|P17593|POLG_EMCVB GENOME POLYPROTEIN OS=ENCEPHALOMYOCARDITIS VIRUS (STRAIN EMC-B NONDIABETOGENIC) PE=3 SV=1
-13.584583	TLALSQVV	SP|P03304|POLG_EMCV GENOME POLYPROTEIN OS=ENCEPHALOMYOCARDITIS VIRUS PE=1 SV=1
-13.584583	TLALSQVV	>GI|9626693|REF|NP_056777.1| HYPOTHETICAL PROTEIN EMCVGP1 [ENCEPHALOMYOCARDITIS VIRUS]
-13.584583	TLALSQVV	>GI|25121612|REF|NP_740409.1| PROTEIN 3AB [ENCEPHALOMYOCARDITIS VIRUS]
-13.743453	ELGLAILV	SP|P26661|POLG_HCVJ8 GENOME POLYPROTEIN OS=HEPATITIS C VIRUS GENOTYPE 2B (ISOLATE HC-J8) PE=1 SV=3
-13.806443	VLALAPEV	>GI|139472812|REF|YP_001129361.1| ORF70 [HUMAN HERPESVIRUS 8]
-13.891999	EMAIPGQV	SP|Q03053|POLG_CXB5P GENOME POLYPROTEIN OS=COXSACKIEVIRUS B5 (STRAIN PETERBOROUGH / 1954/UK/85) PE=3 SV=3
-13.904892	VMSLSGKV	>GI|238801615|REF|YP_002922020.1| POLYPROTEIN [WESSELSBRON VIRUS]
-13.904892	VMSLSGKV	>GI|119952253|REF|YP_950478.1| POLYPROTEIN [SEPIK VIRUS]
-13.942427	FLGIPESV	SP|Q0GBX5|L_RABVD LARGE STRUCTURAL PROTEIN OS=RABIES VIRUS (STRAIN CHINA/DRV) GN=L PE=3 SV=1

8.7.1 Viral database results continued

Score	Peptide	Protein
-14.003154	ILALAPAV	>GI|9629287|REF|NP_044487.1| CAPSID TRIPLEX SUBUNIT 2 [HUMAN HERPESVIRUS 2]
-14.105484	TLALPSNV	1W9Z A[BANNA VIRUS]
-14.105484	TLALPSNV	>GI|23238122|REF|NP_694460.1| VP9 [BANNA VIRUS STRAIN JKT-6423]
-14.118223	TLALAPVV	>GI|9629398|REF|NP_044619.1| CAPSID TRIPLEX SUBUNIT 2 [HUMAN HERPESVIRUS 1]
-14.153498	VLAIALVV	>GI|52139283|REF|YP_081558.1| MEMBRANE GLYCOPROTEIN UL119 [HUMAN HERPESVIRUS 5]
-14.153498	VLAIALVV	SP|P16739|UL119_HCMVA VIRAL FC-GAMMA RECEPTOR-LIKE PROTEIN UL119 OS=HUMAN CYTOMEGALOVIRUS (STRAIN AD169) GN=UL119/UL118 PE=2 SV=2
-14.288697	AMAIAKSV	SP|P07298|VNCS_PAVHU NON-CAPSID PROTEIN NS-1 OS=HUMAN PARVOVIRUS B19 (ISOLATE AU) GN=NS1 PE=3 SV=1
-14.288697	AMAIAKSV	>GI|356457873|REF|YP_004928144.1| NON-STRUCTURAL PROTEIN NS1 [HUMAN PARVOVIRUS B19]
-14.288697	AMAIAKTV	>GI|23343516|REF|NP_694863.1| NS1 PROTEIN [HUMAN ERYTHROVIRUS V9]
-14.288697	AMAIAKSV	SP|Q9PZT1|NS1_PAVHV NON-STRUCTURAL PROTEIN 1 OS=HUMAN PARVOVIRUS B19 (STRAIN HV) GN=NS PE=1 SV=1
-14.353902	FLGLMCSV	>GI|139424540|REF|YP_001129506.1| BILF1 [HUMAN HERPESVIRUS 4 TYPE 2]
-14.474466	ALGIASLV	>GI|20260782|REF|NP_620108.1| POLYPROTEIN [LANGAT VIRUS]
-14.474466	ALGIASLV	>GI|25121533|REF|NP_740301.1| NONSTRUCTURAL PROTEIN NS4B [LANGAT VIRUS]
-14.488209	ALAISGHV	>GI|312164871|REF|YP_003896059.1| VP2 [GREAT ISLAND VIRUS]
-14.625151	ELAIPEAV	>GI|295413964|REF|YP_003587868.1| ORF1 [TORQUE TENO VIRUS 3]
-14.666174	ELGLGGRV	>GI|30984453|REF|NP_851885.1| CAPSID MATURATION PROTEASE [MACACINE HERPESVIRUS 1]
-14.666174	ELGLGGRV	>GI|83722594|REF|YP_443872.1| CAPSID MATURATION PROTEASE [PAPIINE HERPESVIRUS 2]
-14.674795	ALALAGGV	>GI|139424484|REF|YP_001129449.1| BPLF1 [HUMAN HERPESVIRUS 4 TYPE 2]
-14.729165	VLALGSFV	>GI|51557555|REF|YP_068389.1| ENVELOPE GLYCOPROTEIN E [SUID HERPESVIRUS 1]
-14.736338	EMSISTWV	SP|Q02512|VE1_HPV17 REPLICATION PROTEIN E1 OS=HUMAN PAPILLOMAVIRUS TYPE 17 GN=E1 PE=3 SV=2
-14.787583	FMSLAHCV	>GI|12085037|REF|NP_073439.1| 54R PROTEIN [YABA-LIKE DISEASE VIRUS]
-14.787583	FMSLAHCV	>GI|157939677|REF|YP_001497049.1| HYPOTHETICAL PROTEIN TANV_54R [TANAPOX VIRUS]
-14.901285	FLALMPTV	>GI|9625894|REF|NP_040142.1| RIBONUCLEOTIDE REDUCTASE SUBUNIT 1 [HUMAN HERPESVIRUS 3]
-14.910185	EMSLPPWV	>GI|56403980|REF|YP_145795.1| PA POLYMERASE SUBUNIT [THOGOTO VIRUS]
-14.925875	ILALGLLV	>GI|51557552|REF|YP_068386.1| ENVELOPE GLYCOPROTEIN G [SUID HERPESVIRUS 1]
-14.945093	VMAVSTCV	SP|P06437|GB_HHV1K ENVELOPE GLYCOPROTEIN B OS=HUMAN HERPESVIRUS 1 (STRAIN KOS) GN=GB PE=1 SV=2
-14.945093	VMAVSTCV	3NWA A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	4BOM A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	3NW8 A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	3NWF A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	SP|P06763|GB_HHV23 ENVELOPE GLYCOPROTEIN B OS=HUMAN HERPESVIRUS 2 (STRAIN 333) GN=GB PE=1 SV=1
-14.945093	VMAVSTCV	>GI|9629297|REF|NP_044497.1| ENVELOPE GLYCOPROTEIN B [HUMAN HERPESVIRUS 2]
-14.945093	VMAVSTCV	2GUM A[HUMAN HERPESVIRUS 1]

