Functional characterisation of multiple sclerosis-associated single nucleotide polymorphisms in the Eomesodermin region



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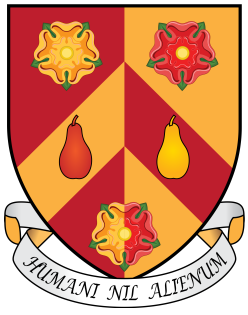
A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Oxford



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Abstract

Multiple sclerosis (MS) is a common inflammatory neurological disease affecting young adults, causing significant disability and increased mortality. Genome wide association studies (GWAS) have uncovered over 100 single nucleotide polymorphisms (SNPs) associated with MS susceptibility, most of which lie outside transcribed DNA and therefore do not have obvious consequences. SNPs in two such non-coding DNA segments upstream of the *EOMES* gene, encoding Eomesodermin (Eomes) have been consistently associated with MS, as well as with the two other autoimmune diseases rheumatoid arthritis (RA) and ankylosing spondylitis (AS). Eomes has been shown to be important for embryonic and neural development, as well as in the immune system, the latter of which was the focus of this investigation.

The association of genetic polymorphisms in the *EOMES* region with MS was interrogated using computational methods. Imputation analysis enabled detection of new variants, which aligned with regions of epigenetic significance and possible regions of DNA-interaction, implying an effect on regulation of gene expression.

Subsequently, Eomes expression was characterised in peripheral blood cell populations, with the highest expression detected in natural killer cells and effector/memory T cell subsets, including MAIT cells, an innate-like lymphocyte population. To investigate potential genotype dependent changes in Eomes expression and the effect on downstream pathways, studies were performed using samples from healthy donors prospectively genotyped for the two MS associated loci. Despite observing significant differences in expression between genotype groups in a pilot study, findings in a larger cohort did not reach the threshold for significance. Finally, the presence of MAIT cells was investigated in the context of MS and these cells were detected in MS lesions.

Collectively, in this D.Phil project I have illustrated potential ways in which the functional relevance of disease-associated SNPs in non-coding regions can be studied. This has been thoroughly investigated through computational studies, analysis of gene expression in peripheral blood cells, and in genotype-dependent studies of both expression and downstream function.

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List of abbreviations

3C	chromatin conformation capture
AIRE	autoimmune regulator
AITD	autoimmune thyroid disease
AL	acute lesions
APC	antigen presenting cell
APC	allophycocyanin
AS	ankylosing spondylitis
BBB	blood brain barrier
BCR	B cell receptor
CAL	chronic active lesion
CCR*x*	CC chemokine receptor x (where x is a number)
CXCR*x*	CXC chemokine receptor x (where x is a number)
CD	Crohn's disease
CDx	cluster of differentiation (where x is a number)
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CMC1	COX assembly mitochondrial protein 1
CNS	central nervous system
CMV	cytomegalovirus
CNV	copy number variant
CSF	cerebro-spinal fluid
Ct	threshold cycle
DC	dendritic cell
dCt	difference between Ct of gene of interest and housekeeping gene
DMT	disease modifying treatment
DN	double negative
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELF	ectopic lymphoid follicle
Eomes	Eomesodermin
eQTL	expression quantitative trait locus

FACS	fluorescence activated cell sorting
FAM	6-carboxyfluorescein
FasL	fas ligand
FcR	fc receptor
FITC	fluorescein isothyocyanate
FOXO1	forkhead box O1
FoxP3	forkhead box P3
g	standard gravitational unit of acceleration
GrzB	granzyme B
GWAS	genome-wide association study
Het	individual heterozygous for the alleles at a single nucleotide polymorphism
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
ICOS	inducible T cell co-stimulator
IFN-	interferon
IGAS	International Genetics of Ankylosing Spondylitis consortium
IM	infectious mononucleosis
Ig	immunoglobulin
IL-x	interleukin (where x is a number)
ILxR	interleukin x receptor (where x is a number)
IMSGC	International Multiple Sclerosis Genetics Consortium
Indel	insertion/deletion
LCL	lymphoblastoid cell line
LD	linkage disequilibrium
LFB	luxol fast blue
LPS	lipopolysaccharide
MAF	minor allele frequency
MAIT	mucosal-associated invariant T cells
MBP	myelin basic protein
mDC	myeloid dendritic cell
MEF	molecules of equivalent fluorochrome
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein

MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
N	number of individuals/replicates per group
NAWM	normal appearing white matter
NFκB	nuclear factor kappa B
NGS	next generation sequencing
NK	natural killer
(i)NKT	(invariant) natural killer T
OR	odds ratio
PAMP	pathogen associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed-death 1
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PerCP-Cy5.5	peridinin-chlorophyll-protein complex cyanine 5.5
Pfn	perforin
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PML	progressive multifocal leukoencephalopathy
Prot	individual(s) homozygous for the protective allele of a single nucleotide polymorphism
PPMS	primary progressive multiple sclerosis
RT-PCR	real-time quantitative polymerase chain reaction
RA	rheumatoid arthritis
RNA	ribonucleic acid
Rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RRMS	relapsing remitting multiple sclerosis
rs237	the rs2371108 SNP
rs669	the rs669607 SNP
SEM	standard error of the mean
S1P	sphingosine-1-phosphate
Seq	sequencing

SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SPMS	secondary progressive multiple sclerosis
STAT	signal transducer and activator of transcription
Stim	stimulated
TAMRA	tetramethylrhodamine
TEC	thymic epithelial cell
T1D	type 1 diabetes
TCF-1	T cell factor 1
TCR	T cell receptor
Th	helper T cell
Tn	naïve T cell
Tcm	central memory T cell
Tem	effector memory T cell
Temra	terminally differentiated effector memory CD45RA-expressing T cell
Tfh	follicular helper T cell
TLR	toll-like receptor
Tm	melting temperature
Treg	regulatory T cell
Trm	resident memory T cell
TNF-α	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
TNFRSF1A	tumour necrosis factor receptor superfamily 1A
TSA	tissue specific antigen
TSS	transcription start site
UBC	ubiquitin-C
UC	ulcerative colitis
UTR	untranslated region
UV	ultraviolet
WIMM	Weatherall Institute of molecular medicine
WTCCC	Wellcome Trust Case Control Consortium
WTCHG	Wellcome Trust Centre for Human Genetics, Oxford

Chapter 1

Introduction

1.1 Multiple Sclerosis

1.1.1 Epidemiology

Multiple sclerosis (MS) was first described as "la sclerose en plaques" by Jean-Martin Charcot in the 19th century. It is a highly complex disease of the central nervous system (CNS), comprising autoimmune, inflammatory and neurodegenerative components with early inflammatory demyelination and gradually accumulating axonal loss. One of the most common central nervous system diseases, affecting up to 2.5 million people worldwide, it has a prevalence of approximately 1 in 1000 in the UK, rising to approximately 3 in 1000 in some areas such as the Shetland Islands. The incidence is greater in women than men, with a ratio of roughly 2:1 (Compston 2005). A recent study suggested that the incidence of MS in the UK is falling, although the prevalence is increasing due to lower all-cause mortality rates (Mackenzie et al. 2014). With peak onset in the third and fourth decades, MS is a leading cause of disability in young people worldwide, with consequent impact on employment, earnings and quality of life (Kobelt et al. 2006, Wiberg et al. 2015). Furthermore, the mortality rate is significantly increased in MS patients compared with the general population, with an average reduction in life expectancy of between 5 and 15 years. Although median survival from diagnosis is lower in progressive forms, due to the later onset overall survival does not differ significantly from relapsing-remitting disease (Bronnum-Hansen et al. 1994, Scalfari et al. 2013, Jick et al. 2014).

1.1.2 Clinical features

MS is highly variable in both course and symptomatology. The vast majority of patients, around 85%, exhibit from the outset a relapsing and remitting form of the disease (RRMS), in which nervous system function is affected during isolated attacks lasting a few weeks which resolve to leave little, if any, permanent disability (Palace 2001, Coles 2009). These attacks are accompanied by the appearance of lesions on MRI scans of the CNS (Renowden 2014). Between attacks the patient's condition remains stable, although with increasing disease duration, ever-greater degrees of residual disability may persist on resolution of the attacks. The majority of these patients will eventually enter a progressive phase of the disease termed secondary progressive MS (SPMS), in which disability gradually accumulates without resolution. 15-20% of MS patients have a progressive form of the disease from the outset, termed primary progressive MS (PPMS), and a small number of patients show a progressive-relapsing form of disease, where relapses and periods of remission are superimposed on a background of gradually increasing disability (Coles 2009) (Figure 1.1).

Although the lesions of MS, described below, have a predilection for the periventricular white matter, any region of the CNS can be affected, with a wide range of possible symptoms. The most common syndromes on presentation are optic neuritis due to optic nerve inflammation, sensory loss and/or weakness due to spinal cord involvement, and double vision/cranial nerve signs due to brainstem lesions (Miller et al. 2012). However, cerebellar, autonomic and cognitive function may be affected from the outset or may become involved with increasing disease duration and lesion load as well as visual and sensorimotor pathways. This range of possible symptoms may cause a degree of diagnostic difficulty, and in this regard the development of MRI scanning has had a huge impact. The incorporation of MRI findings into diagnostic criteria (Table 1.1), consequent to the ability to discriminate between lesions of different ages on a single

MRI scan, has enabled diagnosis at a patient's first presentation (Polman et al. 2011). Differentiation of RRMS from its mimics depends on the demonstration of CNS lesions disparate in time and space; the coexistence of lesions that enhance after the injection of gadolinium contrast with those that do not enhance but are visible on non-contrast sequences elegantly portrays an inflammatory process both contemporaneous and historic.

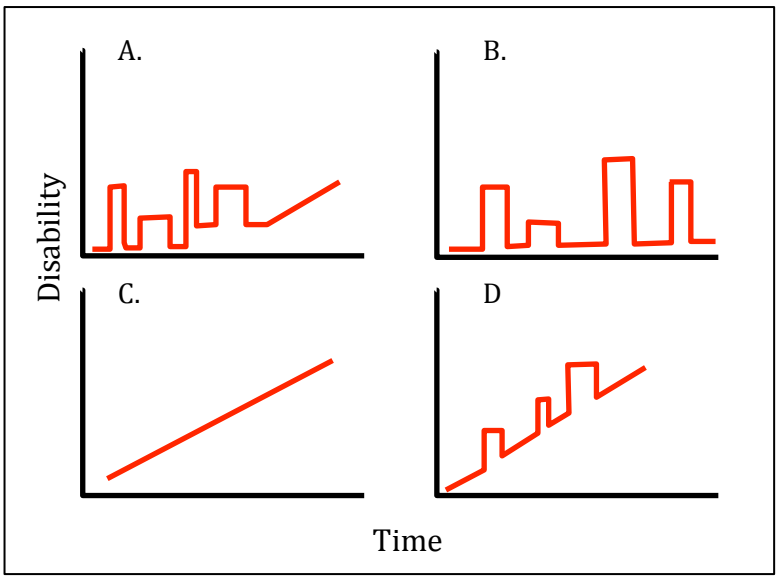


Fig 1.1 Patterns of MS. A. Initial relapsing remitting followed by secondary progressive phase. B. Benign relapsing remitting. C. Primary progressive. D. Relapsing progressive.

Clinical presentation	Additional information required for MS diagnosis
≥2 attacks with clinical evidence of ≥2 lesions; or clinical evidence of 1 lesion with historical evidence of prior attack	None
≥2 attacks; clinical evidence of 1 lesion	DIS: ≥1 MRI T2 lesion in at least 2 MS typical areas
1 attack; clinical evidence of ≥2 lesions	DIT: simultaneous presence of Gad-enhancing and non-enhancing lesions; or new T2 or Gad-enhancing lesions on follow-up MRI
1 attack; clinical evidence of 1 lesion (clinically isolated syndrome)	DIS **and** DIT as above
Insidious neurological progression suggestive of PPMS	1 year of disease progression plus 2 of 1. Evidence for DIS in the brain: ≥1 MRI T2 lesion in MS-typical areas 2. Evidence for DIS in spinal cord: ≥2 T2 lesions 3. OCBs in CSF

Table 1.1. McDonald criteria for diagnosis of multiple sclerosis. DIS: dissemination in space; DIT: dissemination in time; MRI: magnetic resonance imaging; OCBs: oligoclonal bands; CSF: cerebrospinal fluid; PPMS: primary progressive multiple sclerosis; Gad: Gadolinium

1.1.3 Treatment

Treatment of multiple sclerosis can be divided into acute treatment of relapses, predominantly with high-dose corticosteroids, and treatments aimed at ameliorating the disease course, termed disease-modifying treatments (DMTs). The development of the majority of DMTs has been predicated on the concept of MS as a primary autoimmune disease, the hypothesis being that preventing relapses and reducing inflammatory activity would result in less disability. While there has been great success in reducing the number of relapses that patients suffer, this has not translated into an equivalent reduction of disability later in the disease course for the most commonly used treatments (Confavreux et al. 2000, Confavreux et al. 2006, Leray et al. 2010). Furthermore, there is a lack of treatments that slow or prevent accumulation of disability for patients with progressive forms of the disease. In recent years there has been a greater focus on developing neuroprotective treatments, but in comparison to the burgeoning number of approved immunological DMTs progress has been slow. In addition, MS treatment has historically not been started until the second relapse, and there is speculation that commencing powerful anti-inflammatory treatment early in the disease course may still reduce disability; initial trials of alemtuzumab, an anti-CD52 monoclonal antibody mentioned below, had encouraging effects on disability progression (Coles et al. 2006). Of the great number of DMTs that have been approved by drug administrators over the last few years, all are primarily immunomodulatory.

β-Interferon was the first DMT to demonstrate efficacy in MS, now over 20 years ago, when it was observed to reduce the annualised relapse rate of treated patients by around a third compared with placebo (IFNB Multiple Sclerosis Study Group 1993). It was first used as a treatment due to its known anti-viral effects and the theory that MS relapses were related to viral persistence (Jacobs et al. 1982). Its mechanism of action is

now known to be rather more complicated, involving complex effects on the transcriptional machinery of lymphocytes resulting in changes to antigen presentation, cytokine production and expression of regulatory cell-surface molecules (Markowitz 2007, Dhib-Jalbut et al. 2010). Despite initial optimism regarding its effect on disability, the association between treatment and delayed disability progression is unclear (Shirani et al. 2012). However, due to its known efficacy and good side effect profile, it remains first line treatment for active RRMS (NHS England Clinical Reference Group for Neurosciences 2014).

Glatiramer acetate, a drug consisting of multiple polymers of four amino acids in varying ratios, was developed around the same time. It was initially conceived to be similar to myelin basic protein (MBP), a major constituent of the myelin sheath, and was found to ameliorate experimental autoimmune encephalitis (EAE), the mouse model of MS (Teitelbaum et al. 1989). Subsequent clinical trials showed a reduction in relapse rate similar to β-interferon (Johnson et al. 1995). Its mechanism of action is likely to involve induction of immune tolerance in auto-reactive T cells due to a degree of structural similarity with MBP (Dhib-Jalbut 2003).

Two current treatments target cell trafficking in order to reduce the ability of lymphocytes to migrate to the CNS. Natalizumab, a monoclonal antibody directed against CD49d (also known as α4-integrin), which is involved in the extravasation of most leukocytes, is licensed for highly active MS and has reduced relapse rate by over 50% in clinical trials as well as reducing sustained progression of disability (Polman et al. 2006, National Institute for Health and Care Excellence 2007, Butzkueven et al. 2014). However, its use is limited by side effects, the most serious of which include development of progressive multifocal leukoencephalopathy (PML), a devastating monophasic demyelinating disease caused by the JC virus, which can reactivate in the context of reduced CNS immune surveillance.

Fingolimod, an oral treatment licensed more recently, is an analogue of sphingosine-1-phosphate (S1P), and acts by modulating lymphocyte homing, sequestering potentially disease-causing lymphocytes in secondary lymphoid organs and thereby reducing relapse rate (Kappos et al. 2010, Choi et al. 2011, Calabresi et al. 2014). There is also some evidence that S1P modulators act on S1P receptors within the CNS, possibly increasing remyelination; a second-generation S1P modulator, Siponimod, is currently being assessed in phase III trials in SPMS (Lee et al. 2010, Jackson et al. 2011).

Other treatments act to reduce the number of activated or total lymphocytes. Alemtuzumab, a monoclonal antibody directed against CD52, a surface molecule expressed by mature B and T lymphocytes as well as monocytes and some dendritic cells, causes a long-lasting depletion of peripheral blood lymphocytes (Hu et al. 2009). In trials this achieved a reduction in relapse rate of over 50% when compared with interferon-β as well as promising results on sustained accumulation of disability (Kurtzke 2000, Cohen et al. 2012). However, its use is limited by adverse effects, the most serious of which is the emergence of autoimmunity; 30% of patients develop thyroid disorders, 1% develop immune thrombocytopaenic purpura, and a smaller number may develop glomerulonephritis (Coles et al. 2012, Jones et al. 2014). A newly developed oral inhibitor of de-novo pyrimidine synthesis, teriflunomide, has recently been approved for treatment of MS. It targets rapidly proliferating activated lymphocytes, seemingly without compromising normal immune function (Confavreux et al. 2014, National Institute for Health and Care Excellence 2014).

Other monoclonal antibodies demonstrating efficacy include daclizumab (anti-CD25), which was recently shown to reduce disease activity in trials (Giovannoni et al. 2014) and ocrelizumab (anti-CD20), which has shown some promise in early studies and is currently undergoing further trials (Bar-Or et al. 2008, Naismith et al. 2010, Kappos et al. 2011).

Drug repositioning or retargeting has also been a successful strategy for a number of treatments, and has the added benefits of using drugs that have a well-known side-effect profile, or are available off-patent, for greater cost effectiveness. Dimethyl fumarate was used in psoriasis for several decades before it was observed to ameliorate the course of EAE, and has subsequently been shown to reduce relapse rate in MS; in addition, it may have neuroprotective effects (Fox et al. 2012, Gold et al. 2012, Phillips et al. 2013). Following observations regarding the role of acid-sensing ion channels in neuronal injury, the diuretic amiloride was shown to reduce neuronal death and ameliorate the course of EAE (Friese et al. 2007, Vergo et al. 2011). A small trial of its use in PPMS showed beneficial effects on MRI parameters; further trials are underway (Arun et al. 2013).

As well as the success stories there are those of failure; anti-TNF-α treatment, despite efficacy in other autoimmune disease and in EAE, caused worsening of MS during clinical trials (Lenercept MS study group 1999). The same fate befell atacicept, a B cell modulator (Kappos et al. 2014).

The development of treatment for MS therefore remains challenging due to its basis on our limited understanding of the disease. The major unmet need is of treatment that will halt or prevent disability, and this issue is closely related to the question of when to commence treatment. Early treatment appears to reduce the risk of having a second relapse, but whether this also confers a reduced risk of disability developing remains to be determined (Kappos et al. 2006). Ultimately, the development of effective therapies specifically targeting autoimmune and neurodegenerative processes depends on developing our understanding of these two interrelated aspects of disease.

1.2 Immunopathogenesis of multiple sclerosis

1.2.1 MS pathology

The hallmark of multiple sclerosis pathology is the inflammatory lesion, the characteristics of which predominantly depend on the age of the lesion, although there may also be differences between lesions found in different patterns of disease (Bruck et al. 2002). Typically the majority of lesions occur in the white matter of the CNS, but cortical lesions are now recognized to be common and frequently extensive (Filippi et al. 2012); they may be juxtacortical, intracortical or subpial (Kutzelnigg et al. 2005, Calabrese et al. 2010).

The early, acute MS lesion is typified by an inflammatory cell infiltrate, with macrophages containing myelin components, parenchymal infiltration by lymphocytes, predominantly CD8+ T cells, and relative preservation of axons (Lassmann 2011). Inflammatory cells are distributed evenly throughout the early acute lesion, which may have an indistinct border; as the lesion ages and enlarges the centre of the lesion becomes less active, and in chronic active lesions a well-demarcated border of ongoing inflammatory activity can be observed between the demyelinated centre and normal appearing white matter outside the lesion (Lucchinetti et al. 2000, Lassmann 2011). Breakdown of the blood brain barrier (BBB) is highest around active lesions, facilitating the transmigration of lymphocytes, which are often found in a perivascular distribution. However, BBB disruption may persist even in inactive lesions (Hochmeister et al. 2006, Lassmann et al. 2012). Grey matter lesions differ from white matter lesions in that there are fewer T cells and a lesser degree of BBB breakdown (Filippi et al. 2012).

Immunoglobulin and complement deposition is also seen in MS lesions. Some studies report widespread deposition of these molecules whereas in others they are

thought to be limited to a single pattern of lesions (Lucchinetti et al. 2000, Sadaba et al. 2012).

Transection of axons has been observed in MS lesions at all stages (Trapp et al. 1998). The mechanisms of axonal injury are unknown but may include excitotoxicity, for instance due to immune-cell derived glutamate (Pitt et al. 2000) or direct cytotoxicity of immune cells (Medana et al. 2001, Haile et al. 2011). Energy failure due to mitochondrial dysfunction induced by inflammatory mediators may underlie neurodegenerative pathways, leading to disruption of ion transport, influx of Na+ and Ca2+ ions, and ensuing cytoskeletal damage (Waxman 2006, Friese et al. 2007). Although axons are thought to be susceptible to injury in the absence of myelin (Waxman 2006), it has been shown that axonal damage can occur in the absence of demyelination, secondary to reactive species released by immune cells (Nikic et al. 2011).

A significant challenge has been to define the earliest processes in the formation of the MS lesion; it has been postulated that oligodendrocyte death may be an initial event in the absence of lymphocytic infiltration, but this is controversial and disputed (Barnett et al. 2004, Lucchinetti et al. 2004). The heterogeneity of the MS lesion has led to attempts to step away from the traditional acute/chronic active/inactive paradigm and towards reclassification by pattern of immunopathogenesis rather than lesion age (Lucchinetti et al. 2000). However, this is also disputed (Breij et al. 2008). It is clear that the MS lesion is a highly complex environment with multiple cell types playing different roles.

In addition to the clearly defined lesions of MS, subtle inflammation involving macrophages, microglial activation and axonal loss occurs throughout the normal-appearing white matter (NAWM) (Filippi et al. 2012). Meningeal inflammation is also frequently observed in MS, and may contribute to cortical pathology though findings have been conflicting (Kooi et al. 2009, Magliozzi et al. 2010).

1.2.2 Autoimmunity

Many observations, histological and genetic, give a strong basis for MS as a primary autoimmune disease in which cells of the body's immune system are misdirected against components of the myelin sheath of the central nervous system. In common with many autoimmune diseases there are periods of immune hyperactivity, manifest as relapses, interspersed with periods of relative senescence during which recovery occurs. There is some controversy over how much early inflammation contributes to disability; observational studies suggest that although there are many variables that contribute to accumulation of early disability, including relapses, once a threshold has been reached subsequent disability accumulates at a similar rate regardless of disease type and independent of early relapse rate (Confavreux et al. 2003, Confavreux et al. 2006, Leray et al. 2010, Scalfari et al. 2013). In addition, the age at which irreversible disability occurs is similar in both primary progressive and secondary progressive disease, prompting the line of thought that a common neurodegenerative process with a variable immune response underlies MS (Trapp et al. 2008, Stys et al. 2012).

Inflammation is present at all stages of the disease, and is thought by many to be closely related to neurodegeneration, which can be observed early in the disease course (Ferguson et al. 1997, Trapp et al. 1998, Kutzelnigg et al. 2005, Frischer et al. 2009). Furthermore, the majority of genes associated with susceptibility to multiple sclerosis are immunological (International Multiple Sclerosis Genetics et al. 2013). However, there seems to be little correlation between multiple sclerosis lesion load and axonal loss (DeLuca et al. 2006), and inflammatory cells also contribute to neuroprotective processes via the production of neurotrophic factors (Hohlfeld et al. 2006). A possible unifying hypothesis is that early inflammation sets in train a neurodegenerative process

that continues independently once a threshold has been reached (Stadelmann 2011); a similar process has been observed in a mouse model (Hampton et al. 2013).

1.2.3 Development of lymphocyte tolerance

The autoimmune theory of MS supposes that cells of the adaptive immune system lose tolerance to myelin proteins. Both B and T cells are capable of detecting and reacting to a vast number of antigens. In B cells this occurs via the B cell receptor (BCR), which is similar in structure to secreted immunoglobulin but is membrane bound. In T cells, this occurs via the T cell receptor (TCR) which recognises specific antigens in the context of a human leukocyte antigen (HLA) molecule (Murphy et al. 2012). Both the BCR and TCR have regions termed the variable, diverse and joining regions. A number of different genes encode these regions, which are randomly assorted during lymphocyte development, giving rise to a vast TCR and BCR repertoire, which is able to detect a large number of antigens. While this has the advantage of ensuring that almost any non-self peptide can be detected, the random process of gene rearrangement also has the potential to generate a receptor that will react deleteriously to self-peptide. Therefore a number of mechanisms are in place to prevent self-directed lymphocytes escaping into the periphery. For B cells this process occurs in the bone marrow, during the transition through different stages in B cell development, particularly from pre-B cell to immature B cell, and through a process known as receptor editing (von Boehmer et al. 2010).

For T cells the establishment of tolerance occurs in the thymus via a complex two-part process. Initially T cells undergo positive selection whereby only cells that are able to bind major histocompatibility complex (MHC)-expressing thymic epithelial cells (TECs) receive a survival signal. Subsequently they undergo negative selection, during which cells binding self-antigen with high avidity receive signals causing either apoptosis or differentiation to regulatory cell types (Hogquist et al. 2005). A subset of

TECs in the thymic medulla is able to express peptides that are usually only expressed in specific cell types, termed "tissue specific antigens" (TSAs) (Gallegos et al. 2004). The autoimmune regulator gene (AIRE) is required for generation of tolerance to TSAs. It is thought that central tolerance to myelin proteins could be generated either via AIRE or via cross-presentation of exogenous myelin, i.e. presentation of myelin in the context of an HLA-class I molecule that would normally be presented by an HLA-class II molecule (Perchellet et al. 2008).

In addition to central tolerance, peripheral tolerance mechanisms exist to limit disease in the event of an auto-reactive T cell clone escaping central tolerance. There is evidence that AIRE is expressed in secondary lymphoid organs allowing detection of autoreactive clones and inducing anergy or clonal deletion (Metzger et al. 2011). In addition there are several populations of regulatory immune cells, in CD4, CD8 and B cell compartments, that are able to reduce autoreactivity (Perchellet et al. 2008, Yang et al. 2010).

1.2.4 Mechanisms of tolerance breakdown

In order to induce MS, the myelin-reactive lymphocyte must evade the mechanisms in place to ensure self-tolerance. There are multiple potential ways in which this might occur. Firstly, although myelin antigens are known to be expressed in human thymus, expression of some minor myelin antigens in the thymus may be at a low level or non-existent so that developing autoreactive cells do not encounter their cognate antigen during negative selection (Pribyl et al. 1996, Bruno et al. 2002). Alternatively, thymic expression of some antigens may be restricted to certain isoforms, resulting in a lack of tolerance toward the full-length, abundant form of the protein (Anderson et al. 2000). A T cell that binds a myelin antigen with low avidity, and therefore a high threshold for activation, may "pass" negative selection in the thymus.

This in itself is unlikely to cause MS, as myelin-reactive T cell clones have long been known to be present in the blood of both MS patients and healthy people (Meinl et al. 1993, Saruhan-Direskeneli et al. 1993, Berthelot et al. 2008), although there may be differences in the degree and threshold of activation (Meinl et al. 1993, Greer et al. 1997). However, once in the periphery, an inflammatory milieu with concomitant upregulation of co-stimulatory molecules on antigen-presenting cells may overcome the low avidity: so-called bystander-activation (Vanderlugt et al. 2002).

An alternative hypothesis is that of "molecular mimicry". This hypothesis posits that some lymphocytes have "degenerate" receptors that are able to cross react with multiple antigens of similar structure. Therefore, lymphocytes specific for foreign antigens such as viral or bacterial antigens may also bind and become activated by self-antigen; in vitro this has been shown to stimulate T cell proliferation and the production of autoantibodies by B cells (Wucherpfennig et al. 1995, Lang et al. 2002, McClain et al. 2005).

1.2.5 Leukocyte trafficking to the CNS

Once activated in the periphery, autoreactive cells must home and gain access to the CNS, which has long been considered a site of "immune privilege". A degree of immune surveillance does occur physiologically, and CSF from healthy donors contains a small number of memory T and B cells (Svenningsson et al. 1993, Ransohoff et al. 2012). However, in an inflammatory state activated T cells from the periphery upregulate a number of adhesion molecules such as alpha-4 integrin, CD62L, CXCR3 and CCR5, allowing them to adhere to and cross the blood-brain barrier, a highly specialised endothelial membrane, in far greater numbers (Balashov et al. 1999, Eikelenboom et al. 2002). Blocking one of these adhesion pathways may result in upregulation of

alternative molecules, which has implications for cessation or failure of treatment with natalizumab (Schneider-Hohendorf et al. 2014).