8.7.1 Viral database results continued

Score	Peptide	Protein
-14.945093	VMAVSTCV	4L1R A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	>GI|30984455|REF|NP_851887.1| ENVELOPE GLYCOPROTEIN B [MACACINE HERPESVIRUS 1]
-14.945093	VMAVSTCV	>GI|83722596|REF|YP_443874.1| ENVELOPE GLYCOPROTEIN B [PAPIINE HERPESVIRUS 2]
-14.945093	VMAVSTCV	SP|P24994|GB_HSV2S ENVELOPE GLYCOPROTEIN B OS=HERPES SIMPLEX VIRUS TYPE 2 (STRAIN SA8) GN=GB PE=3 SV=1
-14.945093	VMAVSTCV	SP|P06436|GB_HHV1F ENVELOPE GLYCOPROTEIN B OS=HUMAN HERPESVIRUS 1 (STRAIN F) GN=GB PE=1 SV=1
-14.945093	VMAVSTCV	4HSI A[HUMAN HERPESVIRUS 1]
-14.945093	VMAVSTCV	SP|P08665|GB_HHV1P ENVELOPE GLYCOPROTEIN B OS=HUMAN HERPESVIRUS 1 (STRAIN PATTON) GN=GB PE=3 SV=1
-14.945093	VMAVSTCV	>GI|9629408|REF|NP_044629.1| ENVELOPE GLYCOPROTEIN B [HUMAN HERPESVIRUS 1]
-14.949824	TLSLSLNV	SP|P35262|L_MABVP RNA-DIRECTED RNA POLYMERASE L OS=LAKE VICTORIA MARBURGVIRUS (STRAIN POPP-67) GN=L PE=3 SV=1
-14.949824	TLSLSLNV	SP|P31352|L_MABVM RNA-DIRECTED RNA POLYMERASE L OS=LAKE VICTORIA MARBURGVIRUS (STRAIN MUSOKE-80) GN=L PE=3 SV=2
-14.949824	TLSLSLNV	SP|Q1PD54|L_MABVA RNA-DIRECTED RNA POLYMERASE L OS=LAKE VICTORIA MARBURGVIRUS (STRAIN ANGOLA/2005) GN=L PE=3 SV=1
-14.949824	TLSLSLNV	>GI|678222048|REF|YP_009055228.1| RNA-DEPENDENT RNA POLYMERASE [MARBURG MARBURGVIRUS]
-14.949824	TLSLSLNV	SP|Q6UY63|L_MABVO RNA-DIRECTED RNA POLYMERASE L OS=LAKE VICTORIA MARBURGVIRUS (STRAIN OZOLIN-75) GN=L PE=3 SV=1
-15.021849	ALAIAYLV	SP|Q98803|POLG_YEFVI GENOME POLYPROTEIN OS=YELLOW FEVER VIRUS (ISOLATE IVORY COAST/85-82H/1982) PE=3 SV=1
-15.021849	ALAIAYLV	SP|Q1X880|POLG_YEFVU GENOME POLYPROTEIN OS=YELLOW FEVER VIRUS (ISOLATE UGANDA/A7094A4/1948) PE=3 SV=1
-15.021849	ALAIAYLV	SP|P29165|POLG_YEFV8 GENOME POLYPROTEIN (FRAGMENT) OS=YELLOW FEVER VIRUS (ISOLATE PERU/1899/1981) PE=3 SV=1
-15.021849	ALAIAYLV	SP|Q074N0|POLG_YEFVE GENOME POLYPROTEIN OS=YELLOW FEVER VIRUS (ISOLATE ETHIOPIA/COUMA/1961) PE=3 SV=1
-15.021849	ALAIAYLV	SP|Q1X881|POLG_YEFVN GENOME POLYPROTEIN OS=YELLOW FEVER VIRUS (ISOLATE ANGOLA/14FA/1971) PE=3 SV=1
-15.038671	GMGVSCTV	SP|P26028|HEMA_MEASI HEMAGGLUTININ GLYCOPROTEIN OS=MEASLES VIRUS (STRAIN IP-3-CA) GN=H PE=3 SV=1
-15.038671	GMGVSCTV	>GI|9626951|REF|NP_056923.1| HEMAGGLUTININ PROTEIN [MEASLES VIRUS]
-15.038671	GMGVSCTV	SP|P28081|HEMA_MEASY HEMAGGLUTININ GLYCOPROTEIN OS=MEASLES VIRUS (STRAIN YAMAGATA-1) GN=H PE=2 SV=2
-15.038671	GMGVSCTV	4GJT A[MEASLES VIRUS]
-15.038671	GMGVSCTV	2ZB5 A[MEASLES VIRUS STRAIN EDMONSTON-B]
-15.038671	GMGVSCTV	3INB A[MEASLES VIRUS STRAIN EDMONSTON]
-15.038671	GMGVSCTV	SP|P08362|HEMA_MEASE HEMAGGLUTININ GLYCOPROTEIN OS=MEASLES VIRUS (STRAIN EDMONSTON) GN=H PE=1 SV=1
-15.038671	GMGVSCTV	2RKC A[MEASLES VIRUS]
-15.038671	GMGVSCTV	SP|P35971|HEMA_MEASA HEMAGGLUTININ GLYCOPROTEIN OS=MEASLES VIRUS (STRAIN EDMONSTON-AIK-C VACCINE) GN=H PE=1 SV=1
-15.038671	GMGVSCTV	SP|P06830|HEMA_MEASH HEMAGGLUTININ GLYCOPROTEIN OS=MEASLES VIRUS (STRAIN HALLE) GN=H PE=1 SV=1
-15.082923	RLGISSIV	>GI|9625921|REF|NP_040169.1| TEGUMENT SERINE/THREONINE PROTEIN KINASE [HUMAN HERPESVIRUS 3]
-15.090857	VMGKSVLV	SP|Q8V0N6|POLG_HAV88 GENOME POLYPROTEIN OS=HUMAN HEPATITIS A VIRUS GENOTYPE IIB (ISOLATE SLF88) PE=3 SV=1
-15.090857	VMGLVGGV	>GI|83722596|REF|YP_443874.1| ENVELOPE GLYCOPROTEIN B [PAPIINE HERPESVIRUS 2]
-15.169798	GLGIGALV	SP|O70901|VPU_HV190 PROTEIN VPU OS=HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 GROUP M SUBTYPE H (ISOLATE 90CF056) GN=VPU PE=3 SV=1
-15.182156	YLSLSDPV	>GI|9629396|REF|NP_044618.1| DNA PACKAGING TEGUMENT PROTEIN UL17 [HUMAN HERPESVIRUS 1]
-15.213557	ELALGFKV	>GI|25777551|REF|NP_742092.1| ORF1AB [SIMIAN HEMORRHAGIC FEVER VIRUS]
-15.243933	VMGLSDDE	SP|Q67815|CAPSD_HASV6 CAPSID POLYPROTEIN VP90 OS=HUMAN ASTROVIRUS-6 GN=ORF2 PE=3 SV=1
-15.248486	TMGLLSIV	SP|P21040|C6_VACCC PROTEIN C6 OS=VACCINIA VIRUS (STRAIN COPENHAGEN) GN=C6L PE=3 SV=1
-15.248486	TMGLLSIV	>GI|66275819|REF|YP_232904.1| HYPOTHETICAL PROTEIN VACWR022 [VACCINIA VIRUS]
-15.248486	TMGLLSIV	>GI|9627530|REF|NP_042053.1| HYPOTHETICAL PROTEIN VARVGP009 [VARIOLA VIRUS]
-15.248486	TMGLLSIV	>GI|17974927|REF|NP_536441.1| D11L [MONKEYPOX VIRUS ZAIRE-96-I-16]