1.2.6 CD4+ T cells

CD4+ T cells (also T-helper or Th cells) were long thought to be the primary causative cell in multiple sclerosis due to several lines of evidence. CD4+ T cells bind antigens in the context of HLA class II, which was one of the first detected and strongest genetic associations with susceptibility to MS; this finding was therefore consistent with a significant role for this cell population (Gyodi et al. 1982, Olerup et al. 1991). CD4+ T cells are causative in almost all types of EAE ('t Hart et al. 2011, Attfield et al. 2012).

HLA Class II is present on the surface of antigen presenting cells (APCs) and presents exogenous antigen that has been processed intracellularly and transported to the cell surface. On antigen binding the CD4+ T cell may become activated, giving a signal to the APC and itself undergoing differentiation which, depending on the inflammatory context, may be into any of the T-helper subtypes such as Th1, Th2, Th17, Tfh or Treg (Murphy et al. 2012). Dendritic cells are the most specialised APCs but other cell types can also play this role, including B cells and macrophages (Banchereau et al. 1998, Roche et al. 2015). CD4+ T cells therefore act as a crucial link between not just adaptive and innate immune systems but also between cell-mediated and humoral immune responses.

The archetypal pro-inflammatory CD4+ T cell is the Th1 cell, which requires IL-12 for its differentiation and produces IL-2, IFN-γ and TNF-α, which have proinflammatory activity and activate innate immune cells (Murphy et al. 2012). As evidence for the role of Th1 cells in MS, T cell clones from patients with MS were found to produce large amounts of IFN-γ and TNF-α and correspondingly, administration of IFN-γ to patients with the disease worsened the condition (Hirsch et al. 1985, Panitch et

al. 1987, Panitch et al. 1987, Benvenuto et al. 1992). However, mice lacking IFN-γ receptors can still develop EAE, and so other cell types were postulated to be encephalitogenic. A distinct CD4+ T cell subset driven by IL-23 and producing IL-17 was found to be essential for the development of autoimmunity in certain mice (Langrish et al. 2005). These cells were termed Th17 and have since been determined as the major causative cell type in myelin-oligodendrocyte glycoprotein (MOG) induced EAE, one of the most commonly used models (Langrish et al. 2005, Komiyama et al. 2006, Attfield et al. 2012). In MS, IL-17 has been detected in lesions and may be upregulated in patients with SPMS (Tzartos et al. 2008, Huber et al. 2014). In addition, the number of Th17 cells in the CSF increases during relapse (Brucklacher-Waldert et al. 2009).

Regulatory T cells are more recently characterised and consist of distinct subsets (Duhen et al. 2012). The most numerous regulatory cell population is characterised by the master transcription factor FoxP3 and high surface expression of CD25, and are thought to be able to suppress the activity of effector T cells via the secretion of regulatory cytokines such as IL-10, and by direct cell-cell mechanisms (Thornton et al. 1998). These cells may be functionally altered in patients with MS (Viglietta et al. 2004), as may another regulatory cell type characterized by CD46 expression (Viglietta et al. 2004, Astier et al. 2006). However, it is increasingly evident that Th cells exhibit a degree of plasticity and are able to change phenotype under certain cytokine conditions (Shi et al. 2008, Veldhoen et al. 2008, Dominguez-Villar et al. 2011).

A key interaction between cell-mediated and humoral immunity is via the T follicular helper (Tfh) cell, which is involved in the generation of B cell germinal centres and the stimulation of B cell differentiation and proliferation through the expression of B cell interaction markers such as ICOS and CD40 (King 2009). It is thought that in concert with Th17 cells Tfh cells may be involved in the formation of structures resembling germinal centres, termed ectopic lymphoid follicles, which have been found

in the meninges of multiple sclerosis patients (Serafini et al. 2004, Magliozzi et al. 2007, Peters et al. 2011). Further evidence of their involvement is an increase in Tfh markers in the CSF of MS patients (Romme Christensen et al. 2013).

1.2.7 CD8+ T cells

CD8+ T cells, commonly called "cytotoxic" T cells, recognise antigen in the context of HLA-Class I, which is expressed by all cells in the body, is upregulated following stimulation with inflammatory cytokines, and displays endogenous peptides on the cell surface (Murphy et al. 2012). By binding peptide-HLA complexes CD8+ T cells are able to detect intracellular pathogens such as viruses and certain bacteria, and are able to induce apoptosis via the production and release of perforin and granzymes as well as via binding the Fas receptor (Trapani et al. 2002). They can also produce inflammatory cytokines such as IFN-γ and IL-17(Huber et al. 2009, Romme Christensen et al. 2012).

Although MS was long considered a CD4+ T cell driven disease, the importance of CD8+ T cells became clear through detailed examination of lesions in the brains of post-mortem MS patients, where it could be demonstrated that CD8+ T cells outnumber CD4+ T cells in the perivascular cuffs of both the inflammatory lesions and normal appearing white matter (Booss et al. 1983, Hauser et al. 1986). As evidence of these cells playing a specific role rather than simply undergoing bystander activation, it has been shown that CD8+ T cell clones proliferate and produce inflammatory cytokines in response to stimulation with myelin peptides (Tsuchida 1994). The CD8+ T cell component of the cellular infiltrate is more homogeneous, consisting of fewer clones, than that of the CD4+ T cell component implying greater antigen-specific clonal expansion (Babbe et al. 2000). Furthermore, CD8+ T cells belonging to the same clone can be found in distant sites within the same brain, suggesting that re-activation with

the CNS may occur in response to a common antigen within a patient. Very few CD8+ T cell clones are shared between patients, emphasising the individual, heterogeneous aspect of the disease (Junker et al. 2007).

Further evidence that CD8+ T cells play a pathogenic role comes from EAE experiments; CD8+ T cell clones specific for antigens derived from MOG and MBP can be found in the periphery of wild type mice, and adoptive transfer of these cells induces a CNS inflammatory disease similar to classical EAE (Huseby et al. 2001, Sun et al. 2001). Myelin-specific human CD8+ T cells are able to lyse HLA-class I expressing oligodendrocytes (Jurewicz et al. 1998).

The importance of CD8+ T cells is also underlined by the association of HLA class I alleles with MS, most notably HLA-A2 which confers protection against the disease (Link et al. 2010, Link et al. 2012). Mouse experiments using "humanised" mice, which express both a human T cell receptor and HLA molecules, have elegantly demonstrated this by showing that transgenic expression of HLA-A2 results in amelioration of disease through a reduction in the number and TCR-expression of autoreactive CD8+ T cells (Friese et al. 2008). CD8+ T cells also have the capacity to act in a regulatory fashion, as demonstrated by the adoptive transfer of CD8+ T-cells isolated from animals that had recovered from EAE, resulting in prevention or amelioration of disease (Sun et al. 1988) .

CD8+ T cells can also be classified like CD4+ T cells by the cytokines that they produce; this has led to detection of a Tc17 subset analogous to the CD4+ Th17 population. IL-17+ CD8+ T cells have been detected in MS lesions (Tzartos et al. 2008) and in the early stages of MS appear to be increased in CSF (Huber et al. 2013).

CD8+ T cells may have an indirect role in the generation of CNS inflammatory disease via perforin-mediated breakdown of the blood brain barrier (Johnson et al. 2014) and more recent studies have sought to elucidate the regulatory role of CD8+ T cells, which in mice are able to ameliorate the course of EAE and aid functional recovery (Lee et al. 2008). Human regulatory CD8+ t cells are able to prevent CD4+ T cell

proliferation in vitro and may be reduced during the relapse phase of RRMS (Correale et al. 2010, Suzuki et al. 2012). It is possible that a role is played by tissue resident memory (Trm) CD8+ T cells, a recently characterised subset that survive long-term within the CNS following viral infection and confer protection against reinfection via expression of inflammatory cytokines and granzymes (Wakim et al. 2012).

As well as classification by cytokine production, both CD4+ and CD8+ T cells can be classified as naïve or memory cells. After encountering their cognate antigen in secondary lymphoid organs, naive cells proliferate, generating memory and effector cells. Memory cells persist in the blood of healthy individuals and on the basis of CD45RA and CD62L or CCR7 expression can be subdivided into central memory (Tcm; CD45RA-,CD62L+/CCR7+), effector memory (Tem; CD45RA-,CD62L-/CCR7-) and terminally differentiated memory (Temra; CD45RA+,CD62L-/CCR7-)(Janossy et al. 1989, Richards et al. 1990, Sallusto et al. 2004, Yang et al. 2011). Naïve cells can be defined as CD45RA+ and CD62L+/CCR7+. Overlap between CD62L and CCR7 is not complete, but both molecules are frequently used to define the four populations.

1.2.8 B cells

As outlined above, B cells mature in the bone marrow and express the BCR, through which they are able to bind either soluble antigen, which they may endocytose and present bound to HLA-II, or larger intact molecules which directly deliver an activating stimulus via cross-linked receptors. On stimulation, either directly or via CD4+ T cells, they produce antibody and proliferate within the germinal centre of a lymph node. Further differentiation to antibody-producing plasma cells may occur. Within the lymph node the differentiating B cells undergo somatic hypermutation and affinity maturation, processes that allow the development of a clone with even greater antigen specificity (Klein et al. 2000, Murphy et al. 2012).

One of the most common pathological findings in MS is that of oligoclonal bands of B cell derived immunoglobulin within the CSF unmatched in serum, present in up to 95% of patients at presentation, indicating that B cells are stimulated to produce antibody within the CNS (Johnson et al. 1977, Disanto et al. 2012). Although B cells are present in MS lesions in lower numbers than T cells, there is evidence of clonal expansion in the CSF and parenchyma of MS patients (Colombo et al. 2000, Owens et al. 2003). These expanded clones appear to derive from ectopic lymphoid follicles (ELFs) in the meninges and are present in lesions of white and grey matter as well as CSF. IgG and IgM can be found in MS lesions and may colocalise with staining for complement (Serafini et al. 2004, Lovato et al. 2011).

It has been postulated that B cell dysregulation occurs in MS due to long-term persistence of Epstein-Barr virus (EBV), transcripts of which have been found within B cells in MS lesions and ELFs (Serafini et al. 2007, Serafini et al. 2010).

Several studies show a correlation between markers of B cell activity and disease progression (Magliozzi et al. 2007, Joseph et al. 2009). While this is not proof of a causative role for B cells, further evidence of their involvement comes from trials of monoclonal antibodies directed against CD20, which have shown some promise in MS (Bar-Or et al. 2008, Kappos et al. 2011, Sorensen et al. 2014). However, the role of B cells is likely to be more complex than a purely pathogenic one, as outlined by the failure of atacicept, a soluble synthetic analogue of the B cell surface receptor TACI, which resulted in worsening of pathology (Kappos et al. 2014). This may be due to the removal of not only pathogenic B cells but also the small population of regulatory B cells that are capable of secreting anti-inflammatory cytokines such as IL-10 and IL-35 (Iwata et al. 2011, Shen et al. 2014).

1.2.9 Natural Killer cells, Natural Killer T cells and innate-like lymphocytes

These cell populations sit between the innate and adaptive arms of immunity. Natural Killer (NK) cells do not express the same diversity of receptors as B or T cells, but display a range of receptors on their surface and are able to recognise MHC class I and related molecules (Litwin et al. 1993). By expressing high levels of cytokines and the cytolytic molecules granzyme and perforin they are important in the immune response to viruses and tumours, and display an enhanced memory response on repeated exposure to antigen, a feature of adaptive immunity (Cooper et al. 2001, Cooper et al. 2009, Cooper et al. 2009).

A potential regulatory role of NK cells was postulated when the course of EAE was noted to worsen following depletion of NK cells with monoclonal antibody, most likely due to increased CD4+ T cell proliferation in the absence of NK cells (Zhang et al. 1997). Further animal experiments have demonstrated that this regulatory function likely occurs within the CNS; preventing NK cells from migrating to the CNS increased the Th17 activity of CNS CD4+ T cells (Hao et al. 2010).

Some patients with MS in remission have been found to possess NK cells expressing high levels of CD95; this population was able to inhibit the secretion of IFN-γ by antigen-stimulated CD4+ T cells (Takahashi et al. 2004). More recent characterisation of NK cells in humans suggests two predominant populations exist in peripheral blood, CD56+CD16+ and CD56++CD16-. The latter subset is thought to have a greater role in cytokine production and immunoregulation, and was noted to expand in MS patients following treatment with the anti-CD25 monoclonal antibody daclizumab (Bielekova et al. 2006, Bielekova et al. 2011). They were also demonstrated to kill autoreactive CD8+ T cells (Bielekova et al. 2006), which may occur via granzyme K-dependent induction of apoptosis (Jiang et al. 2011). This subset has also been noted to transiently increase following autologous stem cell transplant in MS (Abrahamsson et al. 2013).

The numbers of NK cells do not appear to be altered in MS patients compared with healthy controls, but there is evidence that NK cells of patients are functionally impaired with a reduced ability both to expand in response to inflammatory cytokines and to produce IFN-γ (Lunemann et al. 2011, Sheridan et al. 2011).

Natural Killer T- (NKT) cells are lymphocytes that express NK markers such as CD56, are restricted to the MHC-class I related molecule CD1d, and detect glycolipid antigens. The best-characterised subtype is type 1 invariant NKT (iNKT) cells whose invariant TCR has been detected in MS lesions, and numbers of which are reduced in MS patients (Illes et al. 2000, Berzins et al. 2011).

Mucosal associated invariant T (MAIT) cells are a population of predominantly CD8+ T cells that express the invariant TCR α-chain Vα7.2 and a limited β-chain repertoire (Gold et al. 2014). These cells recognise antigen in the context of the MHC class I related molecule MR1, which is thought to present products of vitamin B metabolism specific to pathogens (Kjer-Nielsen et al. 2012). They are precommitted to an effector memory phenotype, are able to rapidly upregulate effector molecules, and display a tissue-homing phenotype with expression of CCR5 and CCR6 (Dusseaux et al. 2011). Evidence suggests that their role in immunity is predominantly in the defence against a wide range of bacteria and yeasts (Le Bourhis et al. 2010). These cells and their role in MS are covered further in chapter 6.

1.2.10 Innate cells

The innate arm of the immune system responds non-specifically to pathogens via recognition of pathogen associated molecular patterns (PAMPs), which bind to pattern recognition receptors (PRRs) typified by the toll-like receptors (TLRs) (Medzhitov 2001). Ligand-PRR binding initiates complex signaling pathways resulting in anti-microbial defence mechanisms, including the production of cytokines and effector

molecules such as inducible nitric oxide synthase, which occurs predominantly via activation of nuclear factor kappa B (NFκB). Some TLRs signal exclusively via the adaptor protein MyD88, which associates with the cytoplasmic domain of TLRs, to activate NFκB. However, other TLRs, including TLR4, are able to signal via MyD88-independent pathways to induce effector function (Janeway et al. 2002). Multiple cell populations constitute the innate immune system including granulocytes, monocytes, macrophages, microglia and dendritic cells (Janeway et al. 2002, Murphy et al. 2012).

Antigen presentation is a function predominantly of the innate immune system and can be performed by dendritic cells, macrophages, and microglia (Mack et al. 2003). The presentation of antigens to the adaptive immune system in both the periphery and the CNS moulds the immune response and is clearly of great importance in the pathogenesis of MS. Dendritic cells are the most specialised and effective APCs, able to present antigen via MHC class II or to cross-present via MHC class I (Ganguly et al. 2013). In addition to the MHC/antigen complex, they also provide a number of co-stimuli which are sufficient for T cell polarisation. DCs are found in the lesions and meninges of patients with MS (Serafini et al. 2006, Lande et al. 2008) where they may play an important role in reactivation of T cells; in EAE they were found to be sufficient for T cell mediated brain inflammation in the absence of other MHC class II expressing cell types (Greter et al. 2005). However, they also have a significant role in the development of tolerance to self antigen, and their precise role in the pathogenesis of MS remains to be fully determined (Banchereau et al. 1998).

Macrophages are recruited from the circulating blood as monocytes; once in the brain they undergo differentiation to macrophages with cytoplasmic processes, becoming similar in appearance to microglia, large phagocytic innate immune cells that are resident within the CNS from development (Raivich et al. 2004). The active MS lesion is characterised by infiltration of brain parenchyma by both of these cell types, which phagocytose myelin debris and express MHC class II (Bo et al. 1994). Microglial

activation is particularly seen in progressive multiple sclerosis, where these cells are present at the rim of slowly expanding lesions, but also in cortical lesions and diffusely through the NAWM, possibly contributing to neurodegeneration (Kutzelnigg et al. 2007, Lassmann et al. 2007). In addition to their phagocytic and antigen-presenting roles, microglia and macrophages can produce pro-inflammatory cytokines such as IFN-γ, as well as free radicals such as superoxide, which may contribute to axonal degeneration of EAE and MS (Aloisi et al. 1997, Nikic et al. 2011).

1.3 Aetiology of MS

The aetiology of MS remains a subject of intense debate, with many strongly held and contradictory hypotheses. What is clear is that the development of MS derives from a complex interaction of environmental factors on an equally complex background of genetic susceptibility.

1.3.1 Genetics

It has long been noted that while MS does not show a classical Mendelian pattern of inheritance, its incidence is increased in relatives of patients with MS, indicating a hereditary predisposition. Twin studies showed that concordance in monozygotic twins is significantly higher than dizygotic twins (Ebers et al. 1986, Mumford et al. 1994). In monozygotes the concordance was initially thought to be around 30%, suggesting an important role of environmental factors. More recent, larger studies have shown that the risk of MS in siblings of patients is seven times greater than that of the general population, and that the genetic contribution to disease susceptibility – the heritability – is estimated as 65% (Westerlind et al. 2014). Consistent with this, following the identification of MS associated genetic variants it was found that patients from families with multiple cases carried a greater "genetic burden" than patients from single-case

families (Isobe et al. 2013).

The pattern is therefore not one of inheritance involving a single causative mutation but of variants at many loci, frequent ('common') in the general population, each exerting a moderate effect on disease. This is often said to be consistent with the "common disease, common variant" hypothesis, although the authors of the original paper did not make a statement regarding the number of causative loci or their effect size, and there is ongoing debate as to whether susceptibility to common disease is largely due to common or rare variants (Reich et al. 2001, Yang et al. 2005, Visscher et al. 2012).

Over 110 single nucleotide polymorphisms have been associated with MS in several genome-wide association studies (GWAS) performed over the last decade. Each of these variants is common, with a minor allele frequency of greater than 1-5%, and is estimated to have only a small effect on susceptibility; the odds ratio (fold-increase in risk, OR) for each risk variant is between 1.1 and 1.2 (Sawcer et al. 2011). However, some variants have greater effects than others, particularly those variants in the MHC region of chromosome 6, discussed below.

1.3.1.1. Associated gene regions in MS

By far the most important genetic region in terms of MS susceptibility is the MHC, a highly polymorphic segment of the genome encoding the variable segments of the HLA-class I and class II molecules that present antigen to T cells. Functional HLA-class I consists of three HLA class I α-chains bound to a β-2 microglobulin molecule. It is expressed on all cells, and in complex with antigen is recognised by CD8+ T cells. There are three polymorphic HLA class I genes: HLA-A, HLA-B and HLA–C. HLA class II exists as a complex of two α-chains and two β-chains, encoded by three pairs of α- and β-chain genes: HLA-DR, HLA-DP and HLA-DQ. HLA class II is expressed by antigen presenting cells, and in complex with antigen is recognised by CD4+ T cells.

As discussed above, the variability of these molecules determines the repertoire of antigens they present and the avidity with which they bind the T cell receptor, with consequent effects on self-tolerance and the response to foreign antigens.

Early linkage and case-control studies consistently found an association with this locus (Bertrams et al. 1976, Gyodi et al. 1982, Compston 2005), which was later determined as arising due to association with the HLA-DR2 haplotype DRB1*1501 DRB5*0101 DQB1*0602 (Olerup et al. 1991). Due to linkage disequilibrium (LD, the non-stochastic inheritance of alleles as a result of multiple processes including non-random recombination) further dissection of this haplotype was not achieved until the advent of SNP-typing allowed pinpointing of the association to the HLA-DRB1*1501 allele, which confers an OR of 3.1 (Sawcer et al. 2011). Possession of the HLA-DRB1*1501 allele, as well as influencing MS susceptibility, appears to influence cortical pathology, as evidenced by increased innate cell inflammation in the motor cortical lesions of MS patients possessing the HLA-DRB1*1501 allele (Yates et al. 2015).

Conditioning on this risk allele has allowed detection of other susceptibility-associated variants in the HLA region, namely HLA DRB1*0301 and HLA-DRB1*1303 (Sawcer et al. 2014). An association with the HLA-class I region has been confirmed, with HLA-A*02 conferring protection against MS with an OR of 0.73 (Brynedal et al. 2007, Link et al. 2012). Determining how these variants increase susceptibility to or protect against MS has been challenging, although some insight has been obtained using humanised transgenic mice, demonstrating a protective effect of HLA-A2 (Friese et al. 2008) possibly via alterations in the numbers of circulating CD8 cells, and structural research which suggests a potential role for molecular mimicry (Lang et al. 2002).

The MHC association is estimated to account for 10-60% of the genetic risk of MS (Haines et al. 1998, Oksenberg et al. 2008). The remainder is partly accounted for by the 110 (or so) risk variants that have been detected to date.

1.3.1.2. Genome-wide association studies

The first GWAS were performed in the late 2000s and were made possible by increasing knowledge regarding the degree and type of variation present throughout the human genome, and the technology to enable this variation to be detected and exploited on a grand scale (Sachidanandam et al. 2001, International HapMap 2005). A huge degree of genetic variation occurs between individuals, in different forms including single nucleotide polymorphisms (SNPs), insertion/deletions (indels) and copy number variants (CNVs). Of these, SNPs have proven to be the most useful for studying complex diseases; there are estimated to be around 15 millions SNPs across the human genome (1000 Genomes Project Consortium et al. 2010), the great majority of which have a minor allele frequency (MAF) of over 1%. Although they are not completely uniformly distributed – for instance there is a higher density of SNPs in the MHC locus – their abundance and MAF makes them ideal for detecting loci that are associated with disease. During meiosis, homologous recombination occurs non-randomly across the genome; DNA is inherited as blocks, within which there are high levels of linkage disequilibrium (LD). GWAS take advantage of this, using "tagging" SNPs to detect association of a block of high LD.

Performing a GWAS involves genotyping large numbers of patients and matched controls using SNP-typing arrays, which are now able to type for up to 5 million SNPs. The vast number of SNPs analysed throws up significant problems when determining the false discovery rate and adjusting the p-value accordingly; to account for this and adequately power the studies, several thousand people are required in both patients and control groups. The logistical difficulty involved has been overcome by international consortia working in concert to recruit patients and controls and obtain samples for analysis (International Multiple Sclerosis Genetics et al. 2013).

Generally, GWAS have grown in size as the process of performing them has been refined and the cost has decreased. Prior to 2011, several smaller GWAS had been

performed, detecting around 30 variants (Hafler et al. 2007, Baranzini et al. 2009, Patsopoulos et al. 2011). The two most recent have uncovered over 70 disease-associated variants (Sawcer et al. 2011, International Multiple Sclerosis Genetics et al. 2013). Clearly GWAS have increased our knowledge of genetic architecture of MS, but there remains the problem of how to develop this into a practical understanding of the machinations of the disease.

Firstly, SNPs are present at roughly every 300 bases in the genome; as not every SNP is typed, the remaining markers must be imputed from previously characterised reference genomes (1000 Genomes Project Consortium et al. 2010, 1000 Genomes Project Consortium et al. 2012). This may introduce errors depending on the reliability of the imputation sequence, but provides valuable information on untyped variants and indels. The nature of LD, which facilitates detection of disease associated regions, presents a hurdle when attempting downstream studies; LD blocks are highly variable in size but generally span kilobases and contain many SNPs. It is therefore difficult to determine the causative SNP within an LD block computationally, making the regions harder to study definitively (Reich et al. 2001).

The majority of disease-associated SNPs discovered so far are in non-coding regions, meaning that unless they are tagging rare coding variants, their effect is not on protein function but likely to be on levels of gene expression via alteration of a regulatory or epigenetic mechanism. The region containing a disease-associated SNP may contain several genes, and therefore the affected gene, as well as the nature of the effect, must be determined to uncover the impact of the variant in question.

Many studies have sought to address what proportion of variance in disease susceptibility is accounted for by the SNPs detected by GWAS (Lee et al. 2011, Watson et al. 2012, Isobe et al. 2013). Generally the proportion of heritability accounted for by these SNPs is relatively small, which has given rise to the term “missing heritability”. This appears to be confirmed by studies of the “SNP genetic burden” in families with MS.

Although families with multiple affected members have more disease-associated SNPs, these can still not be used to predict whether a relative will have MS (Isobe et al. 2013). It is thought that the most likely explanation is that many of the SNPs that are typed for on commonly used arrays are not causative, and are not in perfect LD with the causative variants (Watson et al. 2012). However, there are other potential causes of missing heritability including copy number variants (CNVs), epigenetic effects, gene-gene interactions, and rare variants with larger effect sizes that are not well tagged by common SNPs due to differences in allele frequency (Manolio et al. 2009, Cortes et al. 2011, Visscher et al. 2012, Watson et al. 2012). Meta-analyses of GWAS often attain sufficient power to detect further associated variants, demonstrating that some of the missing heritability may simply be due to insufficient power of even large GWAS (Anderson et al. 2011, Bradfield et al. 2011). As GWAS increase in size, and fine-mapping methods improve, it seems likely some of the missing heritability will be accounted for. Alternatively, analyses using alternative statistical methods may suggest that much of the heritability is accounted for by common variants that themselves do not reach statistical significance using standard methods (Lee et al. 2011). The contribution of rare variants is unlikely to be uncovered until large scale whole-genome sequencing studies are undertaken, which are unlikely to be performed for some years yet (Dr Adrian Cortes, personal communication).

An immune hypothesis for MS was strengthened by early GWAS, which discovered markers in predominantly immune-related loci, particularly in T-helper and antigen presentation pathways (Sawcer et al. 2011). Following this, a collaboration was entered into by consortia coordinating GWAS for different immune diseases with the purpose of designing an immune-specific genotyping array to “fine map” the previously detected immune loci. This new array was designated the Immunochip, which genotypes SNPs at high densities in immune-related loci previously detected in GWAS of multiple autoimmune diseases, including autoimmune thyroid disease (AITD), primary

sclerosing cholangitis, Crohn's disease (CD), type 1 diabetes (T1D), psoriasis, ankylosing spondylitis (AS) and coeliac disease. Due to the high-density SNP typing, the Immunochip has greater power to discover causal variants. Thus far, Immunochip studies have revealed much about the common genetic susceptibility underlying many autoimmune diseases. The 2013 MS Immunochip study revealed 48 new immune loci, as well as refining the signal from SNPs in 49 previously discovered loci. Over a fifth of these susceptibility loci are shared with other autoimmune diseases (International Multiple Sclerosis Genetics et al. 2013).

GWAS have greatly furthered our understanding of the genetic architecture of many diseases, but it could be argued that they have not as yet been translated into large-scale advances in understanding disease aetiology. Although over 100 MS-associated variants have been identified (Hafler et al. 2007, Patsopoulos et al. 2011, Sawcer et al. 2011, International Multiple Sclerosis Genetics et al. 2013) there are relatively few publications on the mechanisms and downstream effects of these variants. One of the first SNPs to be associated with susceptibility to MS was rs1800693, intronic in the TNF receptor superfamily 1A (TNFRSF1A) locus (De Jager et al. 2009). Characterisation studies revealed that this variant caused a splicing defect and skipping of exon 6, producing a soluble rather than membrane-anchored form of the receptor, mirroring the effect of soluble anti-TNF-α treatment that resulted in worsening of disease in clinical trials (Lenercept MS study group 1999, Gregory et al. 2012). Other studies have demonstrated effects of genotype at the protein and functional level on IL2RA (Dendrou et al. 2009, Hartmann et al. 2014) and IL7RA (Gregory et al. 2007, Lundstrom et al. 2013). Although challenging, characterising the effects of susceptibility variants offers valuable insight into disease pathogenesis and potential treatment.

1.3.1.3. Epigenetics

The finding that the vast majority of disease-associated variants are in non-coding regions has stimulated much research into the role of regulation of gene expression in altering disease susceptibility. Epigenetics encompasses many complex regulatory mechanisms, including such diverse processes as modification of histone proteins, transcription of non-coding RNA molecules and long-range DNA looping (American Association for Cancer Research Human Epigenome Task et al. 2008). DNA modifications, such as attachment of methyl groups to histones, are thought to be inherited during regular cell division, but are highly tissue specific and are altered during cell differentiation, meaning that epigenetic processes may be different in closely related cell types (Byun et al. 2009, Slieker et al. 2013, Farh et al. 2015).