8.7.2 Self database results

Score	Peptide	Protein
-12.513169	VMGLPWFV	>gi|134288865|ref|NP_003606.3| sodium bicarbonate cotransporter 3 [Homo sapiens]
-12.513169	VMGLPWFV	sp|Q9Y6M7-3|S4A7_HUMAN Isoform 3 of Sodium bicarbonate cotransporter 3 OS=Homo sapiens GN=SLC4A7
-12.513169	VMGLPWFV	sp|Q9Y6M7-4|S4A7_HUMAN Isoform 4 of Sodium bicarbonate cotransporter 3 OS=Homo sapiens GN=SLC4A7
-12.513169	VMGLPWFV	sp|Q9Y6M7-2|S4A7_HUMAN Isoform 2 of Sodium bicarbonate cotransporter 3 OS=Homo sapiens GN=SLC4A7
-12.513169	VMGLPWFV	sp|Q9Y6M7-5|S4A7_HUMAN Isoform 5 of Sodium bicarbonate cotransporter 3 OS=Homo sapiens GN=SLC4A7
-12.525908	VMGLAAGV	sp|Q5JTC6-2|AMER1_HUMAN Isoform 2 of APC membrane recruitment protein 1 OS=Homo sapiens GN=FAM123B
-12.525908	VMGLAAGV	>gi|124244056|ref|NP_689637.3| protein FAM123B [Homo sapiens]
-12.539651	VMALSAVV	>gi|41056259|ref|NP_955361.1| solute carrier family 43 member 3 [Homo sapiens]
-12.709879	IMGLPWFV	>gi|90403614|ref|NP_001035049.1| electroneutral sodium bicarbonate exchanger 1 isoform a [Homo sapiens]
-12.709879	IMGLPWFV	>gi|295821221|ref|NP_001171486.1| sodium-driven chloride bicarbonate exchanger isoform 1 [Homo sapiens]
-12.709879	IMGLPWFV	>gi|90568034|ref|NP_004849.2| electroneutral sodium bicarbonate exchanger 1 isoform b [Homo sapiens]
-12.709879	IMGLPWFV	sp|Q2Y0W8-2|S4A8_HUMAN Isoform 2 of Electroneutral sodium bicarbonate exchanger 1 OS=Homo sapiens GN=SLC4A8
-12.709879	IMGLPWFV	>gi|295821223|ref|NP_001171487.1| sodium-driven chloride bicarbonate exchanger isoform 3 [Homo sapiens]
-12.709879	IMGLPWFV	>gi|155722998|ref|NP_071341.2| sodium-driven chloride bicarbonate exchanger isoform 2 [Homo sapiens]
-12.709879	IMGLPWFV	sp|Q2Y0W8-5|S4A8_HUMAN Isoform 5 of Electroneutral sodium bicarbonate exchanger 1 OS=Homo sapiens GN=SLC4A8
-12.709879	IMGLPWFV	sp|Q2Y0W8-4|S4A8_HUMAN Isoform 4 of Electroneutral sodium bicarbonate exchanger 1 OS=Homo sapiens GN=SLC4A8
-12.72542	VLGLSAAV	>gi|42741659|ref|NP_000918.2| multidrug resistance protein 1 [Homo sapiens]
-12.85143	TMALSVLV	>gi|28373107|ref|NP_777614.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform d [Homo sapiens]
-12.85143	TMALSVLV	>gi|28373113|ref|NP_777617.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform f [Homo sapiens]
-12.85143	TMALSVLV	>gi|24638454|ref|NP_733765.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform b [Homo sapiens]
-12.85143	TMALSVLV	>gi|28373105|ref|NP_777613.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform e [Homo sapiens]
-12.85143	TMALSVLV	>gi|10835220|ref|NP_004311.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 1 isoform a [Homo sapiens]
-12.85143	TMALSVLV	sp|Q93084-7|AT2A3_HUMAN Isoform SERCA3F of Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 OS=Homo sapiens GN=ATP2A3
-12.85143	TMALSVLV	>gi|28373109|ref|NP_777615.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform b [Homo sapiens]
-12.85143	TMALSVLV	sp|P16615-3|AT2A2_HUMAN Isoform 3 of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Homo sapiens GN=ATP2A2
-12.85143	TMALSVLV	>gi|4502285|ref|NP_001672.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform a [Homo sapiens]
-12.85143	TMALSVLV	>gi|27886529|ref|NP_775293.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 1 isoform b [Homo sapiens]
-12.85143	TMALSVLV	>gi|28373103|ref|NP_005164.2| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform a [Homo sapiens]
-12.85143	TMALSVLV	sp|P16615-4|AT2A2_HUMAN Isoform 4 of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Homo sapiens GN=ATP2A2
-12.85143	TMALSVLV	sp|P16615-5|AT2A2_HUMAN Isoform 5 of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Homo sapiens GN=ATP2A2
-12.85143	TMALSVLV	>gi|28373115|ref|NP_777618.1| sarcoplasmic/endoplasmic reticulum calcium ATPase 3 isoform c [Homo sapiens]
-12.860619	AMGLSLLV	>gi|153946391|ref|NP_000827.2| glutamate [NMDA] receptor subunit epsilon-4 precursor [Homo sapiens]

8.7.2 Self database results continued

Score	Peptide	Protein
-12.860619	AMGLSRAV	sp|Q8IZY2-2|ABCA7_HUMAN Isoform 2 of ATP-binding cassette sub-family A member 7 OS=Homo sapiens GN=ABCA7
-12.860619	AMGLSVAV	>gi|25952134|ref|NP_116279.2| protein dispatched homolog 1 [Homo sapiens]
-12.860619	AMGLSRAV	>gi|150417984|ref|NP_061985.2| ATP-binding cassette sub-family A member 7 [Homo sapiens]
-12.86222	FMGLPWYV	>gi|19743827|ref|NP_597812.1| electrogenic sodium bicarbonate cotransporter 4 isoform c [Homo sapiens]
-12.86222	FMGLPWYV	sp|Q9BY07-8|S4A5_HUMAN Isoform 8 of Electrogenic sodium bicarbonate cotransporter 4 OS=Homo sapiens GN=SLC4A5
-12.86222	FMGLPWYV	>gi|125987596|ref|NP_067019.3| electrogenic sodium bicarbonate cotransporter 4 isoform a [Homo sapiens]
-12.86222	FMGLPWYV	sp|Q9BY07-4|S4A5_HUMAN Isoform 4 of Electrogenic sodium bicarbonate cotransporter 4 OS=Homo sapiens GN=SLC4A5
-12.86222	FMGLPWYV	sp|Q9BY07-7|S4A5_HUMAN Isoform 7 of Electrogenic sodium bicarbonate cotransporter 4 OS=Homo sapiens GN=SLC4A5
-12.86222	FMGLPWYV	sp|Q9BY07-2|S4A5_HUMAN Isoform 2 of Electrogenic sodium bicarbonate cotransporter 4 OS=Homo sapiens GN=SLC4A5
-12.872962	VMGIALAV	>gi|4502877|ref|NP_001296.1| claudin-4 [Homo sapiens]
-12.92213	ILGLSAAV	>gi|9961252|ref|NP_061338.1| multidrug resistance protein 3 isoform C [Homo sapiens]
-12.92213	ILGLSAAV	>gi|9961250|ref|NP_061337.1| multidrug resistance protein 3 isoform B [Homo sapiens]
-12.92213	ILGLSAAV	>gi|4505771|ref|NP_000434.1| multidrug resistance protein 3 isoform A [Homo sapiens]
-12.980283	GMGISNRV	>gi|4507925|ref|NP_003871.1| WNT1-inducible-signaling pathway protein 3 isoform 1 precursor [Homo sapiens]
-12.980283	GMGISTRV	sp|P29279|CTGF_HUMAN Connective tissue growth factor OS=Homo sapiens GN=CTGF PE=1 SV=2
-12.980283	GMGISTRV	sp|P29279-2|CTGF_HUMAN Isoform 2 of Connective tissue growth factor OS=Homo sapiens GN=CTGF
-12.980283	GMGISTRV	>gi|4503123|ref|NP_001892.1| connective tissue growth factor precursor [Homo sapiens]
-12.980283	GMGISNRV	>gi|38202241|ref|NP_937882.1| WNT1-inducible-signaling pathway protein 3 isoform 3 [Homo sapiens]
-13.0103	EMGLADVV	sp|Q5SNV9-2|CA167_HUMAN Isoform 2 of Uncharacterized protein C1orf167 OS=Homo sapiens GN=C1orf167
-13.0103	EMGLADVV	>gi|310113616|ref|XP_003119818.1| PREDICTED: uncharacterized protein C1orf167 [Homo sapiens]
-13.0103	EMGLADVV	>gi|310118524|ref|XP_003118897.1| PREDICTED: uncharacterized protein C1orf167 [Homo sapiens]
-13.0103	EMGLADVV	sp|Q5SNV9|CA167_HUMAN Uncharacterized protein C1orf167 OS=Homo sapiens GN=C1orf167 PE=2 SV=2
-13.0103	EMGLACVV	>gi|215599585|ref|NP_001135943.1| integrator complex subunit 12 [Homo sapiens]
-13.0372	TLGLSCGV	>gi|282403491|ref|NP_001164120.1| protein LAS1 homolog isoform 2 [Homo sapiens]
-13.0372	TLGLSCGV	>gi|282403493|ref|NP_001164121.1| protein LAS1 homolog isoform 3 [Homo sapiens]
-13.0372	TLGLSCGV	>gi|13654270|ref|NP_112483.1| protein LAS1 homolog isoform 1 [Homo sapiens]
-13.072474	VLGISAEV	sp|Q8TBF2|PGFS_HUMAN Prostamide/prostaglandin F synthase OS=Homo sapiens GN=FAM213B PE=2 SV=1
-13.072474	VLGISRDV	sp|Q460N5-1|PAR14_HUMAN Isoform 1 of Poly [ADP-ribose] polymerase 14 OS=Homo sapiens GN=PARP14
-13.072474	VLGISAEV	sp|Q8TBF2-4|PGFS_HUMAN Isoform 4 of Prostamide/prostaglandin F synthase OS=Homo sapiens GN=FAM213B
-13.072474	VLGISAEV	>gi|307691194|ref|NP_001182670.1| prostamide/prostaglandin F synthase isoform f [Homo sapiens]
-13.072474	VLGISRDV	sp|Q460N5-4|PAR14_HUMAN Isoform 4 of Poly [ADP-ribose] polymerase 14 OS=Homo sapiens GN=PARP14
-13.072474	VLGISRDV	sp|Q460N5-3|PAR14_HUMAN Isoform 3 of Poly [ADP-ribose] polymerase 14 OS=Homo sapiens GN=PARP14
-13.072474	VLGISAEV	>gi|307691186|ref|NP_001182666.1| prostamide/prostaglandin F synthase isoform c [Homo sapiens]
-13.072474	VLGISLTV	sp|Q12967-2|GNDS_HUMAN Isoform 2 of Ral guanine nucleotide dissociation stimulator OS=Homo sapiens GN=RALGDS