Regions of epigenetic importance can be detected via an array of methods such as bisulfite sequencing to detect methylated regions, chromatin immunoprecipitation and sequencing (ChIP-Seq) to detect protein-DNA interactions, and chromatin conformation capture (3C) techniques to identify long-range DNA interaction (Esteller 2008, Suzuki et al. 2008, Hughes et al. 2013). The importance of epigenetic modifications has been demonstrated via manipulation of these mechanisms to induce changes in gene expression, such as through altering DNA methylation or inducing forced chromatin looping (Maeder et al. 2013, Deng et al. 2014). Other groups have taken advantage of different mouse lines to demonstrate the effect of single-nucleotide polymorphisms on transcription factor binding and gene expression using Capture-C, DNase footprinting and RNA-seq (Hughes et al. 2014).

Epigenetic studies suggest that a high proportion of MS-associated SNPs overlap with regulatory regions as defined by DNase hypersensitivity, a marker of active open chromatin, and by ChIP-Seq; this appears to be true of different cell types involved in MS, including CD4+ T cells, CD8+ T cells, B cells and NK cells (Ernst et al. 2011, Disanto et al. 2012, Disanto et al. 2012, Maurano et al. 2012, Disanto et al. 2014). Comparison of MS

patients with healthy controls suggests that CD8+ T cells of MS patients have higher methylation levels at multiple loci, suggesting altered epigenetic regulation of gene expression, although in this study no difference was found at MS-specific loci (Bos et al. 2015).

These findings appear to validate the results of GWAS and point to the importance of gene regulation rather than gene sequence in the genetic architecture of complex diseases. Taking into account epigenetic markers when investigating a disease-associated variant may provide further evidence of the causal variant as well as the cell type in which the variant is relevant.

1.3.2 Environmental factors

The global distribution of MS, as well as the discordance rates among monozygotic twins, suggest significant environmental factors in the aetiology of MS. It is predominantly a disease of Caucasians, and there is a general increase in prevalence with increasing latitude although some populations, such as the Sardinians and Inuits, buck this trend. Prevalence rates in migrant populations have been particularly closely studied; it appears that migrants from low-risk to high-risk areas assume the risk of the destination population, but the effect of migration in the opposite direction is less clear (Guimond et al. 2014, Berg-Hansen et al. 2015). There is some evidence that the age at migration has a significant effect on whether the risk of the destination population is assumed or not, with risk only changing if migration occurs before adolescence (Kurtzke 1977). This suggests that the risk of MS is established early in life, years before the onset of disease. Furthermore, the complex interplay of environment and genetics can result in diverse ethnic populations in the same region having vastly different rates of disease (Alter et al. 2006). These observations have led to much research into the potential

causative environmental factors, of which the most studied are vitamin D and viruses, particularly Epstein Barr virus (EBV).

1.3.2.1. Vitamin D

The high prevalence of MS in areas of lowest UV exposure led to the hypothesis that low vitamin D levels predispose to the disease. Vitamin D is synthesised in the skin by the action of UV light on 7-dehydrocholesterol, before conversion to its active form via steps in the liver and kidneys. The vitamin D receptor is expressed on neurons and glial cells of the CNS, where it has roles in neuroprotection and neurotrophism (DeLuca et al. 2013). In the immune system vitamin D is thought to have multiple effects including the generation of APCs with a tolerogenic phenotype and polarisation of CD4+ T cells to a regulatory phenotype (Correale et al. 2009, Koch et al. 2013).

Retrospective studies using stored serum samples from large cohorts of nurses and military personnel have provided observational evidence that the risk of MS is increased in individuals with low circulating vitamin D levels (Munger et al. 2004, Munger et al. 2006). Some studies have claimed to show evidence of vitamin D levels on relapse rate and progression (Simpson et al. 2010, Ascherio et al. 2014), but this remains contentious due to potential confounding factors (Papeix et al. 2013). Further corroborative evidence has been sought from the month of birth effect, which postulates that patients with MS are born at different rates through the calendar year due to changing levels in maternal vitamin D through the seasons. This is now thought to be a true effect, although smaller than initially suggested once controlling for confounding factors has been carried out (Disanto et al. 2013, Dobson et al. 2013, Fiddes et al. 2013, Hintzen 2013).

Treatment with high dose vitamin D has so far failed to result in lower relapse rates, although these studies have generally been small, poorly conducted, and further trials are underway to examine this question properly (James et al. 2013, Bhargava et al.

2014). GWAS have identified MS-associated variants in the region of two vitamin D hydroxylases, CYP24A1 and CYP27B1, providing further evidence for an aetiological role of vitamin D (Sawcer et al. 2011, International Multiple Sclerosis Genetics et al. 2013).

1.3.2.2. Viruses

A role of infection as the predominant environmental influence in MS has also been studied, principally the association of Epstein Barr virus (EBV) with the disease. 95% of the general population is seropositive for EBV antibodies, indicating previous infection with EBV, by early adulthood. Most primary infection occurs in childhood, but a proportion occurs during adolescence or later when primary infection causes a more significant illness called infectious mononucleosis (IM). Rates of MS in people who remain seronegative are very low – seronegativity has an odds ratio of 0.06 for developing MS (Ascherio et al. 2007) – whereas a history of IM carries an approximately 2-fold greater risk of developing MS (Nielsen et al. 2007). It is possible that early infection with EBV is indicative of exposure to multiple infections which gives rise to a more tolerant immune system, or conversely that a history of IM is a marker of exposure to multiple infections later in life, any of which might predispose to MS.

The mechanism by which EBV might predispose to MS is unclear, but one theory is that it persists in B cells and plasma cells which become immortalized and dysregulated. There is evidence that CNS-infiltrating B cells express viral antigens but it is unclear if these cells contribute to inflammation (Serafini et al. 2007, Serafini et al. 2010). Other theories are that the T cell response to EBV infection is dysregulated, either through reduced efficiency of viral extermination, leading to viral persistence (Jilek et al. 2012) or a misdirected immune attack due to molecular mimicry (Lang et al. 2002).

1.3.2.3. Other environmental factors

Several other environmental factors have been noted to affect susceptibility to MS. Smoking has long been known to increase the risk; the odds ratio depends on the duration and amount smoked, and there is significant interaction with underlying HLA architecture suggesting a biological interaction, possibly due to priming of lymphocytes as they traffic through the lungs (Hedstrom et al. 2009, Hedstrom et al. 2011, Hedstrom et al. 2013).

Dietary sodium intake has increased dramatically over the past few decades with the advent of mass-produced processed food, and parallels the increase in autoimmune disease incidence over this time. A high-salt diet worsens the course of EAE, and the presence of sodium chloride enhances the differentiation of CD4+ T cells into a pathogenic Th17 subtype *in vitro* (Kleinewietfeld et al. 2013). Initial observational studies suggest that increased salt intake in patients with MS correlates with increased clinical and radiological disease activity (Farez et al. 2015).

Commensal intestinal flora have a complex relationship with the host organism and contribute significantly to the development of the immune system; germ-free mice have altered gut-associated lymphoid tissue and lymphocyte populations (Pabst et al. 2006). EAE has a less severe course in germ-free mice, and in animals colonized with a single species of bacteria the severity of EAE may be better or worse depending on the colonizing species and the type of T cell they induce (Kamada et al. 2013). There is no evidence as yet linking the gut microbiome to multiple sclerosis in human studies.

Clearly, the role of the environment in MS pathogenesis is important, but there is still some distance to cover before that role is precisely defined. Many of the observational studies mentioned above demonstrate correlation but not causation. In time, the effect of certain public health measures – for instance the national recommendation of vitamin D supplementation in pregnancy – on MS incidence may elucidate further the role of these environmental factors.

1.4 Eomesodermin

1.4.1 The Eomesodermin locus

The *EOMES* gene on chromosome 3, also known as *TBR2*, encodes the transcription factor Eomesodermin (Eomes). SNPs in this region have been associated with MS in three separate GWAS (Patsopoulos et al. 2011, Sawcer et al. 2011, International Multiple Sclerosis Genetics et al. 2013) as well as with rheumatoid arthritis (RA) (Okada et al. 2014, Kim et al. 2015) and a trend to association with ankylosing spondylitis (AS) (IGAS et al. 2013). The association with autoimmune diseases other than MS suggests that the role of Eomes is not disease-specific but due to a more general dysregulation of immunity. The associated SNPs are in two regions of high LD, a "downstream" block spanning the *EOMES* gene from the 3-prime UTR to 39kb upstream, and an upstream intergenic region around 300kb from the *EOMES* transcription start site (TSS). Although LD is high within these blocks, there is no LD between them; the signals are independently associated.

The downstream LD block contains SNPs associated with all three diseases. The first association was of rs11129295 with MS; this SNP lies 25kb upstream of the TSS. This association was replicated and further refined using the Immunochip, which determined the highest associated SNP as rs2371108, about 500b downstream of *EOMES*. The variants associated with AS (rs1309849) and with RA (rs3806624) are also both in the downstream LD block and in LD with the MS SNP.

The upstream LD block has thus far yielded association only with MS, suggesting that this association may be disease-specific. The associated SNPs were first identified as rs669607 (Sawcer et al. 2011) and rs170934 (Patsopoulos et al. 2011) but in the Immunochip study this signal was refined to rs1813375. Several SNPs in this intergenic region are in near-perfect LD. *EOMES* is the most proximal downstream gene; upstream

genes include 5-Azacytadine-induced 2, which contributes to the activation of NFkB dependent gene expression, and CMC1, a mitochondrial protein.

1.4.2 Eomes function

EOMES is a highly conserved 6-exon gene on chromosome 3 that encodes a 686-amino acid transcription factor first discovered during developmental studies in Xenopus (Ryan et al. 1996), with its principal feature a T-box domain that binds the DNA sequence TCACACCT (Fig 1.2, 1.3). It has important and diverse roles in vertebrates, and is expressed from very early stages of development as well as later in the immune system. The T-box family contains a number of transcription factors, most of which are expressed during embryonic development, and mutations of which cause significant developmental abnormalities or are embryologically lethal (Minguillon et al. 2003).

1.4.2.1 Role in development

Early studies of Eomes in Xenopus demonstrated its expression in early embryogenesis and its role later in neural differentiation (Ryan et al. 1998). This finding was replicated in mice, where Eomes is an essential part of the transcription factor cascade in regions of neurogenesis, necessary for the transition from radial glial cell to intermediate progenitor cell and finally to neurons that project to the cortex (Englund et al. 2005, Hodge et al. 2008). In adult mice, Eomes is expressed in regions of neurogenesis, namely the subventricular zone and dentate gyrus of the hippocampus, which generate mature neurons that are subsequently integrated into the olfactory bulb and hippocampus, and may be important for learning and memory (Hodge et al. 2008, Deng et al. 2010).

Inactivation of *EOMES* in mice causes disrupted differentiation of cortical neurons and reduced numbers of neuronal progenitors, resulting in microcephaly (Arnold et al. 2008). Possibly mirroring this, a consanguineous Moroccan family with

several members affected by a syndrome of microcephaly and polymicrogyria was found to have a balanced translocation upstream of *EOMES*, with consequent silencing of *EOMES* in affected individuals (Baala et al. 2007).

1.4.2.2 Role in immunity

In the immune system Eomes is expressed by several cell types, with multiple roles in differentiation, maturation and function. It is predominantly expressed by NK cells and CD8+ T cells, and by a smaller proportion of CD4+ T cells as well as iNKT cells and γδ-T cells (Knox et al. 2014).

As described above, following detection of antigen, CD4+ and CD8+ T cells undergo clonal expansion and differentiation into effector and memory cells. In CD8+ T cells Eomes has a role in both the production of effector molecules and in the determination of effector and memory cell fate, where its balance with T-bet, a T-box transcription factor and paralogue of Eomes, is thought to be of importance (Intlekofer et al. 2005).

Its key role in the development of CD8+ memory T cells is illustrated by Eomes knockout mouse models, which have lower numbers of CD8+ memory T cells that show poor long-term survival and an inability to proliferate following subsequent antigen challenge (Intlekofer et al. 2005, Banerjee et al. 2010). During an immune response, determination of effector or memory function occurs early as a result of asymmetric lymphocyte division (Chang et al. 2007); analysis of gene expression during an immune response suggests that Eomes is expressed predominantly by those daughter cells associated with memory function (Arsenio et al. 2014). There are several different populations of CD8+ memory T cells, including a recently defined "stem-cell memory" subset that is thought to be the least differentiated and which gives rise to other memory populations. Among memory cells, Eomes is expressed at increasing levels by

progressively differentiated memory cells, highest by “effector memory” and least by “stem-cell memory” (Gattinoni et al. 2011).

During an immune response CD8+ T cells may become exhausted and up-regulate associated markers such as programmed death 1 (PD-1); these cells also show high expression of Eomes as well as cytotoxic molecules (Paley et al. 2012). However, other groups have suggested that PD-1 may be a marker of regulatory CD8+ T cells (Dai et al. 2010). Non-canonical CD8+ T cells, such as gamma-delta T cells and CD8+ innate-like lymphocytes also express high levels of Eomes (He et al. 2010, Gordon et al. 2011).

Eomes is expressed early in embryonic development with the consequence that a complete Eomes knockout is embryonically lethal. However, conditional knockouts, with loss of Eomes in early haematopoietic precursor cells, not only exhibit CD8 memory cell defects but also loss of NK and NKT cells, lineages that are dependent on the expression of CD122, the IL2R/IL15R β-chain which is a downstream target of Eomes (Intlekofer et al. 2005). Eomes and T-bet appear to play key roles in the differentiation of NK cells, as without T-bet or Eomes, NK cells do not develop. Using conditional knockouts, Eomes was shown to be necessary for both the initial maturation of NK cells and the stabilisation of these cells in the mature state, with lack of Eomes causing mature NK cells to downregulate NK-cell markers and to revert to functional immaturity (Intlekofer et al. 2005, Gordon et al. 2012). Eomes and T-bet drive the differentiation of distinct subsets of NK cells that populate the bone marrow or peripheral organs (Daussy et al. 2014).

In CD4+ T cells Eomes is expressed predominantly in pro-inflammatory Th1 cells, which can produce both IFN-γ and TNF-α (Edwards et al. 2014), and might even be used to differentiate Th1 from Th2 cells (Ono et al. 2014). T-bet is the predominant driver of Th1 differentiation, but Eomes also plays a role, particularly in repressing the transcription of the Th17 transcription factor RORc and cytokine IL-17a by binding to their promoter regions (Ichiyama et al. 2011). In this regard, Eomes can compensate for

T-bet in T-bet knockout cells when other polarising signals are blocked (Yang et al. 2008, Sabet-Baktach et al. 2013).

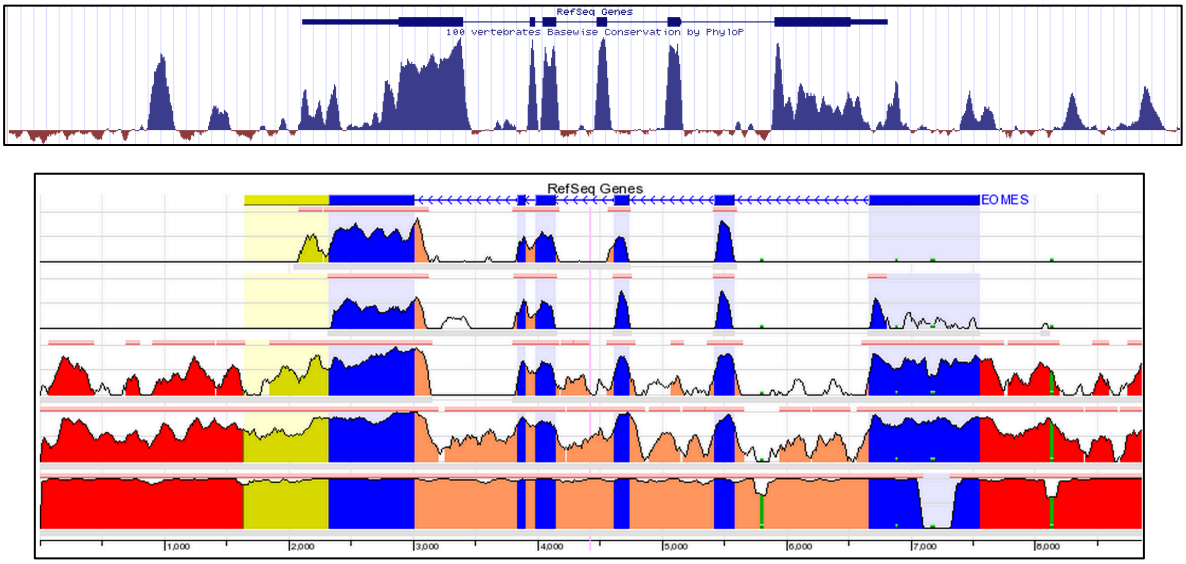
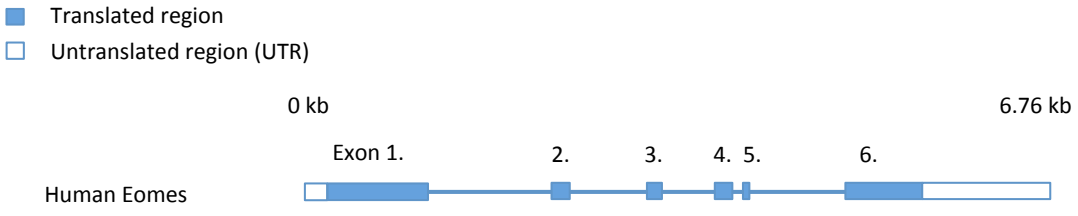


Fig 1.2. Conservation of *EOMES* gene. Upper panel: Conservation of the *EOMES* gene in 100 vertebrates by PhyloP (UCSC genome browser, https://genome-euro.ucsc.edu). Conserved sequences are shown in blue. Lower panel: Conservation of Eomes by pairwise alignment with human sequence (ECR browser; http://ecrbrowser.dcode.org/). Evolutionary conserved regions shown in blue have minimum 70% identical sequence. From top: *gallus gallus; xenopus tropicalis; mus musculus; canis familiaris; pan troglodytes.*

A.



B.

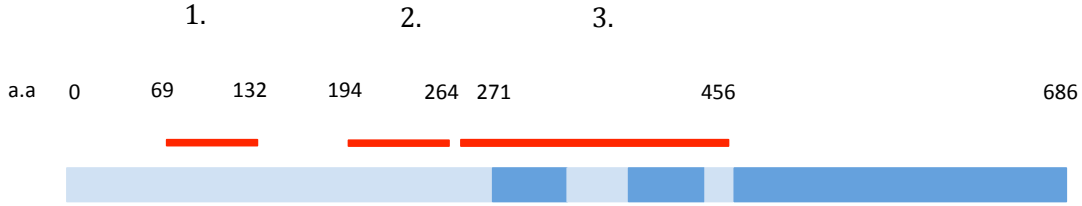


Fig 1.3. Eomes structure. A.Structure of the human *EOMES* gene. B. Eomes protein structure is shown. Red regions overlie protein features. 1. Alanine-rich sequence. 2. Glycine-rich sequence. 3. T-box DNA-binding domain.

1.4.3 Eomes: activation, regulation and downstream targets

The kinetics of Eomes expression must be assessed with those of T-bet, its T-box transcription factor paralogue with which it shares several overlapping functions. Eomes was first noted to be expressed in activated as opposed to naïve CD8+ T cells, where it was necessary for full effector function (Pearce et al. 2003). There are conflicting results from studies investigating Eomes expression following stimulation, which may be due to the time points analysed and the co-stimulatory cytokines used.

Following TCR stimulation alone, either with antigen or with an anti- CD3/CD28 stimulus, Eomes expression appears to be downregulated or unchanged (Agarwal et al. 2009, Cho et al. 2013). However, Eomes expression may be induced at later time points following contraction of the initial immune response and the emergence of a memory population with cytolytic capacity (Cruz-Guilloty et al. 2009). The type of TCR-stimulus may also have an effect. Cells reacting to low-affinity ligands are stimulated to express Eomes, and preferentially differentiate into central-memory T cells (Knudson et al. 2013). In the setting of a low-dose stimulus, the effect of cytokines may be of even greater importance; IL-4 upregulates Eomes to a much higher level in concert with a low-dose rather than high-dose TCR stimulus (Carty et al. 2014).

However, upregulation of Eomes may occur in the absence of a TCR stimulus. "Bystander activation" has been shown to transiently induce Eomes expression, granting these cells short-lived effector function (Marshall et al. 2010). IL-2 is a potent cytokine, produced by both CD4+ and CD8+ T cells, which is required for T cell survival and the generation of memory cells; stimulation with IL-2 causes up regulation of Eomes 3-4 days after the start of culture. This upregulation is blocked by the addition of IL-12, which predisposes to an effector-cell phenotype (Cho et al. 2013). The upregulation of Eomes may only be seen in antigen-specific memory cells weeks after the initial immune

response, after the repressive effect of IL-12 has diminished (Takemoto et al. 2006). However, the addition of either type-1 IFN or IL-12 to a TCR stimulus has also been reported to increase Eomes induction (Agarwal et al. 2009).

The signal transducer and activator of transcription (STAT) family are key effectors of cytokine signaling pathways, and following phosphorylation translocate to the nucleus. STAT3 is required for the optimal differentiation of CD8+ T cells into memory cells following infection, and STAT3 deficient cells show reduced Eomes expression as well as other markers of memory cells (Cui et al. 2011). STAT5 induces Eomes as part of a transcriptional program downstream of common gamma chain signaling; Eomes is a direct target of STAT5, which also acts in tandem with Eomes to induce transcription of effector molecules such as granzyme B and perforin (Pipkin et al. 2010, Grange et al. 2013).

A number of other transcription factors also induce or repress Eomes, either directly or indirectly. FOXO1 has a role in memory and effector determination, in part via induction of Eomes, which is a direct target (Rao et al. 2012, Hess Michelini et al. 2013). Deficiency of T cell factor 1 (TCF-1) causes impaired memory cell development, which may occur due to loss of Eomes expression; TCF-1 binds Eomes regulatory sequences and is required for optimal Eomes expression (Zhou et al. 2010). Inactivation of mTOR, a kinase involved in many cell processes including cell proliferation and survival, leads to upregulation of Eomes via downregulation of T-bet (Rao et al. 2010). Eomes is also constitutively repressed in CD4+ T cells by Ezh2, a polycomb protein and part of a repressor complex that trimethylates histone proteins, preventing DNA transcription (Tumes et al. 2013). A further mode of repression is via microRNA (miR)-29 which targets both T-bet and Eomes, preventing IFN-γ production in CD4+ T cells (Steiner et al. 2011).

In turn, Eomes has many downstream targets in terms of both effector and memory function. In CD4+ T cells it predisposes to a Th1 phenotype by binding to the

promoters of Th17 related genes, inhibiting their transcription (Ichiyama et al. 2011). This repression of type-17 markers also occurs in CD8+ cells, in tandem with T-bet; mice lacking the T-box TFs develop an abnormal Tc-17 phenotype in response to viral infection (Intlekofer et al. 2008). Transfecting CD4+ T cells with Eomes drives the expression of Fas ligand (FasL) and perforin, both mediators of cytotoxicity (Eshima et al. 2012). In CD8+ T cells Eomes binds to two different sequences in the perforin locus as well as to the IFN-γ TSS and CD122 promoter, driving expression of these molecules (Intlekofer et al. 2005, Cruz-Guilloty et al. 2009, Pipkin et al. 2010).

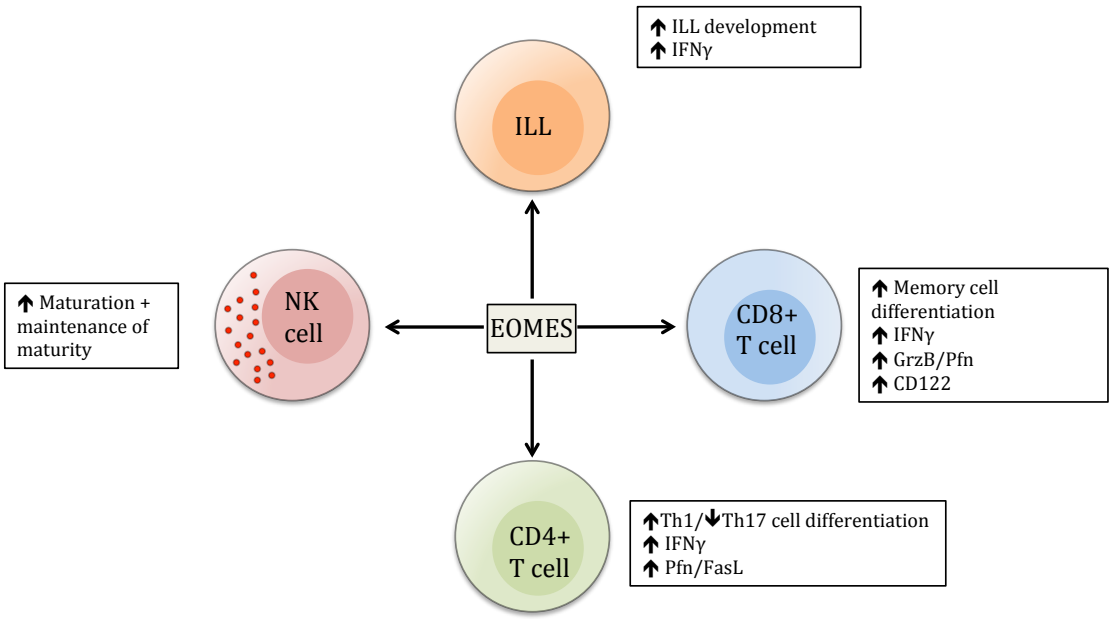


Fig 1.4. Major downstream effects of Eomes in lymphocyte populations. NK: natural killer; ILL: innate-like lymphocytes.

1.4.4 Eomes: role in disease

In disease, Eomes is dysregulated in certain lymphoproliferative syndromes including autoimmune lymphoproliferative syndrome that occurs due to a mutation in the Fas receptor, and the extra-nodal natural killer/T cell lymphoma, nasal type (Kinjyo

et al. 2010, Zhang et al. 2011). There is also evidence that in HIV infection and post-transplant cytomegalovirus (CMV) infection an altered T-bet/Eomes balance correlates with the persistence of virus and an inability to control viral replication, lending weight to the hypothesis that the balance of these two transcription factors is key to the control of chronic infection (Popescu et al. 2014). This is further supported by mouse studies, which show that elimination of either T-bet or Eomes-expressing CD8+ T cell subsets results in loss of control of chronic infection with lymphocytic choriomeningitis virus (Paley et al. 2012). In multiple sclerosis, Eomes and T-bet have both been shown to be expressed at lower levels in whole blood samples of patients when compared with matched controls, although the significance of this and its relevance to susceptibility to disease is not known (Parnell et al. 2014).

1.5 Aims

As outlined, SNPs from two LD blocks in the *EOMES* region are independently associated with multiple sclerosis susceptibility. SNPs in the region have also been associated with susceptibility to ankylosing spondylitis and rheumatoid arthritis, lending weight to the theory that these variants play a role in susceptibility to certain complex autoimmune diseases. The role of Eomes in the differentiation and function of a number of different immune cells makes it an interesting gene to study, as changes in its expression may have wide-ranging effects on a number of immune functions.

The overarching aim of this D.Phil was to determine the functional effect of the genetic variants in this region. As both variants are in non-coding regions it was hypothesised that the variants would exert an effect on gene expression. An initial aim was to use computational methods to fully characterise the genetic association with multiple sclerosis and other autoimmune diseases, and to examine the link between SNPs associated with disease and those associated with an effect on gene expression.

The study also aimed to characterise Eomes-expressing immune cell populations as well as changes in Eomes expression under culture and stimulation conditions. This would inform the third part of the study, which aimed to detect genotype-dependent changes in Eomes expression and downstream pathways.

Lastly, the project sought to characterise the role of MAIT cells, a functionally distinct population of CD8+ T cells that carries a semi-invariant TCR, in MS by examining CNS lesions for these cells. As these cells are cytokine-producing effector cells, genotype dependent changes in Eomes expression were also investigated in this population.

Chapter 2.

Materials and Methods

2.1. Computational studies

2.1.1 Analysis of multiple sclerosis associated variants

In order to further examine the genetic association of variants in the *EOMES* region with multiple sclerosis, genotype data on individuals of European ancestry from the *IMSGC 2013* study was obtained. This work was performed with Dr Adrian Cortes, who provided files of genotype data from the Eomes region for further association analysis. Dr Cortes had previously performed quality control (QC) and principal component analysis on this data set as part of a larger study. Data from 36,239 individuals passed QC comprising 16,691 cases and 19,548 controls. Genotype data from the *EOMES* locus was used to examine genetic association; these analyses and further examination of the associated SNPs were performed by the candidate.