8.7.2 Self database results continued

Score	Peptide	Protein
-13.072474	VLGISRDV	>gi|154813199|ref|NP_060024.2| poly [ADP-ribose] polymerase 14 [Homo sapiens]
-13.072474	VLGISAEV	>gi|307691184|ref|NP_001182665.1| prostamide/prostaglandin F synthase isoform a [Homo sapiens]
-13.072474	VLGISAEV	>gi|307691182|ref|NP_689584.2| prostamide/prostaglandin F synthase isoform b [Homo sapiens]
-13.072474	VLGISRDV	sp|Q460N5-5|PAR14_HUMAN Isoform 5 of Poly [ADP-ribose] polymerase 14 OS=Homo sapiens GN=PARP14
-13.072474	VLGISAEV	sp|Q8TBF2-3|PGFS_HUMAN Isoform 3 of Prostamide/prostaglandin F synthase OS=Homo sapiens GN=FAM213B
-13.072474	VLGISAEV	>gi|307691192|ref|NP_001182669.1| prostamide/prostaglandin F synthase isoform e [Homo sapiens]
-13.074471	FLGLSPHV	>gi|22907034|ref|NP_659434.2| folliculin isoform 1 [Homo sapiens]
-13.074471	FLGLSPHV	Folliculin Homo sapiens
-13.074471	FLGLSGLV	>gi|148886707|ref|NP_001092142.1| hephaestin-like protein 1 precursor [Homo sapiens]
-13.180612	GMALSVLV	>gi|4507017|ref|NP_001851.1| probable low affinity copper uptake protein 2 [Homo sapiens]
-13.180612	GMALSKGV	>gi|53933282|ref|NP_001005518.1| olfactory receptor 6C65 [Homo sapiens]
-13.246321	VLGLPQHV	>gi|312176416|ref|NP_001185901.1| uncharacterized protein KIAA1522 isoform 2 [Homo sapiens]
-13.246321	VLGLPQHV	>gi|112734870|ref|NP_065939.2| uncharacterized protein KIAA1522 isoform 1 [Homo sapiens]
-13.246321	VLGLPQHV	sp|Q9P206-3|K1522_HUMAN Isoform 3 of Uncharacterized protein KIAA1522 OS=Homo sapiens GN=KIAA1522
-13.25906	VLGLAVRV	>gi|63175654|ref|NP_659496.2| L-fucose kinase [Homo sapiens]
-13.25906	VLGLAVRV	sp|Q8N0W3-2|FUK_HUMAN Isoform 2 of L-fucose kinase OS=Homo sapiens GN=FUK
-13.25906	VLGLASIV	>gi|46249410|ref|NP_004735.2| lecithin retinol acyltransferase precursor [Homo sapiens]
-13.25906	VLGLAAEV	>gi|28212272|ref|NP_777573.1| pumilio domain-containing protein C14orf21 [Homo sapiens]
-13.269185	ILGISGCV	>gi|62865631|ref|NP_001017372.1| long-chain fatty acid transport protein 6 [Homo sapiens]
-13.272803	VLALSTEV	>gi|195947374|ref|NP_001124333.1| claudin-5 [Homo sapiens]
-13.272803	VLALSTEV	sp|O00501|CLD5_HUMAN Claudin-5 OS=Homo sapiens GN=CLDN5 PE=1 SV=1
-13.366381	GLGLSGVV	>gi|189095248|ref|NP_001121159.1| adiponectin receptor protein 1 [Homo sapiens]
-13.366381	GLGLSTPV	sp|Q5SV97|CA170_HUMAN Uncharacterized protein C1orf170 OS=Homo sapiens GN=C1orf170 PE=2 SV=3
-13.371097	EMAISKTV	>gi|42764687|ref|NP_073143.2| dual specificity protein phosphatase 6 isoform b [Homo sapiens]
-13.371097	EMAISKTV	>gi|42764683|ref|NP_001937.2| dual specificity protein phosphatase 6 isoform a [Homo sapiens]
-13.371097	EMAISKTV	DUAL SPECIFICITY PROTEIN PHOSPHATASE 6 Homo sapiens
-13.38152	AMGLPEAV	>gi|29789287|ref|NP_203755.1| inositol 1,4,5-triphosphate receptor-interacting protein precursor [Homo sapiens]
-13.394259	AMGLALYV	>gi|4504079|ref|NP_003792.1| glycosylphosphatidylinositol anchor attachment 1 protein [Homo sapiens]
-13.394259	AMGLALYV	sp|O43292-2|GPAA1_HUMAN Isoform 2 of Glycosylphosphatidylinositol anchor attachment 1 protein OS=Homo sapiens GN=GPAA1
-13.394259	AMGLALLV	>gi|55770854|ref|NP_000826.2| glutamate [NMDA] receptor subunit epsilon-3 precursor [Homo sapiens]
-13.408002	AMALSGHV	>gi|13899269|ref|NP_113627.1| transcription factor SOX-7 [Homo sapiens]
-13.421525	FLGISIGV	sp|O95859-2|TSN12_HUMAN Isoform 2 of Tetraspanin-12 OS=Homo sapiens GN=TSPAN12
-13.421525	FLGISIGV	>gi|6912528|ref|NP_036470.1| tetraspanin-12 [Homo sapiens]
-13.422342	FMALANGV	>gi|195927044|ref|NP_001124313.1| RNA 3'-terminal phosphate cyclase isoform a [Homo sapiens]

8.8. TNF capture assay results – Raw data

8.8.1 Multiple sclerosis

1. LS20460

LS20460 $CD4^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
13	CASSPGQVGGAF	1-1	29.23	19
7-3	CASSIRGSRGELF	2-2	21.54	14
6-1	CASSEGPSRVSYEQY	2-7	20.00	13
11-3	CASSLRGLAGSYEQY	2-7	10.77	7
20-1	CSAGVGGYEQY	2-7	9.23	6
7-3	CASSNTGDTEAF	1-1	3.08	2
11-3	CASSLGGGNYNEQF	2-1	1.54	1
15	CATSEDRAYNEQF	2-1	1.54	1
7-2	CASRTAVSTGELF	2-2	1.54	1
29-1	CSVEGGAETQY	2-5	1.54	1
			100	65

LS20460 $CD8^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSLQGANYEQY	2-7	41.56	32
19	CASRTTSGSYNEQF	2-1	18.18	14
7-3	CASRLTSGGTDTQY	2-3	18.18	14
7-9	CASSSRQARRHEQY	2-7	9.09	7
13	CASSPGQVGGAF	1-1	3.90	3
24-1	CATSDSRESGARETQY	2-5	2.60	2
19	CASTMTSGSLWEQY	2-7	1.30	1
19	CASSPTSGAFNEQF	2-1	1.30	1
27	CASSLQGAKYEQY	2-7	1.30	1
27	CASSLQGAKNEQY	2-7	1.30	1
27	CASSLQGAKDEQY	2-7	1.30	1
			100	77

2. EB21570

EB21570 CD4^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
5-4	CASSTPGQPNTGELF	2-2	70.42	50
7-2	CASSSWTSGRTDTQY	2-3	29.58	21
			100	71

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EB21570 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
5-6	CASSAYRGPNTGELF	2-2	21.13	15
2	CASSDPGGPGNEQF	2-1	14.08	10
5-6	CASSLRQGADTQY	2-3	14.08	10
7-3	CASSSETYSTDTQY	2-3	11.27	8
28	CASSSGQDPFYEQY	2-7	8.45	6
7-2	CASSLGGGQGLDWTEAF	1-1	5.63	4
5-6	CASSLGGRAGSRTDTQY	2-3	4.23	3
12-3/12-4	CASSLGDGGLIYNEQF	2-1	4.23	3
19	CASRDGPSYEQY	2-7	4.23	3
12-3/12-4	CASSFGDGGVIYNEQF	2-1	1.41	1
30	CAWSQIRRPYNEQF	2-1	1.41	1
27	CASSLFTGDRSGQY	2-7	1.41	1
7-9	CASSSTTAGDQPQH	1-5	1.41	1
5-5	CASSLYTSGSNEQF	2-1	1.41	1
20-1	CSARGEGLSYEQY	2-7	1.41	1
19	CASTFGQAGEAF	1-1	1.41	1
12-3/12-4	CASSLAYNEQF	2-1	1.41	1
7-9	CASSSGPDEQF	2-1	1.41	1
			100	71