The plink program (http://pngu.mgh.harvard.edu/~purcell/plink/) was used to perform unconditional and conditional analyses of genetic association (International Multiple Sclerosis Genetics et al. 2013). Genetic association was determined by logistic regression, with genotype as the independent variable and principal components included as covariates. Alleles of frequency greater than 0.5% were included. A p-value of lower than 5×10^{-8} was considered to be significant; this is the conventional threshold for genome-wide significance in GWAS, after adjustment for the number of SNPs tested on genotyping microarrays and to minimize false-positive associations. (Barsh et al. 2012)

2.1.2 Imputation analysis

Imputation of untyped SNPs was performed on genotype data by Dr Adrian Cortes as specified above, using reference genomes from the 1000genome database to infer non-typed variants (1000 Genomes Project Consortium et al. 2012, Delaneau et al. 2014). This involved phasing of the data (estimation of haplotypes), performed with SHAPEIT (https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html) and imputation of non-typed SNPs, performed with IMPUTE2 (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html) using default parameters.

The imputed genotype data was then used by the candidate to perform frequentist tests of association assuming an additive effect model using SNPTEST (Marchini et al. 2007); the additive (rather than multiplicative) model is the most commonly assumed model of susceptibility due to SNP genotype.

2.1.3 Epigenetic data

Available epigenetic data from primary cells was obtained from the blueprint epigenome project (http://www.blueprint-epigenome.eu). Associated SNPs were defined as those with a logistic regression p-value within two orders of magnitude of the lead SNP in that region. Any of the associated SNPs were considered as potential causal variants. These SNPs were aligned with tracks of epigenetic marks and colocalisation was assessed by localization of the SNP within the boundary of the reported epigenetic mark.

2.2 Capture-C

2.2.1 Samples

Peripheral blood was obtained from two donors with genotypes as shown in Table 2.1. An aliquot of peripheral blood mononuclear cells (PBMCs) from each donor was processed ex vivo as below. For donor A012 an aliquot of cells was also cultured for 24 h in Richmond Park memorial institute medium (RPMI) (supplemented with L-glutamine, penicillin, streptomycin and 10% human AB serum) in the presence of 30U/ml IL-2, anti-CD3/CD28 dynabeads (Gibco) in a 1:2 bead to cell ratio and 100ng/ml lipopolysaccharide (LPS, Sigma-Aldrich). After 24 h cells were harvested, washed in PBS and processed as in 2.2.2.

Table 2.1. Genotype of donor samples included in Capture C study

Donor	Genotype at rs11129295	Genotype at rs669607
A012	prot	risk
A145	risk	het

2.2.2 Generation of 3C library

This was performed as previously described (Hughes et al. 2013) and is summarised in Fig 2.1.

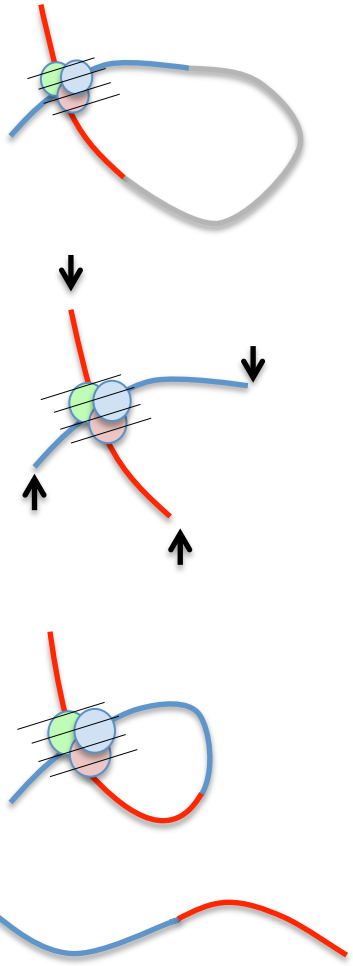
Briefly, mononuclear cells were obtained from peripheral blood as described in section 2.3.4. 10^7 cells were fixed in 2% formaldehyde for 10 minutes at room temperature before quenching with cold 1M glycine and resuspension in cold lysis

buffer (10mM Tris, 10mM NaCl, 0.2% Igepal CA-630 (Sigma Aldrich), cOmplete protease inhibitor (Roche). Samples were subsequently snap frozen and stored at -80°C.

Thawed samples were washed in ddH_2O, dounce homogenized, suspended in restriction buffer (0.25% sodium dodecyl sulphate, 1X restriction buffer (New England Biosystems) and agitated at 1400rpm at 37°C on an Eppendorf Comfort Thermomixer. After an hour, Triton-X100 (Sigma-Aldrich) was added to a final concentration of 1.5% and samples were agitated for a further hour. *Dpn*II restriction enzyme (New England Biosystems) was subsequently added to a concentration of 500U/ml and a restriction digest was performed for 12h at 37°C and 1400 rpm.

The enzyme was then heat inactivated and ligation of DNA ends was performed with T4 DNA ligase (Thermo Scientific) in 1X ligation buffer (Roche) overnight at 16°C and 1400 rpm on the thermomixer. This was followed by an overnight incubation at 65°C with proteinase K to remove any bound protein. Following Rnase treatment a phenol/chloroform method was used to extract the DNA, which was precipitated with ethanol. The resultant DNA libraries were assessed against undigested and uncrosslinked controls using gel electrophoresis and RT-PCR with custom designed Taqman primers and 3'FAM-5'TAMRA probes (Eurofins) shown in Table 2.1. Primers were designed against sequences spanning a *Dpn*II restriction site and against sequences between restriction sites to enable calculation of relative digestion efficiencies. Using these RT-PCR assays, digestion efficiencies were estimated to be in excess of 80%.

Generation of Capture-C library



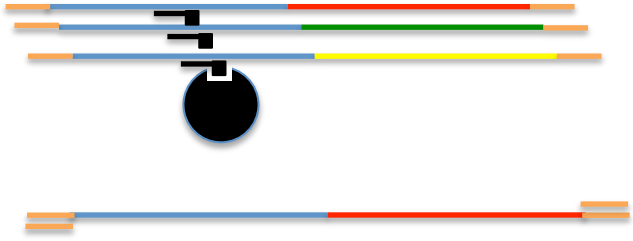
1. Formaldehyde cross-linking of interacting DNA sequences and transcription factors

2. Restriction enzyme digestion with *Dpn*II

3. Ligation of ends with DNA ligase

4. De-crosslinking and DNA isolation with phenol/chloroform to give single strand of interacting sequences

Capture-C



5. Capture-C: ligation of adapters, hybridization with biotinylated oligos and pulldown with streptavidin beads

6. Amplification with primers against adapters and paired-end sequencing

Fig 2.1. Capture-C: generation of library and capture of target sequences with hybridization oligonucleotides.

Table 2.2 Primers used for analysis of digestion and capture efficiency.

Primer set	Component	Sequence
EOMES 1	Forward primer	GGTAACTTCCCCTTCCTCCC
	Reverse primer	GGAAGGAAAAGGAGAGGGCT
	Probe	TGGCGCGCCCTGAATCCAGC
EOMES 2	Forward primer	GCCCTCTCCTTTTCCTTCCT
	Reverse primer	GCAGCTAGTGACAGCCCATA
	Probe	TAGCCCGCGCCTTTCTGCCC
EOMES 3	Forward primer	TTTGTCCCCATCCACCCAC
	Reverse primer	GTCACCTCCACCAGCCAA
	Probe	TAGCCCGCGCCTTTCTGCCC

2.2.3 Enrichment of target DNA sequences using bait oligonucleotides and sequencing

The DNA libraries were quantified using a Qubit fluorometer and 5µg was used as input for the next stage of the protocol. DNA shearing, end repair, addition of 'A' bases, adapter ligation and purification steps with AMPure beads were performed according to the Sureselect target enrichment system protocol steps 1-12 (https://www.genomics.agilent.com/files/LitItems/G3360-90010_SureSelect_IlluminaSingle_2.0.pdf). NEBNext multiplex oligonucleotides (oligos) for Illumina sequencing were used to adapter-ligate and amplify the libraries. Analysis of quality at each step was analysed with an Agilent Bioanalyser using a DNA1000 chip as per the user protocol.

1.5µg of adapter-ligated sample was used for DNA cleanup, hybridization to biotinylated custom-designed oligos (Table 2.2) (Integrated DNA Technologies) and LM-PCR amplification according to the NimbleGen SeqCap EX Library SR User's guide (http://www.nimblegen.com/products/lit/06588786001_SeqCapEZLibrarySR_UGuide_v4p2.pdf). Two sets of overlapping oligos were designed to capture regions of the *EOMES* promoter either side of a *Dpn*II restriction site (GATC). The resulting

hybridized sample was used to repeat these steps to perform a second round of capture. Validation of this method has previously been performed by the Higgs/Hughes lab (personal communication). The quality of the resulting target-enriched DNA library was assessed by Bioanalyser and the capture efficiency by RT-PCR. All output libraries fell within expected output guidelines. DNA was subsequently diluted to the optimum concentration for sequencing. Paired-end sequencing was carried out on an Illumina Miseq with a 150bp read length. Sequencing reads were mapped to the Hg18 build of the human genome and analysed for capture peaks aligning with known variants.

Preparation of samples, hybridization and amplification steps were performed by the candidate. Application of analysis scripts, previously developed by the Higgs/Hughes laboratory, and alignment to the genome was performed by Dr Adrian Cortes and Dr James Davies (Higgs/Hughes laboratory). Further analysis of Capture-C peaks with regard to colocalisation with MS-associated SNPs was performed by the candidate.

Table 2.3 Custom designed oligos to the *EOMES* promoter region. *Dpn*II restriction sites highlighted in red.

No.	Sequence
A1	AAAGCGCCGTGAGTTGGAAAGCAGGAGGTGGAAACTAACCCAGAAGCCCTCTCCTTTTCCTTCCTCGTACCTCTTGCTCCTCAGGACCCCCACCCTCCTCCCAGGCTCACACGCTGGAAG
A2	GCCGTGAGTTGGAAAGCAGGAGGTGGAAACTAACCCAGAAGCCCTCTCCTTTTCCTTCCTCGTACCTCTTGCTCCTCAGGACCCCCACCCTCCTCCCAGGCTCACACGCTGGAAGAAGGT
A3	GAGTTGGAAAGCAGGAGGTGGAAACTAACCCAGAAGCCCTCTCCTTTTCCTTCCTCGTACCTCTTGCTCCTCAGGACCCCCACCCTCCTCCCAGGCTCACACGCTGGAAGAAGGTGACTT
A4	GGAAAGCAGGAGGTGGAAACTAACCCAGAAGCCCTCTCCTTTTCCTTCCTCGTACCTCTTGCTCCTCAGGACCCCCACCCTCCTCCCAGGCTCACACGCTGGAAGAAGGTGACTTA**GATC**
B1	**GATC**TTTGTCCCCATCCACCCACTAGCCCGCGCCTTTCTGCCCTTTTTCAATTGCCAAAGCATCGGTCAAGTTGACCACTTGGAAACTTATGGGCTGTCACTAGCTGCTTTATATATTTG
B2	TTGTCCCCATCCACCCACTAGCCCGCGCCTTTCTGCCCTTTTTCAATTGCCAAAGCATCGGTCAAGTTGACCACTTGGAAACTTATGGGCTGTCACTAGCTGCTTTATATATTTGCGGCC
B3	CCCATCCACCCACTAGCCCGCGCCTTTCTGCCCTTTTTCAATTGCCAAAGCATCGGTCAAGTTGACCACTTGGAAACTTATGGGCTGTCACTAGCTGCTTTATATATTTGCGGCCAGGGA
B4	CCACCCACTAGCCCGCGCCTTTCTGCCCTTTTTCAATTGCCAAAGCATCGGTCAAGTTGACCACTTGGAAACTTATGGGCTGTCACTAGCTGCTTTATATATTTGCGGCCAGGGAGGGGC
B5	GGAAACTTATGGGCTGTCACTAGCTGCTTTATATATTTGCGGCCAGGGAGGGGCTTCCCGGCCTCACAGCCCGGAGACCCTCCTATTGGCTGGTGGAGGTGACACTAATTCAATTAGGCA
B6	CTTATGGGCTGTCACTAGCTGCTTTATATATTTGCGGCCAGGGAGGGGCTTCCCGGCCTCACAGCCCGGAGACCCTCCTATTGGCTGGTGGAGGTGACACTAATTCAATTAGGCATTTAC
B7	GGGCTGTCACTAGCTGCTTTATATATTTGCGGCCAGGGAGGGGCTTCCCGGCCTCACAGCCCGGAGACCCTCCTATTGGCTGGTGGAGGTGACACTAATTCAATTAGGCATTTACTAATT
B8	GTCACTAGCTGCTTTATATATTTGCGGCCAGGGAGGGGCTTCCCGGCCTCACAGCCCGGAGACCCTCCTATTGGCTGGTGGAGGTGACACTAATTCAATTAGGCATTTACTAATTGGATC

2.3 Peripheral blood studies

2.3.1 Samples

Lymphocyte cones were purchased from the NHS Blood and transplant service. Fresh blood samples for characterisation experiments were donated by healthy individuals who had given their consent under ethical approval from the National Research Ethics Service, Oxfordshire (REC B) for the project entitled "Studying the molecular mechanisms of action of genes with relevance to multiple sclerosis in healthy controls"; REC ref 10/H0605/5. Vacuette Safety Blood Collection Sets and sodium heparin-coated tubes were used to obtain the samples by qualified experienced phlebotomists.

Donors for genotype-dependent studies were obtained from the Oxford Biobank, in collaboration with Professor Fredrik Karpe, Research Nurse Jane Cheeseman and Bioresource Coordinator Dr Matt Neville (Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford). All samples were obtained from donors of self-reported white European Caucasian origin who declared themselves free of intercurrent infectious, chronic inflammatory, or autoimmune disease, and who were not taking immunomodulatory therapies. Following the genotyping of stored DNA, donors were recalled on the basis of genotype allowing enrichment of the cohort for donors homozygous for risk or protective alleles as described below.

The flow cytometry work was performed with the guidance of Dr Calliope Dendrou, Dr Hayley Evans, Dr Susanna Brauner and Dr Kate Attfield.

2.3.2 The use of a genotype selectable biobank

2.3.2.1 Power calculation

To estimate the number of donors necessary to recruit for the study, power calculations were performed taking into account previous studies of this type on SNPs with similar odds ratios (Dendrou et al. 2009, Gregory et al. 2012) as well as the results of a pilot study. The *a priori* hypothesis regarding Eomes expression was that expression would be significantly different by genotype in cell populations in which Eomes was likely to play a significant role; this was taken to be those cells that demonstrated substantial expression of Eomes as opposed to low or negligible expression, namely MAIT cells, CD56++ NK cells, and the three memory populations of CD8+ T cells. Both median fluorescence intensity (MFI) and proportion of cells expressing Eomes above isotype control (Eomes+ cells) were measured. Although these measures give slightly different information (see section 2.4.5) they are both measures of expression, and as such were not considered as independent variables. For these 5 cell populations and two independent genetic associations in the region this resulted in an adjusted p-value significance threshold of 0.005 (0.05/10). Using this significance threshold, and considering a possible difference in expression of 30% between homozygous genotype groups and a standard deviation of around 50% of the median value, ~30 individuals per genotype group would give the study approximately 70% power.

The power would be dependent upon inter-individual variability; a greater standard deviation would lower the power of the study. This might vary for different measures of Eomes expression and hence multiple measures were included as outlined below.

2.3.2.2. Recruitment of healthy donors

The allele frequencies for each of the SNPs genotyped (Table 2.3) meant that without prior genotyping, large numbers of individuals would be required to generate adequate numbers of homozygotes in order to power the study appropriately. Therefore, a collaboration was entered into with the Oxford Biobank; genotyping of the biobank cohort was carried out, and donors of the required genotype were recalled to give blood samples. Comparison of the genotype frequencies in the normal population, in our genotype-selected cohort, and the fold enrichment are detailed in table 2.4.

Table 2.4 Enrichment of cohort for homozygous individuals. Frequencies quoted are based on alleles being in Hardy-Weinberg equilibrium. MAF=minor allele frequency.

SNP	MAF	Expected frequency (%)		Observed Frequency (%)		Fold enrichment	
		Risk	Prot	Risk	Prot	Risk	Prot
rs669607	0.38	14.4	38.4	47	53	3.3	1.38
rs2371108	0.47	22	28.1	50	50	2.3	1.78

2.3.2.3. Pilot study and main study

A pilot study, to obtain initial genotype dependent expression data and provide rationale for extension into a larger cohort, was performed on retrospectively genotyped blood samples from healthy donors. In this pilot study a limited number of assays was performed, and analysis of Eomes expression was limited to CD4+ and CD8+ T cell populations and their subsets. The study was then expanded to a prospectively genotyped main cohort as outlined above. The numbers of donor samples in each genotype group for both the pilot and main cohort are outlined in table 2.5.

Table 2.5. Numbers of donors in each genotype group in pilot and main cohorts used for genotype-dependent studies.

	Pilot	**Main**
Total donors	55	62
rs237 risk	5	31
rs237 het	33	0
rs237 prot	18	31
rs669 risk	12	29
rs669 het	31	0
rs669 prot	12	33

Due to changes to the staining panels used on studies of the pilot and main cohorts, compensation values were changed and isotype control values were different between the two cohorts (CD8+ Tcm, new 2.25 v old 3.3; CD8+ Tem 2.2 v 3.3; CD8+ Temra 1.9 v 3.2; $p<0.001$ in each case). Therefore it was felt that measures relying on normalisation to isotype control could not be merged across cohorts. However, numbers of Eomes+ cells could still be accurately measured across both cohorts, and these results pooled, due to the nature of the bimodal population. Therefore, '% Eomes+' analyses of CD4+ and CD8+ T cells and their subsets include combined data from both the pilot study and the main study, and include a heterozygote group. These analyses include data from 117 individuals; downstream pathway analyses containing data from the main cohort, containing solely homozygotes, included data from 62 individuals. These numbers of individuals are comparable to other studies of this type (Dendrou et al. 2009, Couturier et al. 2011, Gregory et al. 2012).

2.3.3 Genotyping of human blood samples

2.3.3.1. Extraction of DNA

Genotyping of lymphocyte cones and the pilot cohort from the Oxford Biobank was performed following genomic DNA (gDNA) isolation. A QiaAmp DNA Blood midi kit (Qiagen) was used to obtain gDNA from 1-2 ml of blood following the manufacturer's protocol. The quality and quantity of gDNA obtained was determined using a Nanodrop 2000, ensuring that absorbance was between 1.7-2.1.

Genotyping of the second cohort of samples from the Oxford Biobank was performed by the Biobank, using previously lyophilized genomic DNA.

2.3.3.2. SNP genotyping

SNP genotyping was performed using the Applied Biosystems TaqMan SNP assays as per the manufacturer's instructions. These use allele-specific probes tagged with a different fluorochrome (VIC or FAM) and a non-fluorescent quencher. Fluorescence emitted from each probe on binding to newly generated sequences is detected and can be measured as a surrogate for amplification of the corresponding allele.

Upon receipt, assays were diluted to a 20X working stock in TE buffer (10mM Tris-HCl, 1mM ethylenediaminetetraacetic (EDTA) acid, pH8, RNase/DNase free sterile-filtered water) and aliquotted for storage at -20C. Reactions were performed in MicroAmp Optical 384-well reaction plates, sealed with an adhesive film lid, using an Applied Biosystems 7900HT Real-Time PCR System using the program in Table 2.6, and were performed in duplicate to ensure reliability. The reaction volume was 5µl, containing 0.25ul Taqman SNP genotyping assay (20X), 2.25µl gDNA and 2.5µl Taqman universal PCR mastermix (2X). Non-concordant duplicates were repeated and excluded if still non-concordant. On each plate, 2 wells were allocated as negative controls that

contained RNase/DNase free water in place of a DNA template. Raw data were exported from SDS software (Applied Biosystems) and analysed in Microsoft Excel.

Table 2.6 PCR settings for SNP genotyping

Enzyme activation & denaturation	95°C	10 min	1 cycle
Denaturation	92°C	15 s	40 cycles
Annealing & extension	60°C	1 min	

2.3.4 Isolation of mononuclear cells from peripheral blood

Peripheral blood in Vacuette tubes was agitated to ensure thorough mixing with sodium heparin prior to dilution 1:1 with room temperature, autoclaved phosphate buffered saline (PBS). 25ml of this mixture was then carefully layered onto 15ml of lymphoprep (Axis-Shield) before centrifugation at 800 x *g* for 20 minutes at room temperature in a Multifuge Heraeus centrifuge with the brake set to zero. The mononuclear cell layer was removed using a sterile pipette and washed twice with ice cold sterile PBS supplemented with 1% heat-inactivated human AB serum (Sigma Aldrich) at 1300rpm for 10 minutes. Cells were then kept on ice until use. Cell count and viability were estimated using trypan blue on either a haemocytometer or a Countess Automated Cell Counter (Life technologies). Viability was generally above 90%.

2.3.5 Cell stimulations for Eomes characterisation

PBMCs were obtained as described in section 2.3.4. CD4+ and CD8+ T cells were obtained by positive selection using CD4+ or CD8+ microbeads (Miltenyi) used as per the manufacturer's protocol. Cell viability and purity were assessed by flow cytometry and were both typically above 90%.

For initial studies of Eomes expression changes following TCR stimulation, isolated cells were suspended at 1 x 10^6 cells/ml in a U-bottom 96-well plate in RPMI supplemented with 10% hAB serum, penicillin/streptomycin, L-glutamine, 30U/ml IL-2 and a 1:1 ratio of anti-CD3/CD28 dynabeads (Gibco). At each time point cells from one well were washed, dynabeads were removed, and cells were lysed in RLT buffer (Qiagen) or stained for FACS analysis. For studies of IL-15, cells were cultured as above with or without the addition of 100ng/ml IL-15 at the start of culture. Cells were harvested at 0,24,48 and 72 h, lysed in RLT buffer (Qiagen) prior to RT-PCR analysis.

For comparison of different stimuli, isolated cells were suspended at 1 x 10^6 cells/ml in a U-bottom 96-well plate in complete RPMI (as detailed above) with 30U/ml rIL-2 (Peprotech) and either no further stimulus, 5 μg/ml LPS (InvivoGen), 500 U/ml IFN-γ (Peprotech) or anti-CD3/CD28 dynabeads (Gibco) in a 1:1 bead:cell ratio. Cells were harvested at 2,4, 18, 24, 48 and 66 h and lysed in RLT prior to RT-PCR analysis.

2.3.6 Fluorescence activated cell sorting of lymphocyte cones for RNA isolation.

CD8+ T cells were isolated from PBMCs by positive selection with CD8 microbeads (Miltenyi) used as per the manufacturer's protocol. After assessing viability, 20 million cells were stained for CD8, CD45RA and CCR7. Cells were sorted into four populations on a Beckman Coulter MoFlo with the help of Dr Craig Waugh, FACS facility, WIMM.

2.3.7 RNA extraction and cDNA synthesis

Cells were lysed in RLT buffer (Qiagen) and RNA was extracted using the RNeasy mini kit (Qiagen), adhering to the manufacturer's protocol. Briefly, ethanol was added to the lysate before applying it to a spin column with a silica membrane. Washing with

centrifugation was then performed to remove any contaminating substances before eluting the RNA with RNAse/DNAse free water. The concentration of RNA was measured using a Nanodrop ND-100 (Thermo scientific) and stored at -80°C.

RNA (0.5 µg or 1 µg) was used for cDNA synthesis by reverse transcription. The quantitect cDNA kit (Qiagen) was used as per the manufacturer's protocol. Briefly, up to 1 µg RNA was briefly incubated with gDNA wipeout buffer (Qiagen) and nuclease-free water to a total volume of 14 µl to remove genomic DNA. This reaction was then combined with reverse transcriptase, primer mix and RT buffer to a total volume of 20 µl in polyethylene tubes and used in the following program on a Bio-Rad Peltier thermal cycler: 42°C for 15 min, 95°C for 3 min, then 4°C. cDNA was stored at -20°C for future use.

2.3.8 Quantitative PCR

Primers for Eomes, T-bet and ubiquitin C (UBC) were designed using primer3, version 0.4.0 (www.frodo.wi.mit.edu/). cDNA sequences for the relevant gene were obtained from Ensembl and primers/probes were designed so that one of the oligonucleotides crossed an exon-exon boundary to exclude genomic DNA or pre-mRNA. Primers were designed so that their length was 18-22 nucleotides, melting temperature (Tm) was 58-60°C, GC content was 20-80% and the 5 nucleotides at the 3' end had 1-2 G or C bases. Probes were designed so that their length was 18-24 bases and Tm was 68-70°C. Primers and high performance liquid chromatography (HPLC)–purified probes were ordered from Eurofins, where it was specified that probes should be labelled with FAM and TAMRA at the 5'- and 3'- ends respectively. On receipt oligonucleotides were reconstituted in TE buffer. The sequences used are detailed in Table 2.6. For each primer/probe set a cDNA titration experiment was performed to plot a standard curve

of threshold cycle values and enable calculation of efficiency at a range of cDNA concentrations; this was between 95 and 105% for the concentrations used.

Table 2.7 Primers used for RT-PCR

Target gene	Primer1	Conc (nM)	Primer 2	Conc (nM)	Probe	Conc (nM)
Human *EOMES*	CGCATGTTTCC TTTCTTGAG	300	TTGTTGCCCTG CATGTTATT	300	AACCACTGGCG CTTCCAGGG	200
Human TBX21	CAGGAAGTTTC ATTTGGGAAA	300	ACTGGAGCACA ATCATCTGG	300	CAACAAGGGGG CGTCCAACA	200
Human UBC	TCGTCACTTGA CAATGCAGAT	300	CATCTTCCAGC TGTTTTCCA	300	TCCCTCCTGAC CAGCAGAGGC	150

Assays were carried out in triplicate in a 384-well polyethylene plate sealed with an adhesive film lid (Microamp, Applied Biosystems). Each reaction volume was 20 µl, containing 2 µl cDNA, primer/probe volumes as above, 10 µl Taqman Fast universal PCR mastermix 2X and nuclease free water at the required volume. The assays were performed on an Applied Biosystems Fast Real-Time PCR system, using the program detailed in table 2.8, and analysed with SDS version 2.4. Threshold for detection was automatically set. Data were exported for further analysis with Microsoft Excel and Graphpad Prism. To calculate expression levels, average Ct (housekeeping gene) was subtracted from average Ct (target gene) to obtain dCt, which is a measure of relative expression. Relative expression was ultimately plotted as 2^{-dCT} which provides the fold difference in gene expression.

Table 2.8 PCR settings for RT-PCR expression analysis

Enzyme activation	50°C	2 min	1 cycle
Initial denaturation	95°C	10 min	1 cycle
Denaturation	95°C	15 s	40 cycles
Annealing & extension	60°C	1 min	

2.4 Flow cytometry: genotype-dependent studies

2.4.1 Staining of unstimulated PBMCs for surface antigens and transcription factors

After isolation, cells were resuspended in PBS containing 1% hAB serum at a concentration of 10 x 10^6/ml. Tubes for staining with NK cell markers were additionally supplemented with 10% mouse serum. Fluorescently conjugated antibodies were added to 100ul resuspended cells and incubated at 4°C for 30 min. Cells were washed once in 30x volume of ice-cold PBS/1% hAB serum at 1300rpm for 5 min.

250 µl fix/perm solution (FoxP3/transcription factor staining buffer set, eBioscience) was added and cells were incubated at 4°C for 30 min. Cells were subsequently washed once in permeabilisation buffer (eBioscience) and resuspended in permeabilisation buffer at a concentration of 10x10^6/ml. Fluorescently conjugated antibodies to transcription factors were then added and cells were incubated at 4°C for 30 min. Cells were washed in ice-cold permeabilisation buffer by centrifugation as before and resuspended in ice cold PBS/1% hAB serum.

2.4.2 Stimulation and staining for cytokines

Isolated PBMC were suspended at 6 million cells/ml in a 24 well plate in RPMI with 10%hAB, L-glutamine, Penicillin/streptomycin supplemented with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) 50 ng/ml, ionomycin (Sigma-Aldrich) 500 ng/ml and GolgiPlug (BD-Biosciences), 1µl/ml. Cells were harvested after 3 h, washed in cold PBS/1% AB serum and resuspended at 10x10^6/ml. Surface antibodies were added to resuspended cells and incubated at 4°C for 30 min, then washed once in ice-cold PBS. 250µl of cytofix/cytoperm solution (BD Biosciences) was added and incubated at 4°C for 30 min, then washed twice in 1ml cytoperm buffer (BD Biosciences) by centrifugation as

before. Fluorescently conjugated antibodies against cytokines were added and incubated at 4°C for 30 min, washed in 2 ml ice-cold cytoperm buffer and resuspended in ice-cold PBS/1% hAB serum.