EB21570 matching CD8^{+} CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASTPSGANVLT	2-6	36.99	27
27	CASSFGGLEKLF	1-4	8.22	6
7-2	CASSLGGGQGLDWTEAF	1-1	6.85	5
11-2	CASSPYPSGRDVEQF	2-1	6.85	5
28	CASSLRLYEQY	2-7	6.85	5
5-5	CASSVVGALNQY	2-4	5.48	4
7-9	CASSLVERAEAF	1-1	4.11	3
7-3	CASSLTTNTEAF	1-1	4.11	3
5-5	CASSLTETGFNQPQH	1-5	2.74	2
28	CASTPRGGGYQPQH	1-5	2.74	2
6-2/6-3	CASSYVGLAEETQY	2-5	2.74	2
7-9	CASRGGRDAEKLF	1-4	2.74	2
6-6	CASLDGSTNEKLF	1-4	2.74	2
20-1	CSATDLASHQETQY	2-5	1.37	1
7-9	CASSDQDKGTDTQY	2-3	1.37	1
9	CASSFGTGNTEAF	1-1	1.37	1
20-1	CSARGRGVQPQH	1-5	1.37	1
29-1	CSVRGLAGVQY	2-7	1.37	1
			100	73

3. LH18836 (CD4+ only)

LH18836 CD4+TNF+

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3/12-4	CASSLRGGELF	2-2	53.75	43
12-3/12-4	CASRTGVNTEAF	1-1	21.25	17
6-1	CASTLGIGHEQY	2-7	12.50	10
9	CASSGEGKRLH	1-6	5.00	4
12-3/12-4	CASRRTLDSTYEQY	2-7	2.50	2
12-3/12-4	CASSLEGHRSYEQY	2-7	2.50	2
20-1	CSARAEGRETQY	2-5	1.25	1
12-3/12-4	CASSVRGGELF	2-2	1.25	1
			100	80

4. MW21576

MW21576 CD4+TNF+

TRBV	CDR3	TRBJ	Freq (%)	Count
18	CASSPRPQGSSYNSPLH	1-6	98.4	63
7-3	CASSLSSNQPQH	1-5	1.6	1
			100	64

MW21576 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
7-3	CASSPGQGQDEQY	2-7	33.8	26
7-3	CASSLGTGIYNEQF	2-1	29.9	23
3-1	CASSPSHRDIWDTQY	2-3	26.0	20
24-1	CATSDLPPTGDTGELF	2-2	7.8	6
5-5	CASSHRTSGSTDKQY	2-3	1.3	1
5-5	CASSQRTSGSTDTQY	2-3	1.3	1
			100	77

MW21576 matching CD8^{+} CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
7-3	CASSPGQGQDEQY	2-7	27.06	23
27	CASSGLGRREQY	2-7	21.18	18
6-2/6-3	CASSLGGTGWTEQF	2-1	10.59	9
20-1	CSAREAGELF	2-2	8.24	7
24-1	CATSDLPPTGDTGELF	2-2	4.71	4
13	CASSRPFGRPYNEQF	2-1	4.71	4
2	CASRQLAGGDNEQF	2-1	4.71	4
7-8	CASSLGQAYEQY	2-7	4.71	4
6-5	CASGSGYYGYT	1-2	4.71	4
29-1	CSARLAGDSTDTQY	2-3	3.53	3
3-1	CASSLLAGGLTDTQY	2-3	2.35	2
11-2	CASSLDPGWSAGGIAKNIQY	2-4	1.18	1
7-3	CASSPGQGQGEQY	2-7	1.18	1
14	CASSQAGIHGYT	1-2	1.18	1
			100	85

5. SA23376

SA23376 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
21-1	CASTPSRQGLIDIQY	2-4	55.6	35
20-1	CSARGNTIY	1-3	41.3	26
21-1	CASTPFRQGVIDIQY	2-4	1.6	1
21-1	CASTPSRQGMIDIQY	2-4	1.6	1
			100	63

SA23376 $CD8^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSEMGTGTFDGYT	1-2	68.7	46
7-9	CASSLNERLEQF	2-1	9.0	6
2	CASSVVVGELF	2-2	6.0	4
27	CASSWLSGGVRDTQY	2-3	4.5	3
4-2	CASSQDVAEQY	2-7	4.5	3
27	CASSWLSGGVRDAQY	2-3	3.0	2
4-1	CASSPGARLVDTQY	2-3	3.0	2
7-9	CASSYQPATGTDSYNEQF	2-1	1.5	1
			100	67

6. RM22664

RM22664 $CD4^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
3-1	CASSSLADYEQY	2-7	5.5	3
5-1	CASRSGTGTYEQY	2-7	5.5	3
7-9	CASSLAPGFSSGNTIY	1-3	3.6	2
6-6	CASSYNPSGGAYNEQF	2-1	3.6	2
28	CASSLGRRGSPLH	1-6	3.6	2
6-6	CASITWAANTEAF	1-1	3.6	2
6-5	CASNTRASNYGYT	1-2	3.6	2
4-2	CASSQLTGHYGYT	1-2	3.6	2
10-3	CAIRDSLGHEQF	2-1	3.6	2
6-2	CASSPSEVTTLH	1-6	3.6	2
6-6	CASSSLYNEQF	2-1	3.6	2
12-3	CASTTRWIRMGGTKNIQY	2-4	1.8	1
5-1	CASSIRTRGGHSNPIEQF	2-1	1.8	1
7-8	CASSFGEGGQSSGNTIY	1-3	1.8	1
5-5	CASSSPATTGGRTEAF	1-1	1.8	1
5-5	CASSSSATTGGRTEAF	1-1	1.8	1
3-1	CASSQVGGAAANYGYT	1-2	1.8	1
28	CASSFSLGSQNEKLF	1-4	1.8	1
20-1	CSASRRTSTYNSPLH	1-6	1.8	1
12-4	CASSPTDAGGGETQY	2-5	1.8	1
12-3	CASRQMSGAVTGELF	2-2	1.8	1
7-8	CASSLVTGTLTDTQY	2-3	1.8	1
5-1	CASSPGQGMLNNEAF	1-1	1.8	1
4-3	CASSLNPVGPYNEQF	2-1	1.8	1
11-2	CASSRDRTTSDGYT	1-2	1.8	1
7-2	CASSSPQGTGSPLH	1-6	1.8	1
7-2	CASSLEGRSNSPLH	1-6	1.8	1
7-2	CASSLGGARSGEAF	1-1	1.8	1
5-1	CASSLVDGEVDGYT	1-2	1.8	1
29-1	CSVVQRGIGTEAF	1-1	1.8	1
12-3	CASSFTGMNTEAF	1-1	1.8	1
7-2	CASSPGQSQETQY	2-5	1.8	1
6-5	CASRLARAYNEQF	2-1	1.8	1
6-2	CASSFGTPAYEQY	2-7	1.8	1
3-1	CASSQVPSSNEQF	2-1	1.8	1
30	CAWSPRQQETQY	2-5	1.8	1
20-1	CSARDRVFDTQY	2-3	1.8	1
7-8	CASSQGQGQPQH	1-5	1.8	1
28	CASSLGLYEQY	2-7	1.8	1
7-2	CASSLGNNEAF	1-1	3.6	2
18	CASSADSGQY	2-7	1.8	1
			100	55

RM22664 matching CD4^{+} CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSPLAASYNEQF	2-1	5.80	4
20-1	CSATGQGGGYGYT	1-2	4.35	3
7-7	CASSWGLAEETQY	2-5	4.35	3
7-2	CASSWGQGANGYT	1-2	4.35	3
24-1	CAAPGTGWYEQY	2-7	4.35	3
6-1	CASGKAPGEQY	2-7	4.35	3
16	CASSQQAGPSSGTQY	2-3	2.90	2
27	CASSLSISGRAEQY	2-7	2.90	2
7-2	CASSLTVLSTDTQY	2-3	2.90	2
6-2/6-3	CASSSARGNNSPLH	1-6	2.90	2
27	CASSKLAGRDTQY	2-3	2.90	2
12-3/12-4	CASSFSSSGNTIY	1-3	2.90	2
10-3	CAITRQGARNEQF	2-1	2.90	2
12-3/12-4	CASSLGSVYEQY	2-7	2.90	2
28	CASSFTNTIY	1-3	2.90	2
27	CASSSGIGQLPANYGYT	1-2	1.45	1
4-3	CASSQAPIGGAGQETQY	2-5	1.45	1
7-6	CASSQGGLAGATDTQY	2-3	1.45	1
29-1	CSVPGTGEKFNYGYT	1-2	1.45	1
28	CASSPPGSPYQETQY	2-5	1.45	1
15	CATSRNPHRGQETQY	2-5	1.45	1
11-2	CASSSRAATGVYEQF	2-1	1.45	1
9	CASSLTSGGVQETQY	2-5	1.45	1
27	CASSLRGVVQDTQY	2-3	1.45	1
7-6	CASSPGAGSADTQY	2-3	1.45	1
7-2	CASNPLAASYNEQF	2-1	1.45	1
6-2/6-3	CASRLGTGRGNEQF	2-1	1.45	1
6-1	CASKPGASYFEKLF	1-4	1.45	1
5-1	CASSSSTGRQETQY	2-5	1.45	1
29-1	CSVVQRGIGTEAF	1-1	1.45	1
28	CASSFWAAQETQY	2-5	1.45	1
20-1	CSAREPGRSTEAF	1-1	1.45	1
12-3/12-4	CASSLSGTGNTIY	1-3	1.45	1
12-3/12-4	CASRKGRRNTEAF	1-1	1.45	1
10-3	CAVTRQGARNEQF	2-1	1.45	1
29-1	CSVDGTGGVEAF	1-1	1.45	1
20-1	CSVSGTNTDTQY	2-3	1.45	1
20-1	CSSPGDTAYGYT	1-2	1.45	1
12-3/12-4	CASSWDRTYEQY	2-7	1.45	1
12-3/12-4	CASGGNQVNTQY	2-3	1.45	1
5-6	CASSLAGRYEQY	2-7	1.45	1
5-1	CASSDRGAHEQY	2-7	1.45	1
12-3/12-4	CASSPDRYEQY	2-7	1.45	1
12-3/12-4	CASSFRNQPQH	1-5	1.45	1
2	CASRWNQGMQY	2-5	1.45	1
29-1	CSARGNTEAF	1-1	1.45	1
18	CASQTNTEAF	1-1	1.45	1
			100	69

RM22664 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSSGTTKDSPLH	1-6	24.5	13
7-9	CASRRDGGLTEAF	2-5	13.2	7
9	CASSVGTDGTNEKLF	1-4	9.4	5
28	CASRIQEEETQY	1-1	9.4	5
11-2	CASTLGAHNEQF	2-1	7.5	4
20-1	CSARGLAGGHPYEQY	2-7	5.7	3
27	CASSGQGSRYEQY	2-7	5.7	3
7-9	CASSLADGPTEAF	1-1	5.7	3
7-3	CASSTLRARFSNQPQH	1-5	3.8	2
4-3	CASSLNPVGPYNEQF	2-1	3.8	2
11-2	CASSLAAAVEAF	1-1	3.8	2
25-1	CASSELGGDLYEQY	2-7	1.9	1
7-9	CASSTDRGLGGEQF	2-1	1.9	1
27	CASSGQASSYEQY	2-7	1.9	1
7-9	CASSLVQGGPGYT	1-2	1.9	1
			100	53

RM22664 matching CD8^{+} CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSPGQAYEQY	2-7	8.20	5
9	CASSAMTSGGADTQY	2-3	4.92	3
7-7	CASSNEQGLSTDTQY	2-3	4.92	3
4-1	CASTRGTSSYNSPLH	1-6	4.92	3
27	CASSSRDSSGNTIY	1-3	4.92	3
7-9	CASSLSALGNEQF	2-1	4.92	3
27	CASTPGQGYEQY	2-7	4.92	3
28	CASSFEGTSGGTDTQY	2-3	3.28	2
7-2	CASSLGGTGPFNSPLH	1-6	3.28	2
29-1	CSVGTGGTNEKLF	1-4	3.28	2
28	CASSFSTDVGGYT	1-2	3.28	2
7-2	CASSVGTEYNEQF	2-1	3.28	2
6-5	CASRASGSSYEQY	2-7	3.28	2
6-2/6-3	CASSPGIYTYEQY	2-7	3.28	2
4-1	CASSLPGDPYEQY	2-7	3.28	2
29-1	CSVETGVVEAF	1-1	3.28	2
6-2/6-3	CASSSILQGLDTGELF	2-2	1.64	1
4-3	CASSHPTPAGSTDTQY	2-3	1.64	1
4-3	CASSHDTPGGRTDTQY	2-3	1.64	1
28	CASSLTDGRLNQPQH	1-5	1.64	1
27	CASSLDGRALHQPQH	1-5	1.64	1
5-1	CASSLGQGRFTDTQY	2-3	1.64	1
20-1	CSARGLSVRNTEAF	1-1	1.64	1
2	CASSEALRTPYGHT	1-2	1.64	1
30	CAWSLGQPTGELF	2-2	1.64	1
27	CASRTHRASDEQY	2-7	1.64	1
12-3/12-4	CASSPGTGGHEQF	2-1	1.64	1
9	CASSPSGVQETQY	2-5	1.64	1
4-1	CASSQGSEGFEQY	2-7	1.64	1
11-2	CASTWGAHNEQF	2-1	1.64	1
11-2	CASTLGAHNEQF	2-1	1.64	1
7-2	CASSAGRGTTF	1-1	1.64	1
6-6	CASSYRRAEAF	1-1	1.64	1
5-6	CASSLRGNEQF	2-1	1.64	1
6-2/6-3	CASSLSYEQY	2-7	1.64	1
6-5	CASTADTQY	2-3	1.64	1
			100	61

7. HD21265

HD21265 $CD4^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
28	CASSLLSGGDVRHEQY	2-7	28.1	16
4-1	CASSRRDTTNYGYT	1-2	19.3	11
7-2	CASSLRTQPPGELF	2-2	10.5	6
10-3	CAISESVRDGGYT	1-2	7.0	4
5-1	CASSFEWGAGADTQY	2-3	5.3	3
6-6	CASSTGIESRGYT	1-2	5.3	3
20-1	CSARQGVNQPQH	1-5	3.5	2
18	CASSSTGGEYAF	1-1	3.5	2
30-1	CAWSTGGYGYT	1-2	3.5	2
12-3	CASSPPWASGHNEQF	2-1	1.8	1
9	CASSVGGTGVYEQY	2-7	1.8	1
7-2	CASTFSGDVVGIQY	2-4	1.8	1
7-2	CASTLSGDVVGIQY	2-4	1.8	1
4-1	CASSPSVGFAYGYT	1-2	1.8	1
6-5	CSVGGQGAYNEQF	2-1	1.8	1
6-2	CASSYGVPGELF	2-2	1.8	1
5-1	CASSLGPYEQY	2-7	1.8	1
			100	57

HD21265 $CD8^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
6-2	CASSESFSRGQETQY	2-5	24.3	18
27	CASSNRPRDYRSYNEQF	2-1	20.3	15
7-8	CASSITGRYYGYT	1-2	18.9	14
4-1	CASSQDRVGTTLSNQPQH	1-5	12.2	9
4-2	CASSQDSSGGPSSYEQY	2-7	6.8	5
4-2	CASMAGGSSSGANVLT	2-6	4.1	3
5-1	CASSGMGRGTEAF	1-1	4.1	3
6-6	CASSYREEQF	2-1	4.1	3
7-9	CASSAFNSPLH	1-6	2.7	2
2	CASSDVGTEAF	1-1	2.7	2
			100	74

8. MH21407 ($CD4^+$ only)

MH21407 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSASGLSYNEQF	2-1	35.3	24
10-2	CASNSKGYNSPLH	1-6	23.5	16
12-3	CASSPLEGLELF	1-4	16.2	11
7-9	CASSPDRGFGPAYGYT	1-2	8.8	6
6-2	CASRKLAGGTDSYNEQF	2-1	2.9	2
2	CASSDIGGGSSYEQY	2-7	2.9	2
5-1	CASSSLQGQSGGYT	1-2	2.9	2
7-3	CASSLTGDRLGTEAF	1-1	1.5	1
2	CASSGIGGGSSYEQY	2-7	1.5	1
7-9	CASSPPGLADNEQF	2-1	1.5	1
7-2	CASSLQGTGRFEQY	2-7	1.5	1
7-9	CASSSPHTDTQY	2-3	1.5	1
			100	68

9.CT25364 ($CD4^+$ only)

CT25364 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
9	CASSVASGGAYEQY	2-7	69.1	56
7-2	CASSETGGGGQPQH	1-5	19.8	16
2	CASSPGGGYSGNTIY	1-3	11.1	9
			100	81

8.8.2 Idiopathic Intracranial Hypertension

1. HS25204 (CD4$^+$ only)

HS25204 CD4$^+$TNF$^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASTKGRGGSPLH	1-6	45.8	27
7-9	CASSSREVLDEQY	2-7	15.3	9
27	CASSLKEITEAF	1-1	11.9	7
27	CASSLSGNEQF	2-1	11.9	7
5-1	CASSLERPSDTQY	2-3	6.8	4
18	CASSPRDTGELF	2-2	5.1	3
5-4	CASSPWDRGKDTQY	2-3	1.7	1
7-8	CASSLASNTGELF	2-2	1.7	1
			100	59

2. LH25311

LH25311 CD4$^+$TNF$^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
30	CAWSFTSSGANVLT	2-6	48.1	37
20-1	CSARATGWGETQY	2-5	44.2	34
21-1	CASSKEIKGCYGYT	1-2	3.9	3
6-4	CASSPQGGGDTQY	2-3	2.6	2
30	CAWSFTSSGVNVLT	2-6	1.3	1
			100	77