2.4.3 Th17 polarisation assay

CD4+ T cells were obtained from PBMCs by positive selection with CD4 microbeads (Miltenyi), used as per manufacturer's protocol. Purity was assessed by flow cytometry and was typically above 90%. 2.5 x 10^5 cells were plated out in triplicate in a 96-well U-bottom plate in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% hAB serum, penicillin/streptomycin and L-glutamine. For Th1 conditions anti-CD3/CD28 dynabeads (Gibco) were added in a 1:1 bead:cell ratio, with 30 U/ml IL-2 (Peprotech) and 10 ng/ml IL-12 (Peprotech). For Th17 conditions anti-CD3/CD28 dynabeads (Gibco) were added in a 1:50 bead:cell ratio with 30 U/ml Il-2, 10 ng/ml IL-1β, 10 ng/ml TGF-β, 10 ng/ml IL-23 (all Peprotech) and 30 ng/ml IL-6 (Miltenyi). Cytokines and media were refreshed at d3 and d6 for Th17 conditions, and d3, d6 and d7 for Th1 conditions. On d1 and d8, staining for cytokines was performed. Cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin. Cytokine release was blocked with 10 µg/ml Brefeldin A (Sigma Aldrich). After 5 hours cells were harvested, washed in cold PBS/1% AB serum and resuspended at 10x10^6/ml. Surface antibodies were added to resuspended cells and incubated at 4°C for 30 min, then washed once in ice-cold PBS. Cells were fixed in 1% formaldehyde, washed twice with saponin buffer (10% fetal calf serum, 1% bovine serum albumin (BSA), 0.5% saponin (all Sigma Aldrich)). Fluorescently conjugated antibodies against IL-17 and IFN-γ were added and incubated at 4°C for 30 min, washed in 2 ml ice-cold saponin buffer and resuspended in ice-cold PBS/1% hAB serum for analysis by flow cytometry.

2.4.4 Antibodies for flow cytometry

Optimum antibody concentrations were determined by titration. Isotype control antibodies were used at the same concentration as the primary antibody.

Antigen	Fluorochrome	Clone	Supplier
CD8	efluor450 (ef450)	OKT8	eBioscience
CD8	Brilliant Violet 510 (BV510)	RPA-T8	Biolegend
CD4	ef450	OKT4	eBioscience
CD45RA	Fluorescein isothiocyanate (FITC)	HI100	eBioscience
CD45RA	Allophycocyanin-efluor780 (APC-ef780)	HI100	eBioscience
CCR7	Allophycocyanin-Cyanin7 (APC-Cy7)	G043H7	BioLegend
CCR7	Phycoerythrin (PE)	G043H7	BioLegend
IFN-γ	AlexaFluor647 (AF647)	4S.B3	Biolegend
IL-17	FITC	BL168	BioLegend
IL-10	Peridinin-chlorophyll-protein complex-cyanine 5.5 (PerCP-Cy5.5)	JES3-9D7	BioLegend
IL-2	Allophycocyanin (APC)	MQ1-17H12	BioLegend
TNF-α	AlexaFluor488 (AF488)	MAb11	BioLegend
CD62L	PE	DREG-56	eBioscience
CD62L	BV510	DREG-56	BD Biosciences
Granzyme B	AF647	GB11	BioLegend
Perforin	AF488	dG9	BioLegend
PD-1	BV510	EH12.2H7	BioLegend
Eomes	Ef660	WD1928	eBioscience
CD25	PerCP-Cy5.5	M-A251	BD Biosciences
CD122	PE	TU27	BioLegend
CD127	PerCP-Cy5.5	A019D5	BioLegend
CXCR3	APC-Cy7	G025H7	BioLegend
CD3	BV510	OKT3	BioLegend
CD3	APC-ef780	UCHT-1	eBioscience
CD56	AF488	CB56	eBioscience
CD56	APC-ef780	CMSSB	eBioscience
Vα7.2 TCR	PerCP-Cy5.5	3C10	BioLegend
CD161	PE	HP-3G10	eBioscience

CD16	FITC	3G8	BioLegend
CD14	ef450	61D3	eBioscience
CD15	Pacific Blue	W6D3	BioLegend
FOXP3	FITC	206D	BioLegend
T-bet	PerCP-Cy5.5	4B10	BioLegend
CD19	PE	HIB19	BioLegend
mouse IgG1κ	PE	MOPC-21	Biolegend
mouse IgG1κ	ef660	P3.6.2.8.1	eBioscience
mouse IgG1κ	APC ef-780	P3.6.2.8.1	eBioscience
mouse IgG1κ	FITC	MOPC-21	BioLegend
mouse IgG1κ	PerCP-Cy5.5	MOPC-21	BioLegend
mouse IgG1κ	BV510	MOPC-21	BioLegend
mouse IgG2b κ	AF488	MPC-11	BioLegend
mouse IgG2b κ	AF647	MPC-11	BioLegend

Table 2.9 Antibodies used for flow cytometry.

2.4.5 Flow cytometry: data collection

Samples were run on a CyAn ADP Analyser flow cytometer using the Summit program (Beckman Coulter) for data collection. Files were subsequently analysed with FlowJo software version 9.7.6 (Tree Star, Inc., Ashland, OR, U.S.A.). Analyses were performed on a minimum of 500 collected events per gate of interest.

2.4.6 Approach to quantification

Numerous sources of potential variation were considered to allow these to be controlled for. Non-specific background staining due to Fc receptor (FcR) binding and non-specific interaction of antibodies with altered intracellular molecules was minimized by rigorously standardizing all protocols, and performing additional blocking with mouse serum in certain cases. A matched isotype control antibody was used to give an indication of background fluorescence. The isotype control antibody almost invariably stained 4-5 fold lower than any cell population; this may indicate a degree of ubiquitous Eomes expression, or a degree of non-specific staining that was not due to FcR binding.

To assess the degree of variability in inter-individual MFI that was due to non-specific FcR binding, isotype control MFI was plotted against uncorrected Eomes MFI for subsets that showed significant Eomes staining, and a linear regression performed to assess the level of correlation. This revealed significant correlation between isotype control MFI and Eomes MFI for CD8+ Tem (r^2 = 0.4465, p <0.0001) and Temra (r^2 = 0.2169, p=0.0002) populations, and $CD56^{dim}$CD16+ NK cells (r^2 = 0.1681, p=0.0009) (Table 2.10).

This is consistent with the literature which suggest that only a minority of CD8+ T cells express FcRs, in the form of CD16, which arise from effector-memory populations (Bjorkstrom et al. 2008, Clemenceau et al. 2008). Furthermore, no correlation was seen with Eomes MFI in CD56++ cells, which do not express CD16. Altogether, this suggests that isotype control antibody was effectively controlling for Fc receptor expression. Changing flow cytometer function over the duration of the study was monitored with the use of fluorescent calibration beads (Dako). For each day of study a calibration bead normalisation coefficient was determined by plotting the observed MFI of the calibration bead peaks against the molecules of equivalent fluorochrome (MEF) provided by the manufacturer and measuring the slope by linear regression. No correlation was observed between the bead slope and staining MFI, which likely reflects the stability of the flow cytometer over the relatively short time-frame of this part of the study and the greater contribution of other factors to staining variability.

Accordingly, fluorescence intensity was adjusted post-hoc by calculating fold MFI of the Eomes antibody over MFI of the isotype control. No further adjustment was made for the calibration beads.

In total, three measures of Eomes expression were determined and are presented: the proportion of Eomes+ cells in the designated cell subset; the fold increase in Eomes MFI above isotype control MFI in the whole of the designated subset; and the fold increase in Eomes MFI above isotype control MFI in the Eomes+ population of the

designated subset. These measures are not entirely independent measures but give different information regarding Eomes expression. The proportion of Eomes+ cells provides a measure of the number of cells in which Eomes expression has crossed a threshold, whereas the MFI provides a measure of the level of Eomes expression on a cellular level.

Cell subset	Isotype MFI v Eomes MFI		Beads MFI vs Eomes MFI	
	r^2	p-value	r^2	p-value
CD8+ Tcm	0.0600	0.06	0.0104	0.45
CD8+ Tem	0.4465	<0.0001	0.0013	0.79
CD8+ Temra	0.2169	0.0002	0.0024	0.72
CD56++CD16-	0.01040	0.43	0.0070	0.52
CD56+CD16+	0.1681	0.0009	0.0279	0.20

Table 2.10 Correlation of isotype MFI and calibration bead MFI with uncorrected Eomes MFI. (cm: central memory, CD45RA+CD62L+; em: effector memory, CD45RA-CD62L-; emra: terminally differentiated effector memory, RA expressing, CD45RA+CD62L-).

2.4.7 Controlling for other variables

As shown in table 2.10, average age was comparable across the genotype groups included in this study and no correlation was observed between age and Eomes expression in any of the immune cell subsets investigated. However, males and females were not equally distributed between all genotype groups with an excess of females in the rs2371108 prot group (Table 2.11). No significant differences were observed by sex for Eomes expression measured either as MFI or percentage positive cells. However, in some subsets there were trends that approached significance. The effect of sex on other immune parameters is well recognised and accordingly sex was included as a covariate

when performing the final analyses. All donors were of self-reported Caucasian European origin and were current non-smokers.

Group	Mean age (Range)	Males:Females
rs2371108 risk	44.9 (32-61)	20:16
rs2371108 prot	43.7 (34-54)	14:35
rs669607 risk	43.7 (34-61)	21:20
rs669607 prot	45.4 (32-53)	19:26

Table 2.11 Age and gender of donor genotype groups.

2.5 Histopathology

2.5.1 Samples

Serial sections containing snap frozen post-mortem brain tissue were obtained from the MS Society Tissue Bank at Imperial College, London. Ethical approval was granted by the Wales Research Ethics Committee (Ref. No. 08/MRE09/31) and covered all use of tissue provided by the Tissue Bank. Patient characteristics are outlined in table 2.12.

Case No.	Age	Sex	MS subtype	Disease duration (months)	Cause of death	Post-mortem interval (h)	Number of CD8+ T cells analysed in AL	Number of CD8+ T cells analysed in CAL
MS100	46	m	SPMS	105	Broncho-pneumonia	7	n/a	99
MS103	77	f	SPMS	252	Broncho-pneumonia	7	129	153
MS104	53	m	SPMS	132	Urinary tract infection	12	72	62
MS106	39	f	Probable SPMS	252	Broncho-pneumonia	18	n/a	59
MS107	38	m	SPMS	>192	Aspiration pneumonia, pulmonary oedema	19	55	96
MS122	44	m	SPMS	>120	Broncho-pneumonia	16	51	84
MS154	34	f	SPMS	132	Broncho-pneumonia	12	110	68
MS160	44	f	SPMS	189	Aspiration pneumonia, MS	18	102	64
MS180	44	f	SPMS	>120	MS	9	69	106
MS203	53	f	SPMS	214	MS	17	n/a	103

Table 2.12. Patient characteristics, post-mortem brain tissue. AL=acute lesions; CAL = chronic active lesions

2.5.2 Characterisation of multiple sclerosis lesions

Sections were fixed in 4% paraformaldehyde (PFA) for 10 min at 4°C and washed in PBS. Endogenous peroxidase was quenched by incubating with 3% hydrogen peroxide/PBS for 15 min at room temperature. Blocking was carried out with a solution

containing 5% normal serum, 3% BSA, 0.05% Triton-X100 in PBS, prior to incubation with monoclonal mouse anti-HLA-DP, -DQ, -DR (Dako M0775, 1:50) overnight at 4°C.

Detection was performed by incubating with HRP-conjugated polyclonal rabbit anti-mouse Ig (Dako P0260, 1:100) for 2 h at room temperature and using the BD Biosciences Pharmingen DAB substrate kit (ref. 550880, used as per manufacturer's protocol) to complete the staining.

To counter-stain with Luxol Fast Blue (LFB), sections were immersed in LFB solution (0.1% LFB, 0.1% glacial acetic acid in 95% ethanol) overnight at room temperature. The following day the sections were placed an oven at 60°C for 3 h, rinsed in alcohol and tap water, differentiated in 0.05% lithium carbonate and dehydrated by immersing in increasing concentrations of ethanol. Slides were mounted in Pertex mounting medium and viewed under a light microscope (Olympus BX60). Images were taken with a Qimaging QICAM digital camera.

2.5.3 Immunofluorescence and confocal microscopy

Sections containing active white matter lesions were incubated with blocking buffer (5% normal goat serum (Dako), 3%BSA, 0.05% Triton-X100, PBS) at room temperature before incubation overnight at 4 °C with primary antibodies directed against CD8 (Abd Serotec rat monoclonal, 1:100) and Vα7.2 (Biolegend mouse monoclonal, 1:50). Detection was performed using Alexa-Fluor 488 conjugated goat anti-mouse (Invitrogen, 1:300) and Alexa-Fluor 568 conjugated goat anti-rat (Invitrogen, 1:300) antibodies. Blocking for endogenous streptavidin/biotin was performed with a commercially available kit (Vector Laboratories) prior to overnight incubation with anti-CD161-biotin (Miltenyi mouse monoclonal, 1:10) and detection with streptavidin-Alexa-Fluor 647 (Invitrogen, 1:300) antibodies. Slides were mounted with fluorescent mounting medium (Dako).

2.5.4. Approach to image acquisition and analysis.

The analysis was limited to active cerebral white matter lesions that had been identified during lesion characterisation. Images were acquired with a Zeiss LSM 510 Meta laser-scanning confocal microscope through a ×63 objective. To reduce bias, each lesion was scanned and images were taken of cells staining positive for CD8 without reference to CD161 or Vα7.2 staining, which was analysed at a later time post image acquisition. Multiple images were taken to ensure at least 50 cells/lesion type were analysed per patient. In small lesions, images of all of the identified CD8+ T cells might have been acquired; in large lesions with several hundred CD8+ T cells, effort was made to acquire images of randomly selected T cells. Both parenchymal and perivascular CD8+ T cells were included in the analysis. Control sections, to exclude non-specific binding of the secondary antibodies or streptavidin, were stained as above but with the omission of primary antibodies.

Image analysis was performed using Metamorph version 7 software. Individual CD8+ T cells were manually outlined, then analysed for average Vα7.2 and CD161 staining intensity. Background staining of each antibody was determined by measuring the average intensity of an acellular, representative region from each image taken from a section and calculating the average intensity of these regions. The threshold for positive staining over background was derived from preliminary studies in thymus tissue. For CD8 and Vα7.2, positivity was designated as staining intensity five-fold greater than background. For CD161, positivity was designated as staining intensity greater than 3x background. This approach was then rigorously applied to data obtained from brain tissue to obtain the percentage of CD8+ T cells staining positive for Vα7.2, CD161, or both.

2.6 Statistical analysis

2.6.1 Flow cytometry data

Raw flow cytometry data was collected with Summit and analysed with FlowJo as described. The resulting data was organized with Microsoft Excel for Mac 2011 (Version 14.4.8) and GraphPad Prism for Mac OS X (Version 6.0e). Final analyses were performed with IBM SPSS Statistics (Version 22).

For analysis of genotype dependent data, a linear regression analysis was performed using the generalized linear model in SPSS, with number of risk alleles at one SNP locus as the dependent variable, and sex, age and number of risk alleles at the other SNP locus as covariates. A Bonferroni correction for multiple testing was used to adjust the significance threshold of the p-value. Each pathway was considered separately according to the *a priori* hypothesis. For tests of Eomes expression, potential effects of two loci in 5 principal cell types were considered to give a significance threshold of 0.05/10=0.005. For downstream pathways thresholds were adjusted as in table 2.13. This gave a projected power of between 40 and 72% to detect a genotype-dependent difference. For the cytokine assays, as IL17 was not detected in CD8 T-cells and this analysis could not be performed, the adjustment was as follows

CD4+ T-cells: 2 (SNPs) x 4 (cytokines) x 2 (subsets) = 16 analyses

CD8+ T-cells: 2 (SNPs) x 3 (cytokines) x 3 (subsets) = 18 analyses

Total analyses: 16+18 = 34

New threshold: 0.05/34 = 0.0015

Assay	Variables	Cell types	New threshold
Cytolysis	Granzyme B Perforin	CD8+ Tem/Temra	0.05/8 =0.0063
Transcription Factors	FoxP3 T-bet	CD4+ Tem/Tcm CD8+ Tem	0.05/12 =0.0042
Regulatory molecules	PD-1 CD122	CD8+ Tem/Tcm/Temra	0.05/12 =0.0042
Cell population size assays	PD-1+ MAIT NK		0.05/6 =0.0083
Cytokines	IFN-γ, TNF-α, IL-17, IL-2	CD4+ Tem/Tcm (all) CD8+ Tcm/Tem/Temra (IFN-γ, TNF-α, IL-2)	0.05/34 =0.0015

Table 2.13. Bonferroni corrected significance threshold for genotype-dependent analyses.

2.6.2 Immunohistochemistry data

Statistical analysis of immunohistochemistry data was performed in GraphPad Prism. For comparison of two groups a Mann-Whitney U test was performed for non-parametric data with a significance threshold of $p \leq 0.05$.

Chapter 3

Genomic studies of MS associated variants in the Eomesodermin gene region

3.1 Introduction

Knowledge of the genetic contribution to common disease susceptibility has been substantially increased by genome-wide association studies (GWAS), several hundred of which have been performed since the first was published on age-related macular degeneration in 2005 (Klein et al. 2005). They are predicated on a modified version of the common disease/common variant hypothesis: that genetic susceptibility to common diseases with a heritable component is due to the cumulative effect of multiple genetic variants, each individually with a low odds ratio for disease, that exist at relatively high frequency (>~5%) in the general population (Reich et al. 2001, Shields 2011). This is in contrast with the model of rare, monogenic inherited diseases that are often caused by a single, causative gene mutation. Due to the need to recruit a large number of participants in order to detect the subtle effect of any single disease-associated variant, GWAS are often carried out by international consortia performing single nucleotide polymorphism (SNP) genotyping of DNA obtained from thousands of cases and controls. The genotype data obtained are then tested for association, uncovering alleles that are unequally distributed between the case and control groups. Due to the vast amount of variation present in the human genome – many millions of common (frequency > 5%) SNPs

(1000 Genomes Project Consortium et al. 2010, 1000 Genomes Project Consortium et al. 2012) – not all variants are typed on a single genotyping array, particularly as GWAS can take advantage of the fact that due to linkage disequilibrium (LD) an association signal can be detected by genotyping for a single "tagging" SNP in an LD block. However, this tagging SNP may not be the causative SNP, which can complicate attempts to obtain a functional understanding of the biological consequences of the genetic variation in question. This can be partially overcome by imputation analysis using reference genomes, whereby genotype at SNPs not originally assayed can be statistically inferred to obtain a more complete picture of total regional genetic variation.

A 2011 GWAS genotyping for 1.2 million SNPs in almost 10, 000 cases and over 17,000 controls identified a total of 57 loci associated with MS susceptibility (Sawcer et al. 2011). A further large association study in 2013, using the Immunochip, identified 48 further loci (International Multiple Sclerosis Genetics et al. 2013). The Immunochip was designed by several autoimmune disease consortia to detect genome-wide associations, but also to genotype SNPs more densely in previously associated regions in order to better localize the association signals therein. These two studies, the largest primary MS GWAS to date, both identified two independent signals in the vicinity of the *EOMES* gene; the specific lead (most highly associated) SNPs in each study were different, but the signals persistently arose from two independent blocks of high LD, one several hundred kilobases (kb) upstream of the *EOMES* gene, and one spanning the *EOMES* gene from around 25kb upstream of the promoter to the 3-prime untranslated region (3'UTR). This finding, and the role of Eomes in the immune system, provided the starting point for this project. Prior to undertaking further work on the characterisation of the effects of these variants it was necessary to perform studies *in silico* to provide a sound basis for further work. This would give a more complete picture of the associated variants in the region, for instance regarding SNPs within coding regions or splice sites, or those in

other non-coding areas that might have a regulatory effect on the expression of *EOMES* or more distal genes.

Therefore, the overall aim of this chapter was to further delineate the *EOMES* region in order to obtain a more complete picture of the number of disease-associated SNPs, and to assess their relative functional importance by examining their specific localization.

Initially the association signals in the *EOMES* region were clarified by further analysis of association using data from the Immunochip study, with the aim of uncovering other SNPs in the region that were in high LD with the reported "lead" SNPs. This included fine-mapping of the region by imputation analysis to infer the genotype of SNPs not directly assessed using the Immunochip platform.

Following the generation of a credible set, here meaning those SNPs with p-values within two orders of magnitude of the lead SNP, the physical location of these SNPs relative to the EOMES gene was assessed; given the number of variants in non-coding regions that were not proximal to exon-intron splice junctions, evidence of regulatory potential was sought, and alignment of SNPs with regions of known epigenetic significance was performed using publicly available datasets. It was hypothesised that any regulatory effect of the SNPs on Eomes expression would involve long-range chromatin looping as has been demonstrated to occur in other genes (Hughes et al. 2014). Pilot studies in total peripheral blood mononuclear cells (PBMCs) were performed using the novel method Capture-C, which combines high-resolution chromatin conformation capture (3C) and next-generation sequencing (NGS).

Any regulatory effect of disease-associated SNPs in the *EOMES* region would be postulated to lead to genotype-dependent differences in gene expression. Data-mining approaches were used to investigate the existence of SNPs in the *EOMES* region that correlated with a change in Eomes expression (expression quantitative trait loci, eQTLs).

As outlined in Chapter 2, files of non-imputed and imputed genotype data were provided by Dr Adrian Cortes, who performed the quality control and imputation steps. Tests of association, and further analysis of the possible effects of MS-associated SNPs, were performed by the candidate. Colocalisation studies of both other autoimmune disease associated SNPs/eQTLs, and alignments with epigenetic data, were performed by the candidate. For Capture C studies, preparation of samples, hybridisation and amplification were performed by the candidate. Computational analysis of raw data and alignment to the genome were perfomed by Dr Adrian Cortes and Dr James Davies using pre-developed scripts. Further analysis, involving colocalisation of Capture-C peaks with MS-associated SNPs, was performed by the candidate.

3.2 Results.

3.2.1 Disease association analysis of Immunochip data

Raw genotype data from the IMSGC 2013 Immunochip study were obtained for a 700kb region spanning the *EOMES* gene and the PLINK program (http://pngu.mgh.harvard.edu/~purcell/plink/), which performs statistical tests of genotype-phenotype association, was used to analyse the region. Unconditional analysis showed the lead SNP was rs1813375 (OR 1.145, $p = 3.743 \times 10^{-19}$, 95%CI 1.11-1.179), as reported in the IMSGC study, with four additional SNPs in high LD (Table 3.1, Fig 3.1 A). This region of high LD is approximately 300 kb upstream of the *EOMES* gene.

Conditional analysis – controlling for the effect of the lead SNP from the region and analysing for secondary independent signals – revealed a second region of association with MS, more proximal to the *EOMES* gene. The lead SNP in the conditional analysis was rs11129295 (OR 1.1, $p = 1.078 \times 10^{-09}$, 95%CI 1.067-1.134) with eleven other SNPs in the region with p-values within two orders of magnitude (Table 3.1, Fig 3.1 B). Minimum LD in this block calculated by r^2 was 0.645. The highly associated SNPs detected in this region spanned the *EOMES* gene. One SNP, rs71624961, was located in the 3'UTR but no SNPs from this analysis were located in coding regions or introns.

A further conditional analysis did not reveal any remaining association signals in the region (Fig 3.1 C).

Primary signal					Secondary signal				
SNP ID (RA)	OR	RAF	p-value	r^2/D' to lead SNP	SNP ID	OR	RAF	p-value	r^2/D' to lead SNP
rs1813375 (A)	1.145	0.4895	3.74×10^{-19}	n/a	rs11129295 (A)	1.1	0.3749	1.08×10^{-09}	n/a
rs170934 (A)	1.145	0.4897	4.12×10^{-19}	0.999/1.000	rs9880772 (A)	1.096	0.4699	1.60×10^{-09}	0.672/0.997
rs36104507 (G)	1.144	0.4901	5.76×10^{-19}	0.997/1.000	rs12330493 (C)	1.098	0.3779	1.83×10^{-09}	0.987/1.000
rs669607 (C)	1.143	0.49	9.17×10^{-19}	0.997/0.999	rs2371108 (A)	1.096	0.4012	2.26×10^{-09}	0.814/0.954
rs438613 (G)	1.145	0.4897	9.96×10^{-19}	0.997/0.999	rs9866625 (A)	1.095	0.4705	2.29×10^{-09}	0.651/0.982
					rs3806624 (G)	1.093	0.4708	4.21×10^{-09}	0.657/0.987
					rs71624691 (A)	1.092	0.4692	4.96×10^{-09}	0.645/0.975
					rs34269949 (C)	1.091	0.4711	5.58×10^{-09}	0.665/0.994
					rs4680845 (A)	1.089	0.3528	3.77×10^{-08}	0.699/0.877
					rs4680844 (A)	1.089	0.3554	6.16×10^{-08}	0.690/0.867
					rs4680843 (A)	1.089	0.3554	6.51×10^{-08}	0.690/0.866
					rs12493245 (A)	1.085	0.3553	6.79×10^{-08}	0.690/0.867

Table 3.1. Lead SNPs from analysis of genotype data from IMSGC. SNPs were considered "credible" if the p-value was within two orders of magnitude of the lead SNP. RA: Risk allele; OR: Odds ratio; RAF: risk allele frequency

A.

B.

C.

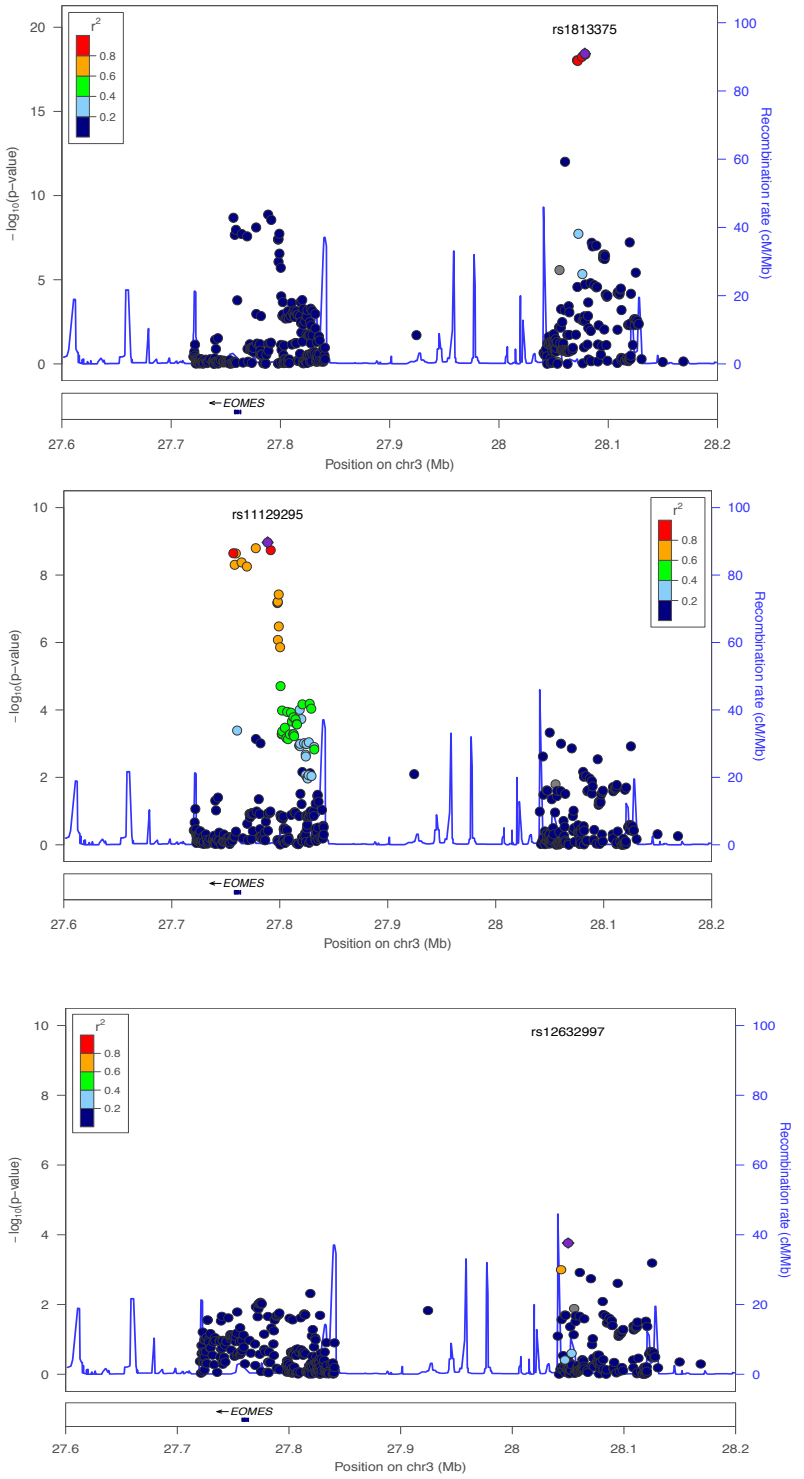


Fig 3.1. Association of *EOMES* region SNPs with MS from IMSGC data using PLINK. A. Unconditional analysis shows primary association signal with lead SNP rs1813375. B. Analysis after conditioning on rs1813375 shows secondary association with lead SNP rs11129295. C. Analysis after conditioning on rs1813375 and rs11129295 does not reveal any further association with genome-wide significance. Colours show LD with lead SNP. Plots were generated with locuszoom (Pruim et al. 2010).

3.2.2 Imputation analysis

In order to obtain a more complete list of MS-associated SNPs, including those not on the Immunochip SNP array, to inform functional analyses, variants were imputed from reference genotypes from the 1000 genome project. As mentioned in Chapter 2, Dr Adrian Cortes provided imputed genotype data for further analysis. In addition to SNPs the imputation analysis also enable the association of small indels to be evaluated.

Association analysis was performed using the SNPTEST program. For both the primary and secondary signals in the region a greater number of highly associated SNPs was obtained. Conditional analysis returned the same primary signal as the analysis of Immunochip data (rs1813375, $p=2.74968 \times 10^{-19}$; OR 1.17, CI 1.11-1.23. Table 3.2, Fig 3.2 A). Conditioning on this primary signal demonstrated the presence, as before, of a second signal arising from a region downstream of the primary signal with respect to *EOMES*, and spanning the *EOMES* locus. The lead SNP for this secondary signal was different to the previously reported lead SNP and was instead found to be rs5847475 ($p=1.16137 \times 10^{-13}$; OR 1.10, 95% CI 1.05-1.15, Table 3.2, Fig 3.2 B). Further conditioning on this SNP did not reveal any additional associations in the region (Fig 3.2 C). rs5847475 is a single-nucleotide insertion that lies upstream of *EOMES*. The second most significantly associated variant was rs368178421, a variable length in-frame insertion in exon 1 of *EOMES*. Two other SNPs within the credible set lie within the *EOMES* gene: rs71624691 within the 3'UTR, and the intronic SNP rs9866625 located100bp proximal to exon 6. The implications of these findings are discussed below.

Table 3.2. Lead SNPs from analysis of imputed genotype data. SNPs were considered "credible" if the p-value was within two orders of magnitude of the lead SNP. * : rs368178421 is a variable length non-synonymous in-frame insertion discussed in section 3.3. +: rs145548114 is a non-coding insertion upstream of *EOMES*. **: information unavailable. RA: risk allele; OR: Odds ratio; RAF: risk allele frequency.

Primary Signal				
SNP ID (RA)	OR	RAF	p-value	r^2/D' to lead SNP
rs1813375 (A)	1.17	0.49	2.75×10^{-19}	n/a
rs170934 (A)	1.17	0.49	3.47×10^{-19}	0.99/1
rs338611 (G)	1.17	0.49	3.58×10^{-19}	1/1
rs173082 (C)	1.17	0.49	3.62×10^{-19}	1/1
rs338603(C)	1.16	0.49	4.37×10^{-19}	1/1
rs36104507 (G)	1.17	0.49	4.38×10^{-19}	1/1
rs388368 (C)	1.17	0.49	5.37×10^{-19}	1/1
rs388448 (G)	1.17	0.49	5.42×10^{-19}	1/1
rs819991 (T)	1.17	0.49	5.90×10^{-19}	1/1
rs413544 (G)	1.17	0.49	6.11×10^{-19}	1/1
rs398390 (C)	1.17	0.49	6.47×10^{-19}	1/1
rs669607 (C)	1.17	0.49	6.87×10^{-19}	1/1
rs438613 (G)	1.17	0.49	7.59×10^{-19}	1/1
rs427289 (T)	1.17	0.49	7.74×10^{-19}	1/1
rs432888 (C)	1.17	0.49	1.05×10^{-18}	1/1

Secondary signal				
SNP ID (RA)	OR	RAF	p-value	r^2/D'
rs5847475 (TA)	1.10	0.40	3.03×10^{-13}	n/a
rs368178421 (*)	1.11	0.40	3.09×10^{-13}	0.89/**
rs12497690 (C)	1.10	0.38	3.71×10^{-12}	0.82/0.94
rs2371108 (A)	1.10	0.40	3.74×10^{-12}	0.84/0.93
rs4680838 (G)	1.10	0.47	3.87×10^{-12}	0.79/1
rs12635205 (G)	1.10	0.47	4.31×10^{-12}	0.79/1
rs9310852 (G)	1.10	0.47	5.82×10^{-12}	0.79/0.99
rs9880772 (A)	1.10	0.47	5.92×10^{-12}	0.79/1
rs11129295 (A)	1.10	0.37	6.60×10^{-12}	0.84/0.96
rs6773363 (C)	1.10	0.47	8.02×10^{-12}	0.76/0.96
rs1353286 (G)	1.10	0.47	8.27×10^{-12}	0.79/1
rs6806966 (T)	1.10	0.37	9.61×10^{-12}	0.83/0.96
rs9866625 (A)	1.09	0.47	1.11×10^{-11}	0.75/0.97
rs12330493 (C)	1.10	0.38	1.71×10^{-11}	0.85/0.96
rs3806624 (G)	1.09	0.47	1.82×10^{-11}	0.78/0.99
rs34269949 (C)	1.09	0.47	2.13×10^{-11}	0.78/0.99
rs6801231 (A)	1.10	0.38	3.21×10^{-11}	0.87/0.96
rs71624691 (A)	1.09	0.47	3.33×10^{-11}	0.75/0.97
rs145548114 (+)	1.12	0.45	3.89×10^{-11}	0.73/0.93

A.

B.

C.

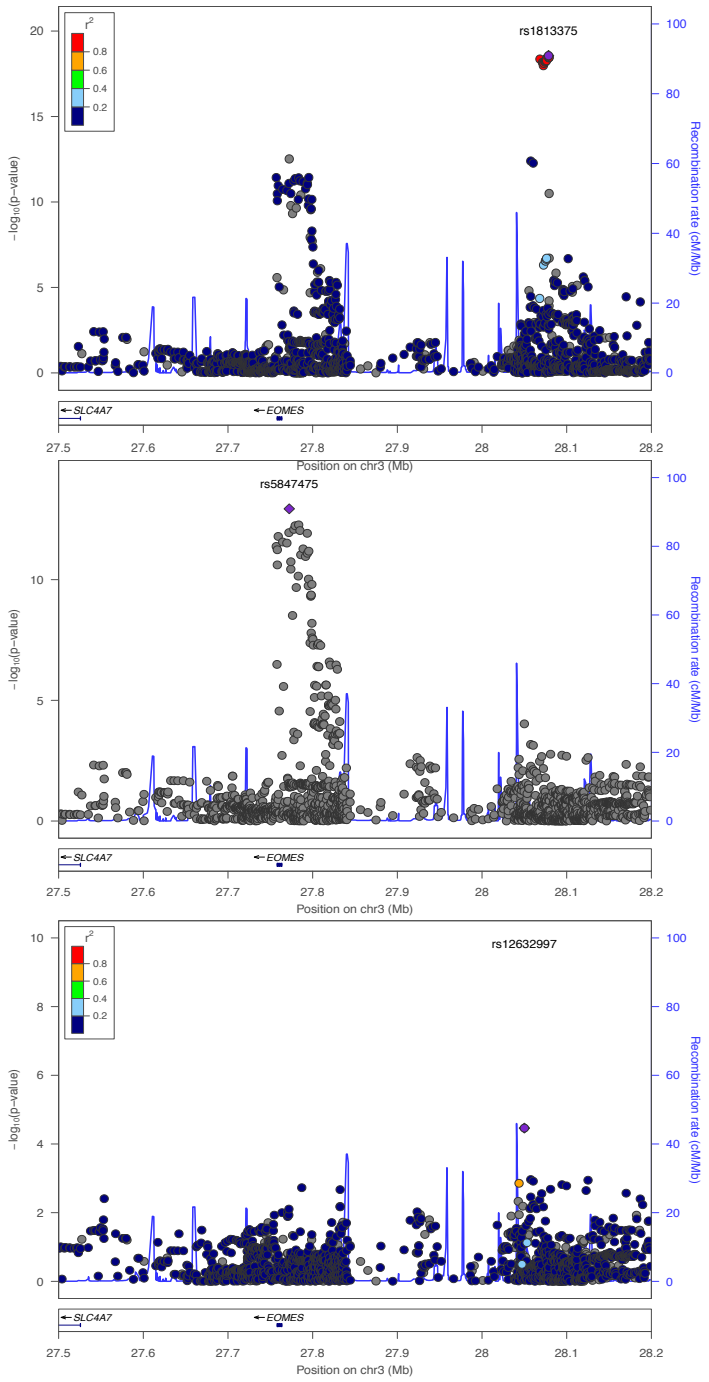


Fig 3.2. Association plot of imputed data using 1000genomes reference genotypes. A. Unconditional analysis shows primary association signal with lead SNP rs1813375. B. Conditioning on rs1813375 shows secondary association with lead SNP rs5847475. C. Conditioning on rs1813375 and rs5847475 does not reveal any further association with genome-wide significance. Plots were created with locus zoom using the Hg19 build of the human genome. Colours show LD with the lead SNP. Grey SNPs indicate insufficient information to show LD.

3.2.3 Colocalisation of MS- and other autoimmune disease- associated SNPs

Apart from multiple sclerosis, variants in the Eomesodermin region have also been associated with the autoimmune diseases ankylosing spondylitis (AS) (IGAS et al. 2013) and rheumatoid arthritis (RA) (Okada et al. 2014, Kim et al. 2015). However, the reported lead SNPs for these diseases are different to the lead SNPs reported for MS (see Table 3.2). For example, rs13093489, reported for AS, reaches association with MS at the genome wide significance level ($p = 1.80 \times 10^{-10}$, OR 1.09, 1.04-1.14) but is not within the cut-off of two orders of magnitude of the lead SNP.

Therefore, evidence was sought to elucidate whether or not all the disease-associated SNPs arose from the same signal, and whether the associations across diseases was concordant, as this might provide functional insight and shed light on a common autoimmune disease susceptibility pathway. Using imputed data an association analysis was performed, conditioning on each of the RA and AS SNPs, to determine their effect on the primary and secondary MS-association signals.

Conditioning on the RA and AS SNPs resulted in a greatly reduced p-value of the lead SNP from the MS secondary signal (Table 3.3, Fig 3.3). This suggests that these disease associations arise from the same signal. Furthermore, the directionality of the effect is the same throughout: the risk allele for MS is also the risk allele for RA and AS. This implies a common susceptibility pathway across these conditions. However, the primary MS signal appears to be disease-specific, and the p-value for the lead SNP rs1813375 was unchanged after conditioning for the other disease-associated SNPs.

SNP rsid	Associated Disease	OR of minor allele	rs1813375 p-value before conditioning	rs1813375 p-value after conditioning	r^2 to rs1813375	rs5847475 p-value before conditioning	rs5847475 p-value after conditioning	r^2/D' to rs5847475
3806624	RA (Kim et al)	1.09	2.75×10^{-19}	4.24×10^{-20}	<0.2/<0.2	3.03×10^{-13}	0.0036	0.78/0.99
9880772	RA (Okada et al)	1.10		3.90×10^{-20}	<0.2/<0.2		0.0095	0.79/1
13093489	AS (Cortes et al)	1.09		3.11×10^{-19}	<0.2/<0.2		0.0004	0.78/0.96

Table 3.3 Conditional analysis of association of imputed data, conditioning on other autoimmune disease associated SNPs.

A.

B.

C.

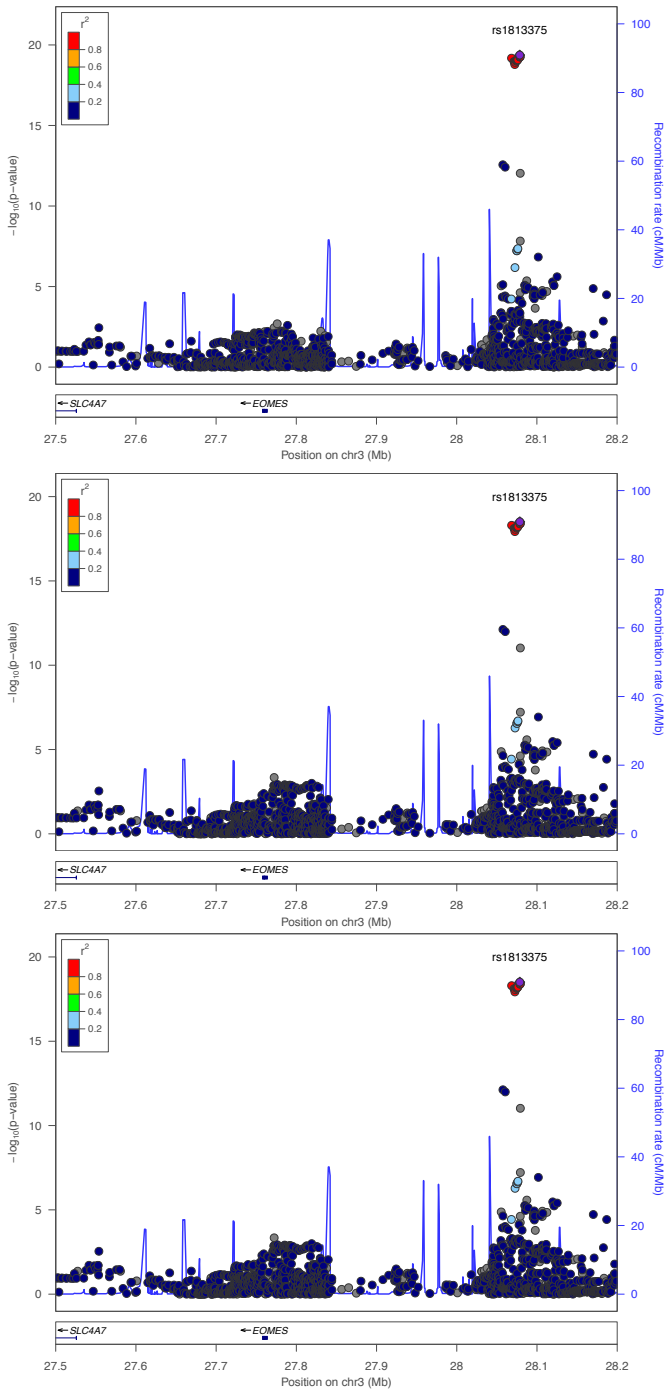


Fig 3.3. Association plots for MS after conditioning on other autoimmune disease SNPs. To examine whether lead SNPs associated with other autoimmune diseases arose from the MS association signal, a conditional analysis was performed for RA-associated variants rs3806624 (A) and rs9880772 (B), and AS-associated variant rs13093489 (C). Note loss of secondary signal peak in all three conditional analyses.

3.2.4 Colocalisation of MS-associated SNPs and eQTLs

Several large eQTL studies have been performed testing the association, at the genome-wide level, between SNP genotype and gene expression as measured by microarray or RNA-seq (Table 3.4). When investigating a disease-associated non-coding SNP, eQTL studies can provide valuable information about the effect of variants on mRNA expression in the cell types included. However, such studies may need to be interpreted carefully; different genotyping arrays and samples are used across studies, and even though a disease-associated SNP emerges as an eQTL, this may not explain the variation in the expression of a given gene as much as a non- associated SNP. This then calls into question whether the eQTL is likely to be the disease-relevant functional consequence of the SNP. Further examining these data can provide valuable information in terms of the cell types in which a SNP may be functionally important, and can provide additional supporting evidence regarding the role of disease-associated variants in affecting gene expression rather than structure or function, for example.

Mining these data revealed several Eomes eQTLs for the MS-associated SNPs in different cell-types, summarised in Table 3.4. eQTLs from Raj *et al*, in CD4+ T cells, and Fairfax *et al*, in LPS-stimulated monocytes, were located a large distance away from the MS-associated SNPs and outside the LD blocks for these variants, suggesting that these eQTLs are highly unlikely to drive the disease associations in the *EOMES* region. For the remaining SNPs a conditional analysis was performed for each eQTL in turn as for the RA and AS SNPs above to more closely evaluate the colocalisation of these eQTLs with the disease association signals (Table 3.4). The threshold for colocalisation was minimal residual association of the lead SNP, defined as $p>0.001$, following conditioning on the eQTL. This threshold was derived by dividing the p-value of least significant SNP in the credible set (3.89×10^{-11}) by the threshold for genome wide significance (5×10^{-8}). In two studies using lymphoblastoid cell lines (LCLs) (Grundberg et al. 2012, Stranger et al.

2012) five Eomes eQTLs were detected in total. Four of these colocalised with the primary association signal. One, rs2371258, did not colocalise with either of the MS signals. Two studies used whole blood (Westra et al. 2013, Battle et al. 2014) and detected six Eomes eQTLs. Three of these (rs11129295, rs13327021, rs12330493) colocalised with the secondary MS signal. Two (rs4680868, rs9817112) showed only a small effect on the secondary MS signal following conditioning (Table 3.5), implying that these variants may have an effect on expression distinct from other variants in the region. One, rs17021298, did not show any association with GWAS signals. No significant effect on the expression of other genes was noted for any of these variants.

Overall, the colocalisation of many Eomes eQTLs with MS-association signals provides further evidence that MS-associated variants in the *EOMES* region may have an effect on disease susceptibility via an effect on *EOMES* expression. The fact that studies on different cell types uncover different eQTLs suggests that regulatory mechanisms in this region may be tissue-specific, and therefore that MS-associated SNPs may have a cell type-specific effect.

Study	Cells analysed	Eomes eQTLs	p-value of eQTL	Direction of expression with minor allele	Location of SNP	LD (r^2)	
						rs181	rs584
eQTLs located within LD blocks containing MS-associated SNPs							
Grundberg et al 2012	LCL	rs388368 rs170934 rs669607 rs438613	7.5×10^{-5} 7.5×10^{-5} 7.5×10^{-5} 7.5×10^{-5}	Decreased Decreased Decreased Decreased	All ~300kb upstream of EOMES TSS	1 1 1 1	<0.2 <0.2 <0.2 <0.2
Stranger et al 2012	LCL	rs388368 rs170934 rs669607 rs438613 rs2371258	7.5×10^{-5} 7.5×10^{-5} 7.5×10^{-5} 7.5×10^{-5} *	Decreased Decreased Decreased Decreased *	All ~300kb upstream of EOMES TSS	1 1 1 1 <0.2	<0.2 <0.2 <0.2 <0.2 <0.2
Westra et al 2014	WB	rs11129295 rs13327021 rs4680868 rs9817112 rs12330493	4.5×10^{-5} 6.3×10^{-5} 1.1×10^{-4} 1.1×10^{-4} 1.1×10^{-4}	Increased Increased Increased Increased Increased	25 kb upstream 20 kb upstream 65 kb upstream 65 kb upstream 28 kb upstream	<0.2 <0.2 <0.2 <0.2 <0.2	0.84 0.82 0.51 0.51 0.85
eQTLs located outside LD blocks containing MS-associated SNPs							
Battle et al 2014	WB	rs17021298	2.3×10^{-6}	*	<500 kb upstream	<0.2	<0.2
Raj et al 2013	CD4+ Tcells	rs1488238 rs1386888	7.8×10^{-6} 3.0×10^{-6}	Decreased Decreased	1 Mb downstream	<0.2	<0.2
Fairfax et al 2014	LPS-treated Monocytes	rs820429	0.00024	*	>200 kb downstream	<0.2	<0.2

Table 3.4 . Summary of eQTL studies where Eomes eQTLs have been detected. * = information unavailable. LCL, lymphoblastoid cell lines; LD, linkage disequilibrium; WB, whole blood; rs181, rs1813375; rs584, rs5847475

Table 3.5. Conditional analysis of association of imputed data, conditioning on known eQTLs. Numbers in bold indicate a substantial effect of conditioning on the p-value, implying colocalisation.

	rs1813375 (primary signal)		rs58474745 (secondary signal)	
eQTL	Unconditional association p-value	p-value after conditioning on eQTL	Unconditional association p-value	p-value after conditioning on eQTL
Grundberg et al 2012				
rs388368		**0.0627**		1.18×10^{-13}
rs170934		**0.269**		1.16×10^{-13}
rs669607		**0.0438**		1.17×10^{-13}
rs438613		**0.0272**		1.18×10^{-13}
Stranger et al 2012				
rs2371258	2.75×10^{-19}	2.04×10^{-18}	3.03×10^{-13}	3.14×10^{-13}
Westra et al				
rs11129295		2.21×10^{-19}		**0.0111743**
rs13327021		2.70×10^{-19}		**0.00103**
rs4680868		2.08×10^{-20}		6.48×10^{-09}
rs9817112		2.00×10^{-20}		1.01×10^{-08}
rs12330493		1.78×10^{-19}		**0.00477**
Battle et al				
rs17021298		3.67×10^{-19}		2.98×10^{-13}

3.2.5 Epigenetic alignment

Analysis of eQTL datasets provided further evidence that the disease association in the *EOMES* region may occur via an effect on Eomes expression. These studies were performed on either whole blood or transformed B cells, and do not give any information as to the mechanism by which gene expression is altered.

To provide further confirmation for the eQTL signals and obtain insight regarding the possible mechanism, SNPs from both credible sets were aligned with epigenetic data obtained from EU Blueprint epigenome project (http://www.blueprint-epigenome.eu/). This project has produced genome-wide epigenetic data on multiple populations of primary cells and cell lines, predominantly from the immune and haematopoietic systems, displayed as peaks aligned with the human genome to enable localization and visualization of regulatory regions.

SNPs from the primary signal aligned only with h3k9 methylation peaks, associated with repression, in both innate and lymphoid populations (Fig. 3.4) (Berger 2007). SNPs from the secondary signal aligned with a number of other epigenetic marks. DNase hypersensitivity site data were only available for monocytes and macrophages, and no significant peaks were observed in this region (Fig 3.5). However, alignment of SNPs in the secondary signal region with methylation and acetylation peaks was observed in these and other cell types (Fig 3.6, 3.7). The distribution of epigenetic marks varied by type of mark, hence different SNPs aligned with different epigenetic marks. These are summarised in table 3.6.

In innate immune cells there was notable absence of a proximal h3k4me3/h3k4me1 peak suggesting that this region may be more important for Eomes expression in lymphoid cells (Figs 3.7).

Table 3.6. Alignment of MS-associated SNPs with epigenetic marks in multiple cell types.

Epigenetic mark	Significance	Aligned SNPs (cell type)
Primary signal		
H3K9me3	Associated with inactive promoters, transcriptional stability	rs427289 (monocyte, macrophages) rs413544 (NK cells) rs413544, rs398390, rs819991, rs388448 (CD4+, CD8+ T cells)
Secondary signal		
H3K27me3	Associated with transcriptional repression, inactive loci	rs2371108, rs36178421 (neutrophils, monocytes)
H3K36me3	Associated with transcriptional stability and mismatch repair	rs9866625 (CD8+ T cells)
H3K27ac	Associated with active/inducible loci	rs2371108, rs36178421 (CD8+ Tem cells)
H3K4me3	Associated with active promoters	rs368178421 (monocytes, macrophages, NK, CD4+ CD8+ T cells) rs3806624 (CD8+ T cells) rs1353286 (CD8+ T cells)
H3K4me1	Associated with enhancer regions	rs2371108 (CD8+ T cells) rs368178421 (neutrophils, monocytes, macrophages) rs3806624 (all cell types investigated) rs34269949, rs1353286, rs5847475 (monocytes, macrophages, NK, CD4+, CD8+ T cells)

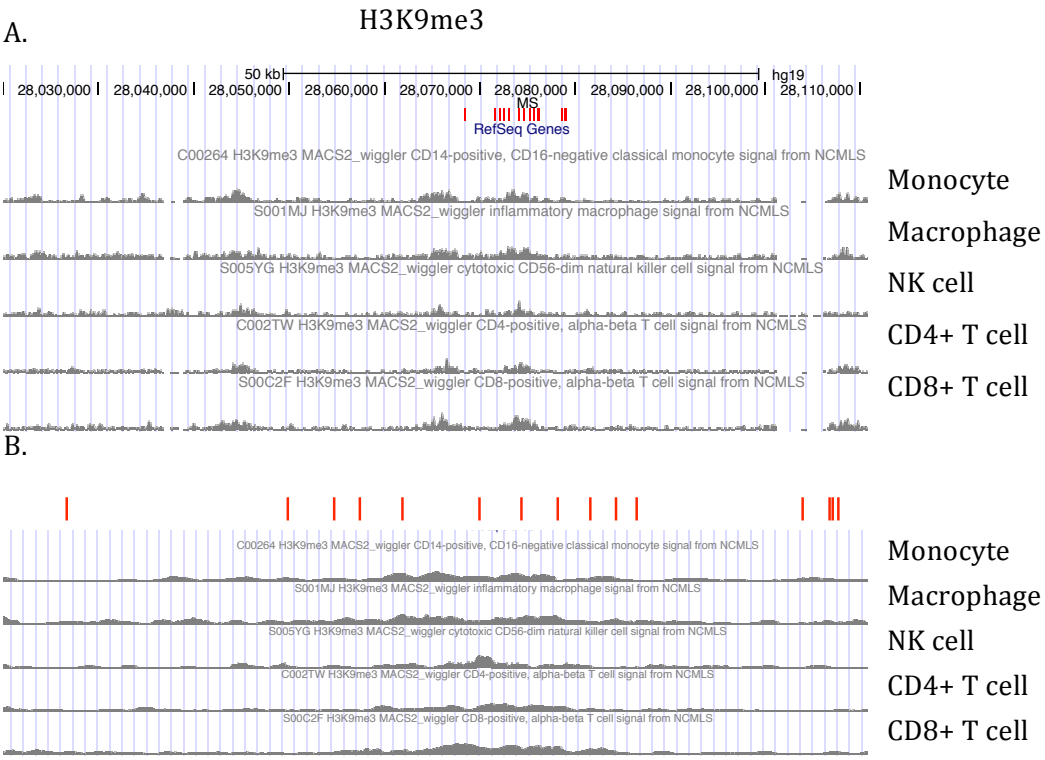


Fig. 3.4 Alignment of MS associated SNPs with epigenetic marks. MS associated variants were uploaded to the UCSC genome browser and aligned with Blueprint project epigenetic data. **A**. Overview of primary signal alignment with h39me3 marks. **B**. Close up of the region. SNPs from left: rs338603, rs669607, rs438613, rs432888, rs427829, rs413544, rs398390, rs819991, rs388448, rs36104507, rs427221, rs388368, rs1813375, rs173082, rs338611, rs170934

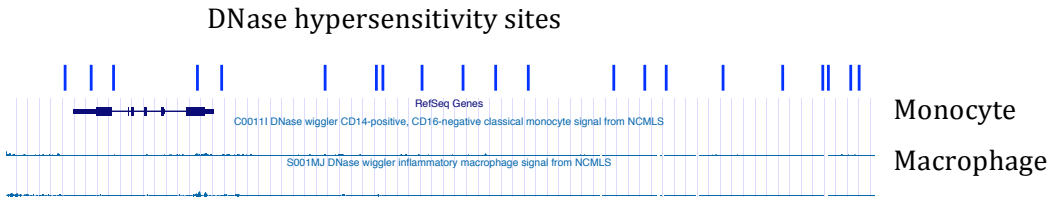
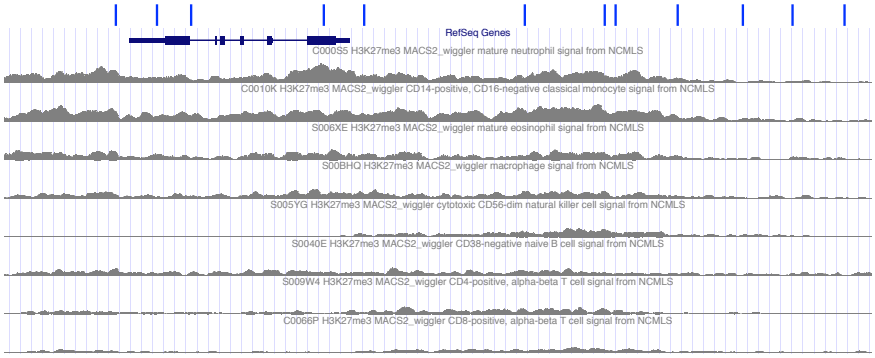


Fig 3.5. Alignment of secondary signal SNPs with DNase hypersensitivity sites. MS associated SNP were aligned with DNase hypersensitivity sites in macrophages and monocytes. SNPs from left: rs2371108, rs71624691, rs9866625, rs368178421, rs3806624, rs34269949, rs1353286, rs5847475, rs6801231, rs34860778, rs9880772, rs12635205, rs4680838, rs9310852, rs145548114, rs11129295, rs12330493, rs6773363, rs6806966, rs13093489, rs12497690

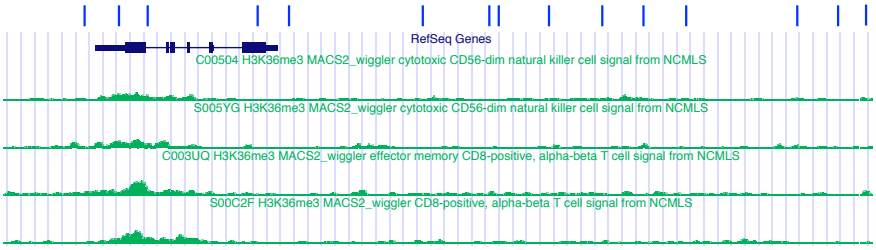
A.