LH25311 CD8$^+$TNF$^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSARAGAYPKNIQY	2-4	77	62
7-6	CASSHMTGDEREQY	2-7	15	12
9	CASSVEGSYEQY	2-7	9	7
			100	81

LH25311 matching CD8$^+$ CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
7-6	CASSHMTGDEREQY	2-7	74.7	62
20-1	CSARAGAYPKNIQY	2-4	25.3	21
			100	83

3. SW25353

SW25353 CD4^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSARTANTGELF	2-2	98.9	87
7-2	CTSSARTANTGELF	2-2	1.1	1
			100	88

SW25353 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSLQGGNYGYT	1-2	70.9	56
13	CASSSRTGNTYEQY	2-7	12.7	10
27	CASSIQGSEAF	1-1	10.1	8
4-1	CASSQDRLAGRSEQF	2-1	3.8	3
6-7	CASSYWTGGHQPQH	1-5	2.5	2
			100	79

4. KA38079

KA38079 CD4^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSLNRGTSDLSTDTQY	2-3	97.8	44
7-2	CSSRINRGSNDLSTDTQY	2-3	2.2	1
			100	45

KA38079 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
15	CATSRELGGHEQF	2-1	43.8	21
5-6	CASSLGRDNRRSYEQY	2-7	18.8	9
5-1	CASSLEGQASSYEQY	2-7	18.8	9
13	CASSLTERETQY	2-5	12.5	6
6-1	CASSSRTGLTTEAF	1-1	4.2	2
7-6	CASSLSTDTQY	2-3	2.1	1
			100	48

5. ML25308 ($CD4^+$ only)

ML25308 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
6-2	CASTQIFRAATYLPYEQY	2-7	22.0	13
5-4	CASSSRLAGGPTDTQY	2-3	20.3	12
27	CASSPILPDRDQRGV	1-1	13.6	8
7-8	CASSHRVAGGPTDTQY	2-3	8.5	5
6-1	CASSLTPAGSSTDTQY	2-3	8.5	5
18	CASSPTSGGGEGEQY	2-7	6.8	4
6-2	CASSYSTSGGNEQF	2-1	5.1	3
6-2	CASSYRLGQGGSLDEQF	2-1	3.4	2
7-3	CASSLWGASGGDTQY	2-3	3.4	2
5-1	CASSLGQITDTQY	2-3	3.4	2
12-4	CASSFVAGRGPGSTDTQY	2-3	1.7	1
6-5	CASRARQGENYGYT	1-2	1.7	1
20-1	CSAKGQGYEQY	2-7	1.7	1
			100	59

6. RY21758 ($CD4^+$ only)

RY21758 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
10-2	CASSEDGMNTEAF	1-1	62.5	30
2	CASSSGFSYEQY	2-7	22.9	11
5-4	CASSSGQSNEKLF	1-4	10.4	5
7-9	CASSVTTHLAKNIQY	2-4	4.2	2
			100	48

7. RC41200 ($CD4^+$ only)

RC41200 $CD4^+TNF^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
30	CAWSPGYQETQYF	2-5	44.0	22
7-3	CASSALTGRMNTEAFF	1-1	16.0	8
6-2	CASSSPGGSTNEQFF	2-1	10.0	5
5-1	CASSSGTGQTTGELFF	2-2	8.0	4
12-4	CASSLYPPGQGRDGELFF	2-2	4.0	2
25-1	CASSIYRGTSTDTQYF	2-3	2.0	1
5-4	CASSSIGTPSYEQYF	2-7	2.0	1
6-5	THYRASSGINAEYF	2-7	2.0	1
5-1	CASKERAGTDTQYF	2-3	2.0	1
5-1	CASRGQGFDEQYF	2-7	2.0	1
7-2	CASSPLVDSPPHF	1-6	2.0	1
25-1	CASSRSSDRGYTF	1-2	2.0	1
15	CATSRNRQETQYF	2-5	2.0	1
20-1	CSAQRGQETQYF	2-5	2.0	1
			100	50

8.8.3 Other Neurological Diseases

1. SE29703 (CD8+ only)

SE29703 $CD8^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSFEGQASSYEQY	2-7	14.7	10
6-5	CASSLQGGNEQF	2-1	14.7	10
6-5	CASSYTETSGNEQF	2-1	11.8	8
4-1	CASSPRAGWDEQF	2-1	10.3	7
2	CASSDLGSGVSRIAKNIQY	2-4	10.3	7
7-9	CASSLTGPGDNEQF	2-1	7.4	5
7-3	CASSLIVSGGEQF	2-1	5.9	4
7-6	CASSLVIGEGVGEQF	2-1	4.4	3
10-3	CAISPGEGTQY	2-5	4.4	3
9	CASSVGIGAALNTEAF	1-1	2.9	2
7-9	CASSQRGTSGTTDTQY	2-3	2.9	2
29-1	CGVERGVGAGELF	2-2	4.4	3
7-3	CASSLVASGGWETQY	2-5	1.5	1
7-3	CASSYGQGQDTQY	2-3	1.5	1
6-5	CASRYRGGADGYT	1-2	1.5	1
12-3	CASSWGPAPEAF	1-1	1.5	1
			100	68

2. JG33488 ($CD4^{+}$ only)

JG33488 $CD4^{+}TNF^{+}$

TRBV	CDR3	TRBJ	Freq (%)	Count
5-1	CASSWTDTGELF	2-2	48.7	37
7-2	CASSPGPYEQF	2-1	22.4	17
24-1	CATSDSGYGYT	1-2	21.1	16
10-3	CAISEGAYSNQPQH	1-5	3.9	3
7-2	CASSLNRGTSDLSTDTQY	2-3	1.3	1
24-1	CASSDCEYGHT	1-2	1.3	1
24-1	CAASGSGQGYT	1-2	1.3	1
			100	76

3. AG20355 (CD8$^+$ only)

AG20355 CD8$^+$TNF$^+$ (N.B. CSF CD8$^+$ sort discarded because of contamination)

TRBV	CDR3	TRBJ	Freq (%)	Count
6-2	CASSPGTARYEQY	2-7	27.5	19
9	CASSFPEGGSSGNTIY	1-3	23.2	16
7-2	CASRETSGLRSEQY	2-7	20.3	14
28	CASTLGGGGAGETQY	2-5	8.7	6
28	CASSINRGADEQY	2-7	7.2	5
27	CAGKTQAAGDAFFTDTQY	2-3	2.9	2
7-2	CASSAPGRSVNNEQF	2-1	2.9	2
9	CASREGDPTDTQY	2-3	2.9	2
27	CASKTQAAGDAFFTDTQY	2-3	1.4	1
27	CASRISGAHNEQF	2-1	1.4	1
28	CASSWGIAYEQY	2-7	1.4	1
			100	69

4. JM25229

JM25229 CD4$^+$TNF$^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
12-3	CASSLGLYEQY	2-7	98.6	73
12-3	CAGSLGLYEQY	2-7	1.4	1
			100	74

JM25229 matching CD4$^+$ CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
20-1	CSAPASRGTGELF	2-2	41.3	33
12-3	CASSLGLYEQY	2-7	21.3	17
11-3	CASSGRAPRTQY	2-3	12.5	10
5-6	CASSLADQPQH	1-5	8.8	7
9	CASSVVGLSDTQY	2-3	5.0	4
7-8	CASSLDMQGINEKLF	1-4	3.8	3
3-1	CASSQDGASRDGTDTQY	2-3	2.5	2
12-3	CASSLGTGKADTQY	2-3	1.3	1
20-1	CSAPASRGAGELF	2-2	1.3	1
5-1	CASSLEGDYTEAF	1-1	1.3	1
5-6	CASSLMGVYEQY	2-7	1.3	1
			100	80

JM25229 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
27	CASSLWARATGELF	2-2	44	27
7-9	CASTQTGDSYGYT	1-2	26	16
5-5	CASSQRTSGSTDTQY	2-3	13	8
27	CASSPKGPRWQPQH	1-5	10	6
7-2	CASSLAYGRLHYGYT	1-2	5	3
28	CASESGTSGSRTDTQY	2-3	2	1
			100	61

5. AB25236

AB25236 CD4^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
3-1	CASSQLLAANEQF	2-1	20.9	9
20-1	CSARDPGQTYEQY	2-7	18.6	8
28	CASRRTGTDYGYT	1-2	18.6	8
18	CASSPLSLTYEQY	2-7	18.6	8
6-2	CASRDLEGILNEQF	2-1	9.3	4
20-1	CSANRGDTQY	2-3	4.7	2
7-2	CASSSPTALRGREQY	2-7	2.3	1
6-5	CASSYMLAGDTQY	2-3	2.3	1
30	CAWGGRGPEAF	1-1	2.3	1
20-1	CSARGDQPQH	1-5	2.3	1
			100	43