H3K27me3



B.

H3K36me3



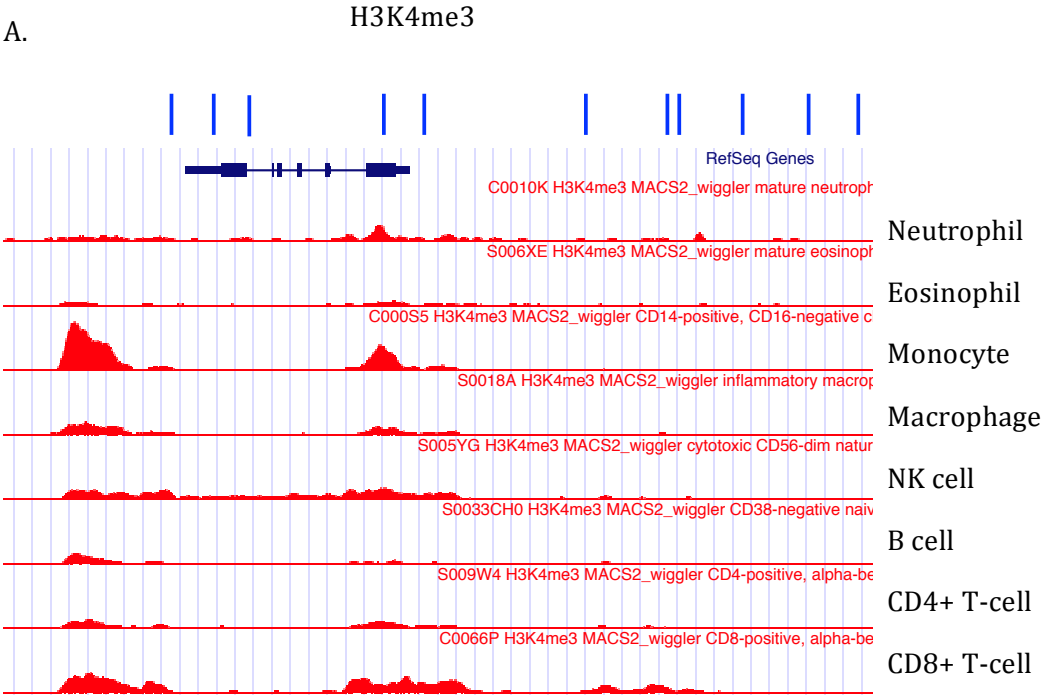
C

H3K27ac



Fig 3.6. Alignment of MS associated SNPs with epigenetic marks. SNPs in the secondary signal were aligned with epigenetic marks from Blueprint. **A**. Alignment with H3K27me3 marks associated with repression or inactive loci. **B**. Alignment with H3K36me3 marks associated with stabilisation of transcription and mismatch repair. **C**. Alignment with H3K27ac marks associated with active or inducible loci. SNPs from left as for Fig 3.5.

A.



B.

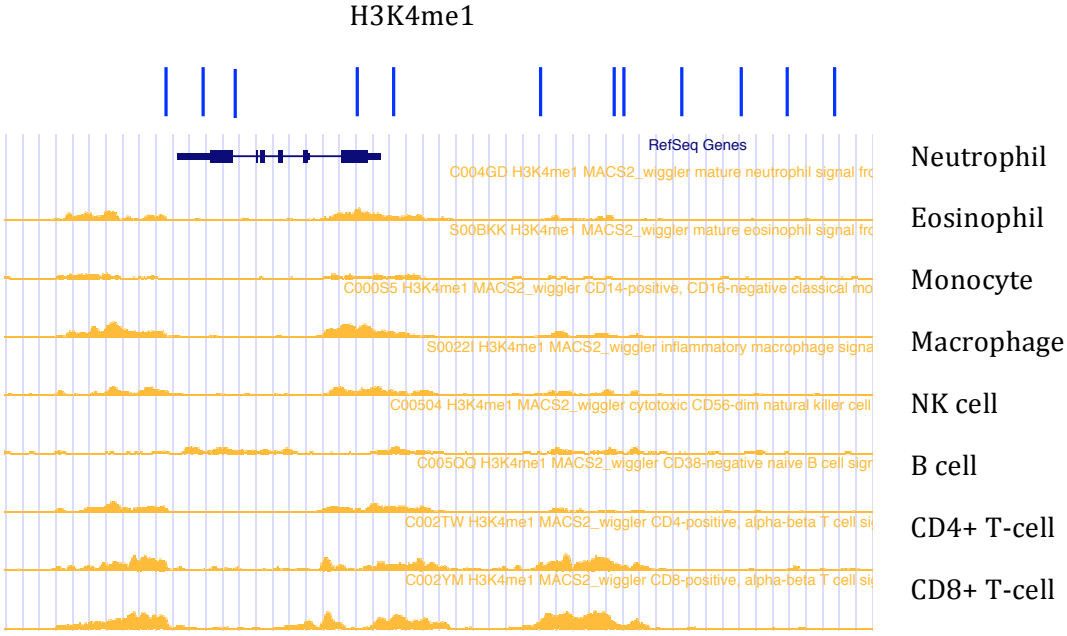


Fig 3.7. Alignment of MS associated SNPs with epigenetic marks.
SNPs in the secondary signal were aligned with epigenetic marks from Blueprint. **A.** Alignment with H3K4me3 associated with promoters. **B.** Alignment with H3K4me1 marks associated with enhancers. SNPs from left as for Fig 3.5.

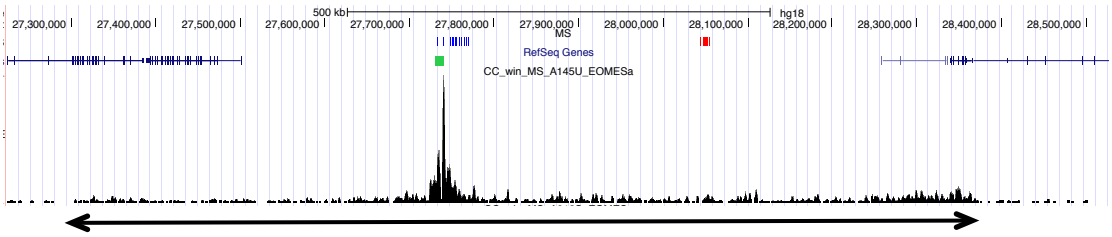
3.2.6 Capture C

Capture-C is a newly developed technique that combines chromatin conformation capture (3C) with enrichment of target sequences using bait oligonucleotides followed by high-throughput sequencing. It enables detection of interacting DNA sequences, for instance enhancer regions that are brought into contact with gene promoters via long-range chromatin looping. In this study, oligonucleotides were designed to "capture" the *EOMES* promoter region and any sequences in close physical proximity to it. These captured sequences were aligned to build 18 of the human genome and visualised on the UCSC genome browser.

Three samples of total PBMCs were used from two individuals, one of which was also stimulated for 24 h with anti-CD3/CD28 dynabeads and LPS prior to the study. In all samples the highest signal was observed proximal to the *EOMES* gene, but further interactions over a region spanning 1Mb (Fig 3.8 A) were also demonstrated. The highest peak of signal is artefactual and corresponds to sequences directly interacting with the bait oligonucleotides. None of the SNPs in the region containing the primary signal, around 300kb upstream of the *EOMES* promoter, aligned with any clear peaks of interaction (Fig 3.8 B). This has several possible explanations, which are discussed in section 3.3.

Around 5kb upstream of the promoter region was a further high signal peak, present in all samples, that aligned with the most associated SNP from the secondary signal (Fig 3.9), and appears to broadly correspond with epigenetic marks in peripheral blood mononuclear cells as shown above. Although this is a preliminary study, this is possible evidence of interaction between regions containing disease-associated variants and the *EOMES* promoter. No differences were observed between stimulated and unstimulated cells here, but to explore this fully would require further work in different cell types and under various stimulation conditions and time points.

A.



B.

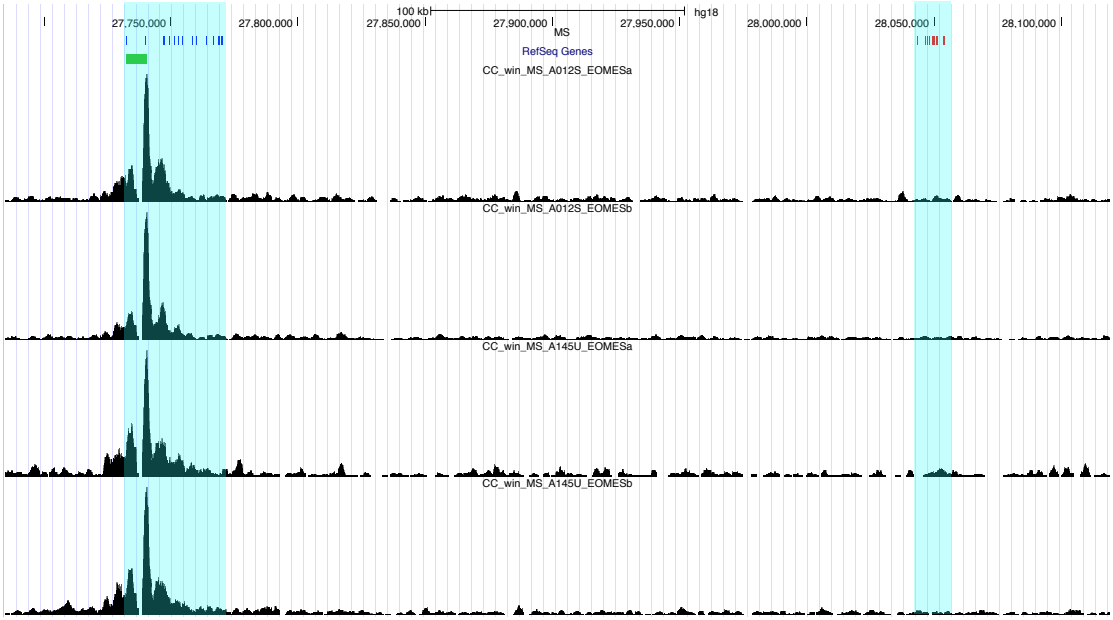


Fig 3.8. Capture-C studies of the *EOMES* promoter region. A. Overview of captured sequence peaks. Span of captured interactions demonstrated by arrow approximating 1Mb. B. Interaction peaks corresponding to regions of primary and secondary MS association signal. Regions of MS association signal highlighted in blue. Green boxes represent the *EOMES* gene. Tracks for two sets of bait oligonucleotides are shown for the stimulated sample (upper two tracks) and one representative unstimulated sample (lower two tracks).

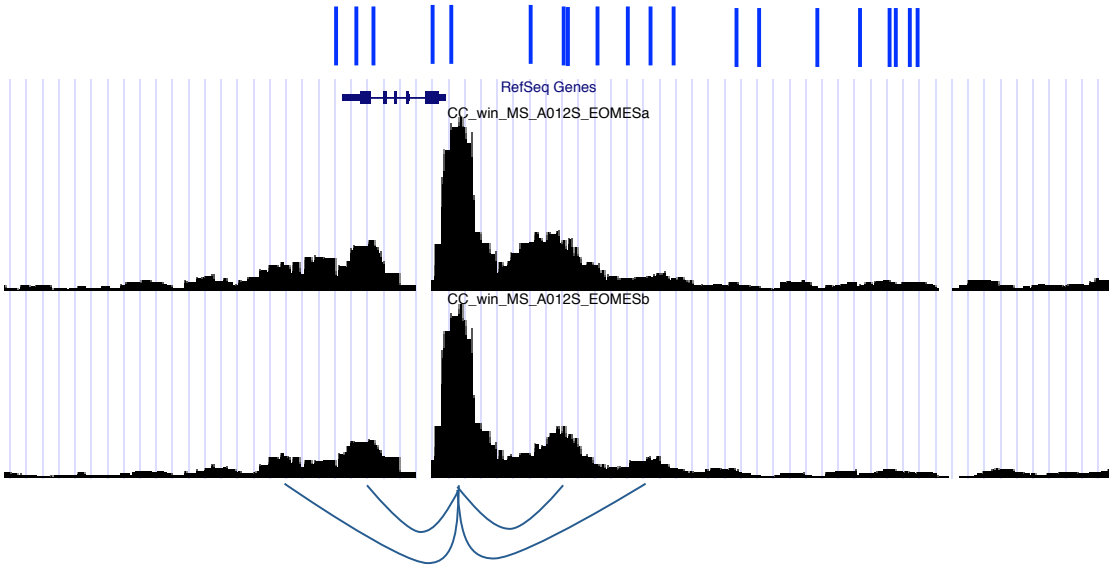


Fig 3.9 Capture-C studies of the *EOMES* promoter region. Focus on region of secondary MS association signal and alignment of SNPs with Capture-C peaks. The figure shows representative tracks for PBMCs from one individual using two different sets of bait oligonucleotides as described in chapter 2. Tracks shown are from one sample of stimulated PBMCs using two sets of bait oligonucleotides. SNP from left: rs2371108, rs71624691, rs9866625, rs368178421, rs3806624, rs34269949, rs1353286, rs5847475, rs6801231, rs34860778, rs9880772, rs12635205, rs4680838, rs9310852, rs145548114, rs11129295, rs12330493, rs6773363, rs6806966, rs13093489, rs12497690.

3.3 Discussion

The over-arching aim of this chapter was to further characterise the two MS association signals in a large region on chromosome 3 that includes the *EOMES* gene. Evidence was also sought that these variants altered susceptibility to disease via an effect on *EOMES* rather than via effects on other genes in the region. Lastly, by examining epigenetic marks in the region and performing initial capture-C studies, the study aimed to determine if there was any preliminary evidence that the variants localize to regions of regulatory importance.

Initially, an analysis was performed on Immunochip genotype data from the *EOMES* region to generate a list of highly associated variants; as expected this showed a primary signal of association, with the lead SNP rs1813375, 300kb upstream of *EOMES*, as reported in the IMSGC 2013 paper, and four other SNPs in high LD. Conditional analysis on rs1813375 revealed a secondary signal; the lead SNP in this analysis was rs11129295, which was initially reported as the lead SNP for this region in Sawcer *et al* 2011 but was more recently revised to rs2371108 in the 2013 Immunochip analysis. This may be due to small differences in the principal component analysis, but these two SNPs are both highly associated with MS and in high LD, and therefore both act as tag SNPs for this secondary signal. Further conditional analysis did not reveal any significant tertiary associations.

Subsequently, an association analysis was performed on genotype data imputed from 1000 genomes reference data. As mentioned above, GWAS do not type for every SNP present in the genome but for a selection of SNPs that "tag" a region of high LD. Although the Immunochip was designed to densely type autoimmune-disease associated regions and enable a degree of fine-mapping, it still misses a considerable portion of genetic variation including untyped SNPs, large variants (such as CNVs) and small indels; only 718 indels are included on the Immunochip, a tiny proportion of the many

hundreds of thousands that exist in the human genome (Mills et al. 2011). Here, association analysis was performed on imputed genotype data using 1000 genomes datasets to infer genotype at untyped SNPs and small indels. This showed the same lead SNP, rs1813375, for the primary signal, but a single base insertion, rs5847475, as the lead SNP for the secondary signal.

The second-highest associated variant in this region was rs368178421, a variable length in-frame insertion within exon 1 of *EOMES* that results in addition of a glycine residue, with or without one or two alanine residues, to the alanine-rich sequence of Eomes after amino acid position 120 (Fig 3.9). This indel is predicted to have a neutral effect by both the SIFT and PROVEAN analysis tools (Ng et al. 2003, Choi et al. 2012). However, these programs may miss subtle effects of this insertion, which would be expected given the low odds ratio for disease of these SNPs. The precise role of the alanine-rich sequence is unknown, but low-complexity regions have been postulated to have a role in regulation of transcription (Rado-Trilla et al. 2012).

Imputation also revealed two other associated SNPs within the Eomes gene: rs71624691 in the 3'UTR, and rs9866625 which is in intron 5, around 100bp from the exon 6 boundary. This SNP does not appear to localize with a splice site as predicted by the SplicePort tool (Dogan et al. 2007), consistent with its distance from the exon-intron junction. Overall, imputation analysis discovered several more MS-associated variants, some of which were within the *EOMES* gene, giving a more complete picture of genetic variation in this region and demonstrating the value of imputation analysis.

SNPs in the *EOMES* region have also been associated with RA and AS. To elucidate a possible common pathway underlying an effect on susceptibility to multiple autoimmune diseases, an analysis was performed conditioning on SNPs associated with RA and AS; this showed that the association with RA and AS is the same signal as the secondary MS association. The direction of the effect on susceptibility is concordant, meaning that risk alleles are the same for all three diseases. Conditioning on the AS and

RA associated SNPs had no effect on the primary MS signal, implying that this association is specific to MS.

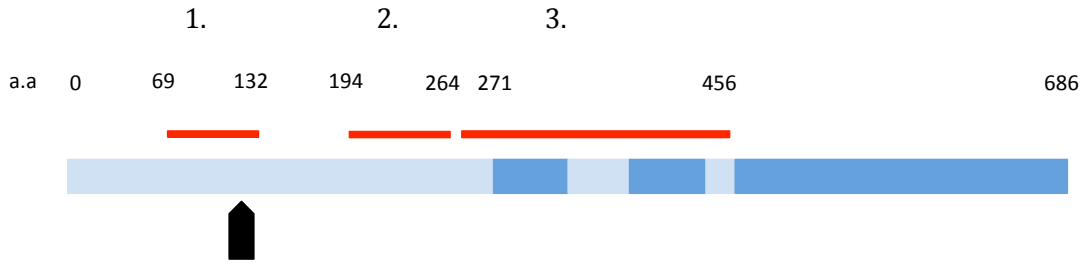


Fig 3.9. Eomes protein with functional domains annotated. 1: alanine-rich region. 2:glycine-rich region; 3: T-box DNA-binding domain. Black arrow denotes position of non-synonymous in-frame insertion rs368178421. a.a: amino acid.

Variants in the *EOMES* region have as yet only been associated with these three autoimmune diseases; this may be in part due to the size of GWAS in other diseases, which may not be large enough to detect an association, but may also imply a cell type or aetiological process common to RA, AS and MS that does not play a role in other diseases in the case of the secondary signal. However, despite several shared loci between MS and the other two diseases, no clear pathways involving Eomes emerge that are solely specific to these conditions. MS stands apart in that the inflammation occurs in a site of "immune privilege", and that there is a prominent neurodegenerative phase that occurs alongside and may even precede the inflammation. It is possible that the secondary signal region is important for gene regulation in immune cells involved in all three diseases, whereas the upstream region plays a role in a cell type of particular importance to MS; this might be an immune cell population, or a CNS cell. In this regard it is interesting that Eomes is expressed in neurons in the developing nervous system, and in adult neurogenesis. In adults, data suggest that the only region of notable Eomes expression is the cerebellum. However, recently released data searchable via the Braineac tool (http://www.braineac.org/) suggest that SNPs in the primary MS signal

are eQTLs for Eomes expression in tissue from the putamen, but not in other regions of the brain. This may reflect that the putamen is a site of adult neurogenesis (Ernst et al. 2014). However, the relevance of this finding to MS remains unclear.

To uncover further evidence of the potential role of the disease associated variants in gene expression and the relevant cell types, eQTL studies were examined for evidence of gene expression correlating with SNP genotype. Firstly, genotype at highly-disease associated SNPs correlated with Eomes expression in three studies, two of LCLs and one of whole blood (Grundberg et al. 2012, Stranger et al. 2012, Westra et al. 2013). Other variants, not within the credible set of SNPs, correlating with Eomes expression were found; conditional analyses demonstrated that the majority of these were associated with either the primary or secondary MS signal, and SNPs within the credible set better explained the correlation. This suggests that as hypothesised, there is a considerable likelihood that disease association occurs via changes in the regulation of Eomes expression. However, two variants that correlate with Eomes expression, rs2371258 in LCLs and rs17021298 in whole blood, did not colocalise with either of the MS association signals. The simplest explanation for this would be that these variants are part of regulatory sequences in cell populations present in whole blood that do not have a major role in either RA, AS or MS. Expression levels in whole blood will reflect not just those cells that express the gene of interest but also the most abundant cells, even if they only express the gene of interest at a low level. This may mean that granulocytes, particularly neutrophils, dominate the signal. The effects of cell activation are also not taken into account, and as these studies measure mRNA, whether the eQTL translates to an effect on protein is unknown.

The creation of LCLs involves transforming B cells with Epstein Barr virus which may influence gene expression and DNA methylation profiling; furthermore, the effects of long term cell culture may also contribute to altered gene expression meaning that these cell lines do not reflect genotype-dependent gene expression in the same way as

primary cells (Carter et al. 2002, Caliskan et al. 2011, Yuan et al. 2015). However, given the possible role of EBV in MS aetiology, it is interesting that studies involving LCLs have detected eQTLs, and that expression correlates only with the primary MS signal in these studies. A more direct effect of EBV on Eomes expression would be intriguing to investigate in future work.

The presence of two independent signals in the *EOMES* region adds extra complexity and raises several possibilities. Previous studies have elucidated independent effects on susceptibility of non-coding variants in a single gene, which may act through different pathways possibly via effects on different functional domains (Couturier et al. 2011, Diogo et al. 2015). With regard to Eomes, the eQTL studies suggest that risk genotype at the two regions has opposing effects on expression; risk genotype at rs1813375 correlated with lower Eomes expression in Stranger *et al*, which studied LCLs, whereas risk genotype at rs11129295 correlated with increased expression in Westra *et al,* which studied whole blood. This appears to further suggest that the two signals have an effect in different cell types or in different states of activation, each of which has a different role in MS pathogenesis.

To further explore the potential regulatory role of each region and the relevant cell types, colocalisation of disease-associated SNPs with epigenetic marks was investigated. Only h3k9me3 marks, in both innate and lymphoid populations, were observed to align with the primary signal. The secondary signal was observed to align with a number of epigenetic marks, predominantly colocalising with those SNPs located proximal to the gene including rs2371108, rs368178421 and rs3806624, in a variety of cell types. ENCODE data from the GM1278 cell line indicate that several transcription factors involved in transcriptional regulation, such as CTCF and TCF12, may bind to *EOMES* and overlie rs368178421.

Notably, an H3K4me1 peak aligning with rs5847475, around 5 kb upstream of *EOMES*, was present in lymphoid cells but absent in monocytes and macrophages,

possibly suggesting a lymphoid-specific regulatory region which may be of relevance as Eomes expression is largely confined to lymphoid cells (Chapter 4). In all, alignment of epigenetic data with MS-associated SNPs suggests that SNPs proximal to the gene are most likely to be functional, particularly rs2371108, rs368178421 and rs3806624, in all cell types, and rs5847475 in lymphocyte populations.

Epigenetic data still have some shortcomings; primary cell data are limited in terms of the number of individuals included, meaning that there is virtually no genotype-specific information available. Furthermore, in some primary immune cell populations the range of epigenetic marks covered is limited, and there are few data on small subsets of cells or activated cells. However, the advent of new techniques such as ATAC-Seq, which enables rapid acquisition of genome-wide information on chromatin state and transcription factor footprints in small numbers of cells, may overcome these problems (Buenrostro et al. 2013).

It has been demonstrated that distant regulatory regions of DNA, bound to transcription factors, are brought into close contact with gene promoters to exert their effect (Gothard et al. 1996). To examine whether either of the MS-associated regions physically interacted with the *EOMES* promoter, we performed capture-C experiments, which suggested several peaks of interaction, the largest of which was a region 5kb upstream of the *EOMES* promoter aligning with rs5847475. However, no significant peaks aligned with the primary association region. As epigenetic regulation is highly tissue specific, these studies may need to be repeated in specific cell populations to detect significant interactions in smaller subsets that are drowned out by more abundant cells in bulk population data. Future work may be able to uncover more information regarding the function of each SNP; the high frequency of *Dpn*II restriction sites means that bait oligonucleotides could be designed specifically for almost every SNP in an LD block, enabling visualization of DNA interactions with individual SNPs rather than with the gene promoter. By performing the studies in the relevant cell

populations with cells from individuals of different genotype, it might also be possible to detect whether these interactions are genotype-specific.

Given the high LD that exists across these regions, determining the causal variant is not required to perform studies of genotype-dependent gene expression, but is relevant in terms of discovering the mechanisms of gene regulation. Increasing the knowledge of regulatory landscapes, and how disease associated variants exert an effect on gene expression and disease susceptibility may eventually lead to the development of new treatments; for instance, reactivation of haemoglobin genes has recently been enabled through forced chromatin looping, bringing them into contact with a powerful distal enhancer (Deng et al. 2014). This remains experimental but holds promise for future therapy of haemoglobinopathies.

Taken together, these results confirm two independent signals of MS-association in chromosome 3p24 in the vicinity of the *EOMES* gene: a distant, upstream primary signal with lead SNP rs1813375 and a more proximal, downstream secondary signal with lead variant rs5847475, a single nucleotide insertion. A non-synonymous variant, rs368178421, was also highly disease-associated and a functional effect of this SNP cannot be excluded. The secondary signal is shared and concordant with the autoimmune diseases RA and AS and corresponds to published eQTLs in whole blood. Alignment with few epigenetic marks was observed for the primary signal, suggesting an effect in an as yet unknown primary cell type or cellular activation state. However, several SNPs in the secondary signal align with epigenetic marks suggesting that dysregulated epigenetic mechanisms could underlie this association with disease.

Chapter 4.

Characterisation of the pattern and kinetics of Eomes protein expression in peripheral immune cells

4.1 Introduction

The aim of the project was to elucidate the potential functional implications of MS-associated genetic variants in the *EOMES* region, tagged by single nucleotide polymorphisms (SNP) rs2371108 (rs237) and rs669607 (rs669). rs669607 was identified in a 2011 GWAS (Sawcer et al. 2011), following which this project was started and genotyping of the Oxford Biobank was performed; as this SNP is in perfect LD with the lead SNP, rs1813375 (see table 3.2), the genotyping was not repeated for rs1813375 as both SNPs 'tag' the primary association equally.

As mentioned in Chapter 2, it was hypothesised that variation at these loci would have an effect on Eomes expression due to changes in epigenetic mechanisms controlling the level of Eomes expression. We postulated that a change of expression resulting in downstream effects would be most likely to occur in those cells normally expressing substantial levels of Eomes; accordingly the primary aim of this chapter was to clarify the pattern of Eomes expression in multiple cell subsets. This information was then used to inform the genotype-specific studies in chapter 5.

Eomes expression has been little explored in humans; however, detailed characterisation of expression has been performed in experimental animals. In mice and other vertebrates it is known to be essential for normal cortical development; a major region of neurogenesis is the subventricular zone, from where neural progenitors arise and transiently express Eomes. Abolition of Eomes expression causes microcephaly and

behavioural abnormalities (Bulfone et al. 1999, Arnold et al. 2008, Arnold et al. 2009). This prominent role in neural development may well be recapitulated in humans, as suggested by a Moroccan family with an unusual translocation that causes silencing of *EOMES* and the condition of microcephaly with polymicrogyria (Baala et al. 2007). In adults it may also be involved in neurogenesis, although this is a much more limited process in mature animals and is restricted to a few small areas such as the hippocampus and subventricular zone (Abrous et al. 2005). In adult human CNS, publicly available microarray data suggest that Eomes expression, at least at the mRNA level, is largely limited to the cerebellum (data from Allen brain atlas http://human.brain-map.org/). Despite predilection for certain regions of the CNS, the lesions of multiple sclerosis are usually widespread, and the neurodegeneration observed from the early stages of disease affects the normal-appearing white matter (NAWM) diffusely (Lucchinetti et al. 2004). This disparity suggested that the role of Eomes in multiple sclerosis susceptibility was more likely to be due to changes in immunity. It is also possible that the link between Eomes and susceptibility is due to changes in both the immune system and CNS, but as it is not possible to sample CNS tissue from healthy controls, the focus of the project was the role of Eomes in the immune system.