AB25236 CD8^{+}TNF^{+}

TRBV	CDR3	TRBJ	Freq (%)	Count
4-2	CASSPVTGSSGAEAF	1-1	56.7	17
7-3	CASSLTLLAGGPGTEAF	1-1	13.3	4
29-1	CSVVRQGAPGGYT	1-2	10.0	3
3-1	CASSQDNVVAGRAGHTDTQY	2-3	6.7	2
7-3	CASSLGVGNSPLH	1-6	6.7	2
7-3	CASSTKQGEYTEAF	1-1	3.3	1
5-1	CASSLELAGYGYT	1-2	3.3	1
			100	30

AB25236 JM25229 matching CD8$^+$ CSF

TRBV	CDR3	TRBJ	Freq (%)	Count
15	CATSRSRGASYEQY	2-7	26.6	17
6-2	CASSSWTGLGNTEAF	1-1	17.2	11
12-3	CASSFDVRGETQY	2-5	15.6	10
2	CASSEEAAKNQETQY	2-5	12.5	8
4-2	CASSLETGTAPEQY	2-7	7.8	5
6-2	CASIQGPETYEQY	2-7	7.8	5
5-1	CASSLELAGYGYT	1-2	6.3	4
27	CASSLHSGQGFYEQY	2-7	3.1	2
10-3	CAISARDGREDTEAF	1-1	1.6	1
7-9	CASSEGVRGYT	1-2	1.6	1
			100	64

6. CJ26014 (CD4$^+$ only)

CJ26014 CD4$^+$TNF$^+$

TRBV	CDR3	TRBJ	Freq (%)	Count
7-2	CASSVMESSYEQY	2-7	96.9	63
7-2	CASSVMESSYERY	2-7	1.5	1
12-4	CASSLGRGSGYT	1-2	1.5	1
			100	65

8.9 Overview of samples included for phenotyping, clonotyping and TNFα capture assay

	Phenotyping	Clonotyping		Vbeta		TNF capture	
		CD4	CD8	CD4	CD8	CD4	CD8
MS							
LC20552	Yes	Yes	Yes	Yes	Yes	No	No
LJ20639	Yes	Yes	Yes	Yes	Yes	No	No
LS20460	No	Yes	Yes	Yes	Yes	Yes	Yes
MJ19588	Yes	Yes	Yes	No	No	No	No
EB21510	Yes	Yes	Yes	Yes	Yes	Yes	Yes
KG19967	Yes	Yes	Yes	No	No	No	No
LH18836	Yes	Yes	Yes	Yes	Yes	Yes	No
NW21326	Yes	Yes	Yes	Yes	No	No	No
MW21576	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CS21983	Yes	Yes	Yes	No	No	No	No
AL22847	Yes	Yes	Yes	Yes	Yes	No	No
MK21405	Yes	Yes	Yes	No	No	No	No
SA23376	Yes	Yes	Yes	Yes	Yes	Yes	Yes
RM22664	Yes	Yes	Yes	Yes	Yes	Yes	Yes
TL22789	Yes	Yes	Yes	Yes	Yes	No	No
CT25364	Yes	Yes	No	No	No	Yes	No
HD21265	Yes	Yes	No	Yes	No	Yes	Yes
MH21407	Yes	Yes	Yes	Yes	Yes	Yes	No
RW21309	Yes	Yes	Yes	Yes	No	No	No
DD22299	No	No	No	No	No	No	No
CG41964	Yes	Yes	Yes	No	No	No	No
IIH							
EC21870	Yes	Yes	No	No	No	No	No
HS25204	Yes	Yes	No	Yes	No	Yes	No
LH25311	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ML25308	Yes	Yes	No	Yes	No	Yes	No
SW25353	Yes	Yes	Yes	Yes	Yes	Yes	Yes
RY21758	Yes	Yes	No	No	No	Yes	No
DC37877	Yes	No	No	No	No	No	No
ES37889	Yes	Yes	No	No	No	No	No
FC24414	Yes	No	No	No	No	No	No
VE25562	No	No	No	No	No	No	No
CC40712	Yes	Yes	Yes	Yes	Yes	No	No
RC41200	Yes	Yes	No	No	No	Yes	No
KA38079	Yes	No	Yes	No	No	Yes	Yes
CC41471	Yes	Yes	No	No	No	No	No
OND							
SE29703	No	Yes	Yes	Yes	Yes	No	Yes
JG33488	Yes	Yes	No	No	No	Yes	No
ND37140	Yes	Yes	Yes	Yes	Yes	No	No
DL37140	No	No	No	No	No	No	No
CS19395	Yes	Yes	No	No	No	No	No
AG20355	Yes	Yes	No	Yes	No	No	No
JM25229	Yes	Yes	Yes	No	No	Yes	Yes
AB25236	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CJ26014	Yes	Yes	Yes	No	No	Yes	No
JJ37566	No	No	No	No	No	No	No
JP24822	No	No	No	No	No	No	No

8.10 Publications and presentations arising from this thesis

Publications

Kalincik T, Brown JW, Robertson N, **Willis M**, Scolding N, Rice CM, Wilkins A, Pearson O, Ziemssen T, Hutchinson M, McGuigan C, Jokubaitis V, Spelman T, Horakova D, Havrdova E, Trojano M, Izquierdo G, Lugaresi A, Prat A, Girard M, Duquette P, Grammond P, Alroughani R, Pucci E, Sola P, Hupperts R, Lechner-Scott J, Terzi M, Van Pesch V, Rozsa C, Grand'Maison F, Boz C, Granella F, Slee M, Spitaleri D, Olascoaga J, Bergamaschi R, Verheul F, Vucic S, McCombe P, Hodgkinson S, Sanchez-Menoyo JL, Ampapa R, Simo M, Csepany T, Ramo C, Cristiano E, Barnett M, Butzkueven H, Coles A; MSBase Study Group. Treatment effectiveness of alemtuzumab compared with natalizumab, fingolimod, and interferon beta in relapsing-remitting multiple sclerosis: a cohort study. Lancet Neurol. 2017;16(4): 271-281.

Willis MD, Pickersgill TP, Robertson NP, Lee RW, Dick AD, Carreño E. Alemtuzumab-induced remission of multiple sclerosis-associated uveitis. Int Ophthalmol. 2016 Oct 11. [Epub ahead of print]

Willis MD, Harding KE, Pickersgill TP, Wardle M, Pearson OR, Scolding NJ, Smee J, Robertson NP. Alemtuzumab for multiple sclerosis: Long term follow-up in a multi-centre cohort. Mult Scler. 2016 Aug;22(9): 1215-23

Presentations

Willis MD, Ladell K, McLaren JE, Llewellyn-Lacey S, Miners K, Price DA, Robertson NP, Wooldridge L. Immunophenotyping of CSF-resident T cells in Multiple sclerosis. British Society of Immunology Annual Congress. Liverpool 2016.

Pace A, Rog D, Mihalova T, Sharaf N, Talbot P, Wilson M, **Willis M**, Robertson N. Alemtuzumab-induced idiopathic thrombocytopaenic purpura in three regional UK MS treatment centres. European Committee for treatment and research in multiple sclerosis. London 2016.

Healy S, Nasser T, **Willis MD**, Muller I, Harding K, Pickersgill T, Wardle M, Dayan C & Robertson NP. Outcomes of Thyroid Autoimmune Disease Following Alemtuzumab Treatment for Multiple Sclerosis. Association of British Neurologists Annual Meeting. Brighton 2016.

Willis M, Cossburn M, Ingram G, Pickersgill T, Barry S, Robertson N. Hypersensitivity pneumonitis following alemtuzumab treatment for multiple sclerosis. Association of British Neurologists Annual Meeting. Harrogate 2015

Williams O, Harding KE, **Willis M**, Pickersgill T, Wardle M, Robertson N. Disease modifying treatments in ms: induction or escalation? Association of British Neurologists Annual Meeting. Harrogate 2015.

Willis MD, Harding KE, Wardle M, Pickersgill T, Tomassini V & Robertson NP. Alemtuzumab: long term follow-up in a single centre cohort.
Association of British Neurologists Annual Meeting. Cardiff 2014.

Willis MD, Ladell K, McLaren JE, Llewellyn-Lacey S, Miners K, Clement M, Price DA, Robertson NP & Wooldridge L. CSF immunophenotyping in patients with neuroinflammatory disease. Association of British Neurologists Annual Meeting. Cardiff 2014.

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