In the immune system, Eomes has been studied to a much greater extent in mice than in humans. It is known to be highly expressed in the lymphoid compartment, particularly in CD8+ T cells and Natural Killer (NK) cells (data from http://www.immgen.org/; (Heng et al. 2008). In CD8+ T cells it is associated with memory cell development and maintenance, and after a delay is upregulated following antigen stimulation with timing consistent with the emergence of the memory population (Cruz-Guilloty et al. 2009). Consistent with this, Eomes knockout mice have fewer long-lasting memory cells, which are also poorly functional (Banerjee et al. 2010). However, it also known to be important in the transcription of effector molecules, and

there appears to be a gradient of Eomes expression in different memory populations, with expression highest in the effector memory and lowest in the stem cell memory subsets (Gattinoni et al. 2011). Atypical, innate-like CD8+ T cells are dependent on Eomes expression for their development and continue to express high levels in maturity (Gordon et al. 2011).

Eomes is also known to be highly expressed in NK cells, consistent with the effector functions of this cell population in anti-viral and tumour immunity, and is required for the development of certain NK populations, as well as for the maintenance of a mature cellular phenotype (Gordon et al. 2012, Daussy et al. 2014). In CD4 T cells Eomes expression is much lower, and it is therefore thought to be less important than its paralogue and related T-box transcription factor, T-bet/TBX21 (Pearce et al. 2003). However, in certain CD4 populations it may still play an important role, for instance in response to viral infection (Edwards et al. 2014) as well as in a small population of cytotoxic CD4 T cells (Qui et al. 2011). In addition, it is expressed in a subset of Th2 cells, where it has effects on cytokine production (Endo et al. 2011). In certain conditions it may also be important for diverting differentiating cells towards a Th1 rather than Th17 phenotype (Ichiyama et al. 2011).

There are few reported studies of Eomes expression in the innate immune system. However, publicly available microarray data from both mouse and human tissues suggests that at the mRNA level there is little expression in the myeloid compartment when compared with lymphoid cells (Su et al. 2004, Heng et al. 2008). However, these experiments look at bulk populations and show solely mRNA level data; there is little data on protein expression at the immune subset level. Eomes expression was assessed in multiple myeloid and lymphoid populations. In this exploratory chapter comparing multiple cell populations, two measure of Eomes expression were used. Eomes expression as median fluorescence intensity (MFI) was measured in whole cell

populations; where there was a clear bimodal distribution of Eomes expression in a cell population, the percentage of cells expressing Eomes was also measured.

As Eomes is upregulated in immune cells following exposure to antigen, a further aim was to examine Eomes expression in CD8+ and CD4+ T cells following a TCR stimulus as well as other modes of activation. This was performed with the aim of identifying conditions in which Eomes was dependably and strongly upregulated, which might be taken forward for further study. Large eQTL data sets have suggested that in some cases, eQTL may only become apparent following stimulation (Fairfax et al. 2014). The kinetics of Eomes expression on stimulation are complex and there are few available data on this subject in humans, particularly at the protein level. It appears that in mice, Eomes expression may be upregulated by IL-2, but that TCR stimulation diminishes this upregulation (Agarwal et al. 2009). However, on culturing cells for longer periods, Eomes expression may subsequently increase with the emergence of the memory cell subset (Cruz-Guilloty et al. 2009). In light of the need for easily reproducible experimental conditions, Eomes expression for up to 72 h following a variety of stimuli was assessed; this is covered further in section 4.3.

4.2 Results

4.2.1 Eomes protein expression in peripheral blood lymphoid subsets

According to the literature, CD8+ T cells and NK cells express the highest levels of Eomes. Therefore, Eomes expression in these populations, as well as in CD4+ T cells, CD3+CD56+ NKT cells and CD19+ B cells, was assessed by flow cytometry. The level of expression was measured as staining MFI/isotype control MFI to control for non-specific staining, for instance due to Fc-receptor expression (see section 2.4.5).

A gate was first drawn around the lymphocyte population based on their forward scatter/side scatter properties, and then the "pulse width" parameter was used to exclude doublets (Fig 4.1). CD8+ T cells and CD4+ T cells were gated on the basis of high expression of CD8 and CD4 respectively (Fig 4.2 A and B). NK cells were defined as CD3-CD56+, and NKT cells as CD3+CD56+ (Fig 4.3). B cells were initially gated as CD19+, and memory and naïve cells were defined as CD27+ or CD27- respectively (Fig 4.4).

This demonstrated that consistent with mouse data and mRNA datasets, expression was highest in NK (mean normalised MFI of total population 24.5, range 11.6-51.2), NKT (26.91, 7.6-89.4) and CD8+ T cell (19.95, 3.9-67.3) populations, with little expression above background in B cells (6.569, 4.3-9.1) and CD4+ T cells (2.031, 1.4-2.6) (Fig 4.5). Interestingly, all subsets except B cells displayed clear bimodal populations with both positive and negative cells (see figs 4.2-4.4). In these instances the MFI may be a poor measure of expression, and therefore the percentage of Eomes+ cells was measured in addition.

Consistent with the MFI data, NK (mean % positive cells 86, range 73-96%) and NKT (76, 39.8-88.3%) cells demonstrated the highest proportion of Eomes+ cells, followed by CD8+ T cells (46, 8.7-83%), which showed the greatest variation of values

for Eomes positivity between donors. Despite a clear positive population on FACS plots, in CD4+ T cells this did not constitute more than 10% of cells in any of the donors (2.3, 1.1-8.7%, Fig 4.5).

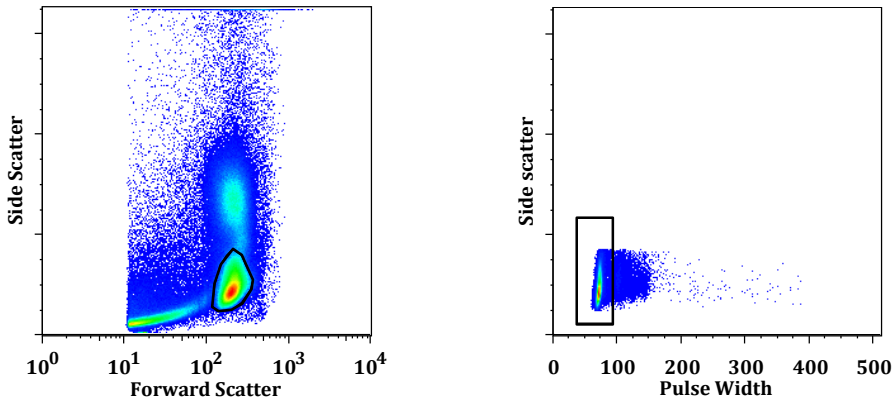


Figure 4.1. Flow cytometry gating strategy for human PBMCs
PBMCs were first gated on their forward scatter/side scatter profile, and subsequently on their pulse width profile to exclude any doublets/triplets formed during the staining process.

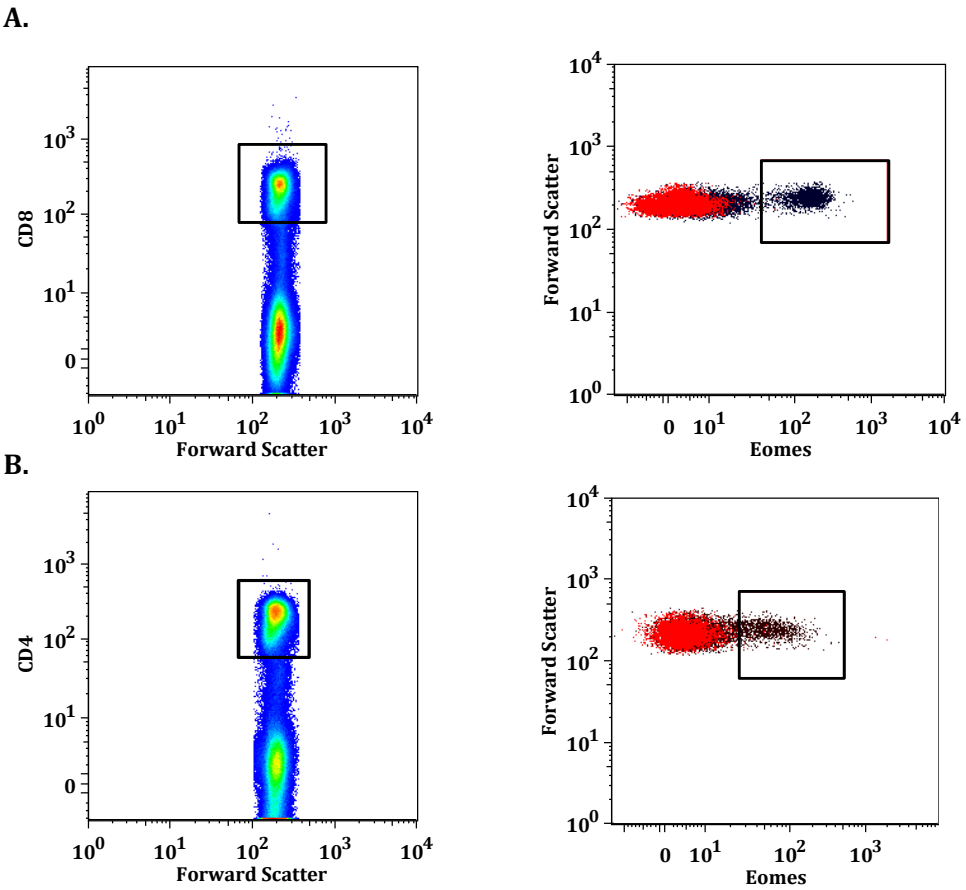


Fig 4.2 Flow cytometry gating of lymphoid subsets. A. CD8 T cells were gated on the basis of high CD8 expression. Intermediate CD8+ cells were excluded. Gating was then performed on the basis of Eomes expression. **B**. CD4+ T cells were gated on the basis of high CD4 expression before gating on the Eomes+ population. Isotype control shown in red.

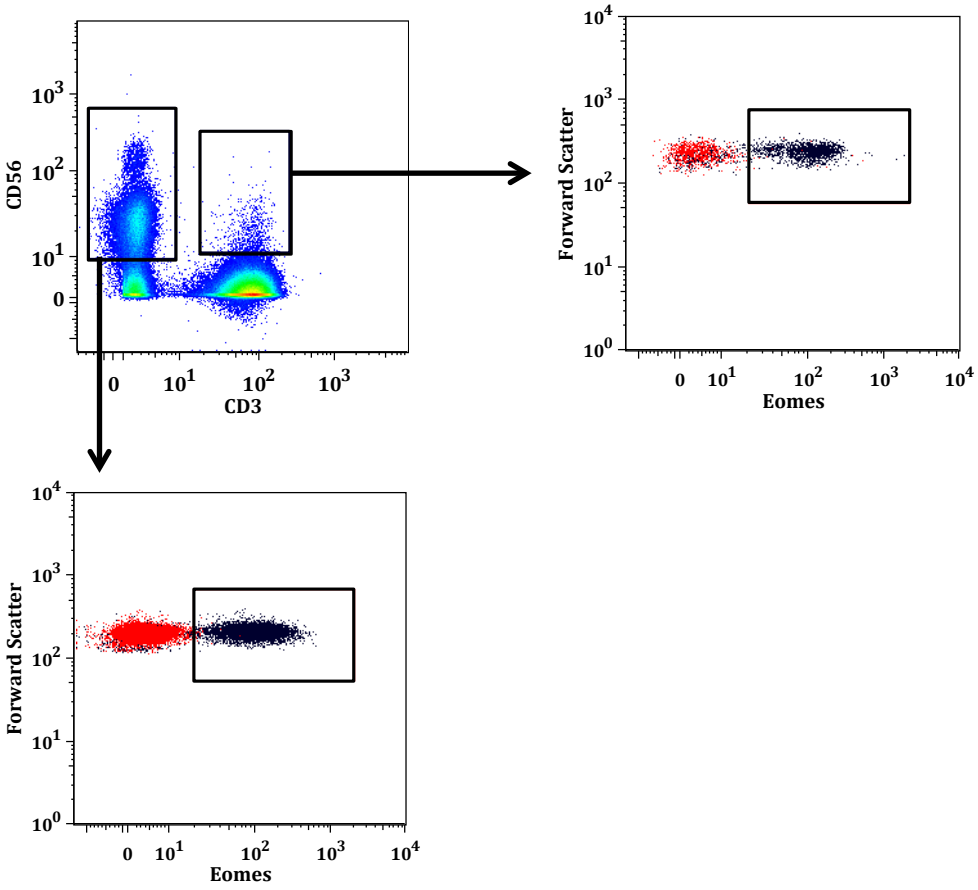


Fig 4.3. Flow cytometry gating of lymphoid subsets. NK cells were identified as CD3-CD56+ and NKT-like cells were identified as CD3+CD56+. Eomes+ populations were then identified.

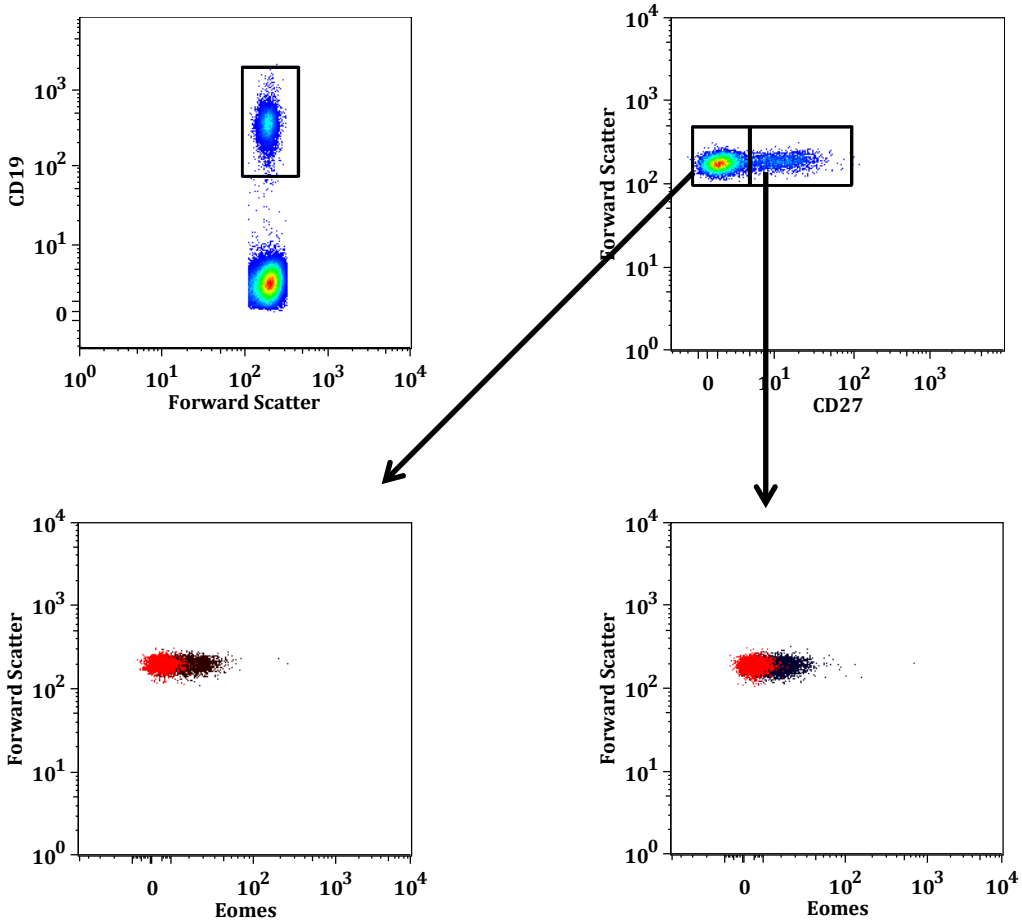
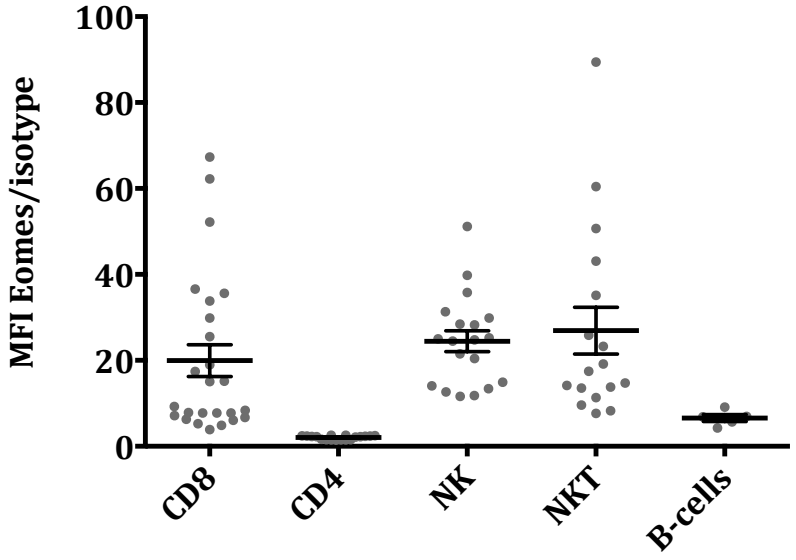


Fig 4.4 Flow cytometry gating of lymphoid subsets. B cells were identified as CD19+ and subsequently identified as memory or naïve B cells on the basis of CD27+ (memory) or CD27- (naïve).

A.



B.

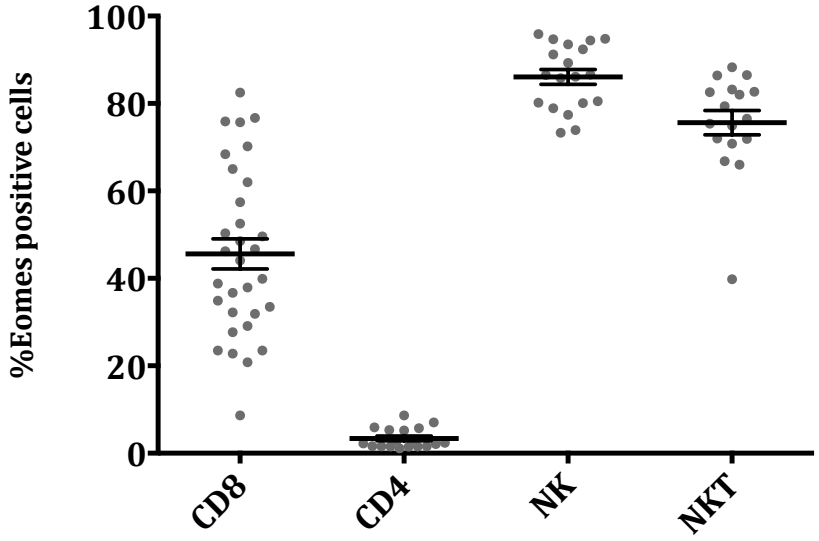


Fig 4.5. **Eomes expression in lymphoid subsets**. **A.** Eomes expression in lymphoid subsets measured as fold expression (MFI) v isotype control. **B.** In subsets with a clear bimodal distribution Eomes expression was measured as percentage positive cells.

4.2.2 Eomes expression in peripheral blood myeloid subsets

Eomes expression was assessed in the major myeloid subsets in peripheral blood by flow cytometry. Two subsets of the monocyte population were assessed, as well as CD15+ granulocytes, consisting predominantly of neutrophils, and two subsets of dendritic cells.

For monocytes an initial gate was drawn around the monocyte and lymphocyte populations, and aggregated cells were excluded. Monocytes were then defined by CD14 and CD16 expression into two subsets: CD14+CD16- and CD14+CD16+ (fig 4.6). For dendritic cells (DCs) all live cells were included, and lin1 antibody cocktail (CD3, CD14, CD16, CD19, CD20, CD56) was used to exclude other populations. DCs were then defined as HLA-DR+ and classified as plasmacytoid DCs (pDCs, CD123+CD11c-) and myeloid DCs (mDCs, CD123-CD11c+) (Fig 4.7). Neutrophils were defined on the basis of size/granularity and CD15 expression (Fig 4.8).

Publicly available data sets suggested that there is little expression in the myeloid compartment and the findings were consistent with this, with little expression in monocytes (mean normalised MFI 7.4, range 3.5-12), mDCs (6.6, 3.2-8.7), pDCs (8.0, 3.9-11) or granulocytes (3.6, 2.7-6.1) (Fig 4.9). On overlaying the populations with the isotype control, a low level of staining above the isotype control was observed in all populations. This did not approach the level of expression observed in the lymphoid subsets, and was consistently a uniform level of expression rather than a bimodal distribution; therefore this was considered likely to be either a low background level of constitutive expression in all cells, or due to non-specific staining and a mismatch in fluorescence between the isotype control antibody and the Eomes antibody.

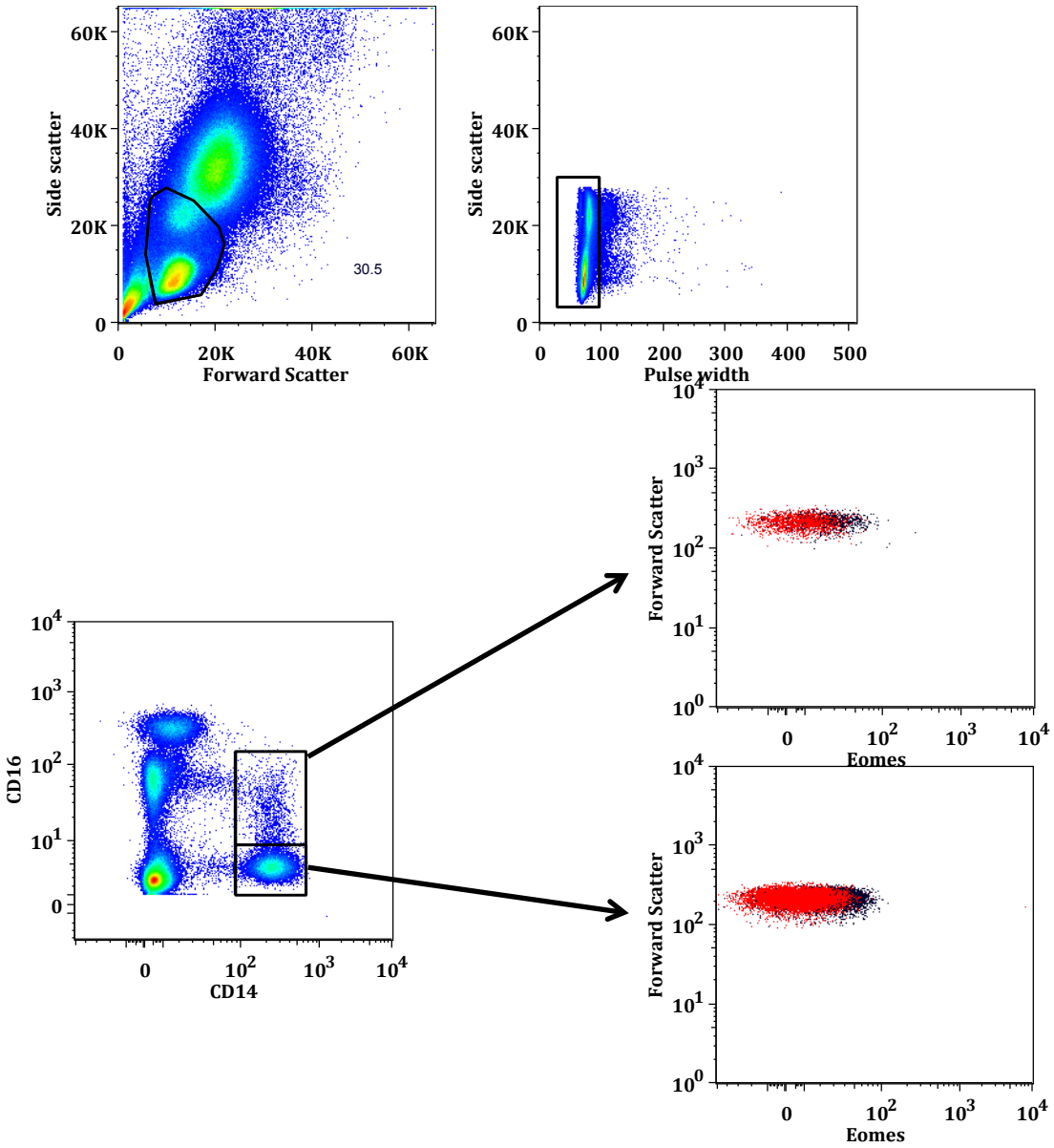


Fig 4.6. Flow cytometry gating of myeloid subsets: Monocytes. A wide gate was drawn to encompass the monocyte and lymphocyte populations. Cells were subsequently gated on pulse width to exclude doublets. Monocytes were identified by CD14 positivity and divided into two subsets: CD14+CD16- and CD14+CD16+.

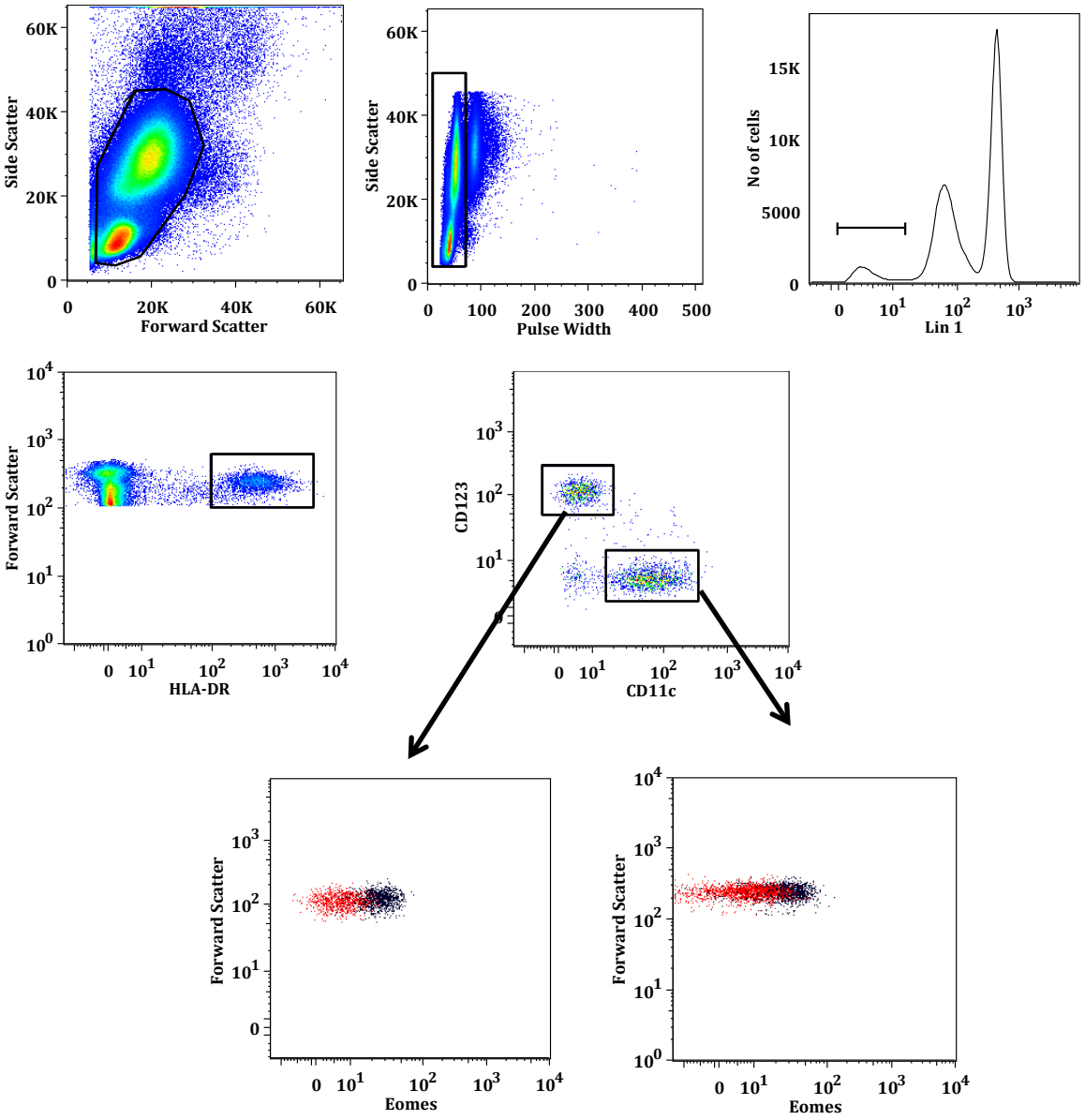


Fig 4.7. Flow cytometry gating of myeloid subsets: Dendritic Cells (DCs). Initially all live cells were included before gating to exclude doublets. A lin1 antibody was used to exclude other immune subsets. Cells were then gated on the basis of HLA-DR positivity and then subdivided into plasmacytoid DCs (CD123+CD11c-) and myeloid DCs (CD123-CD11c+).

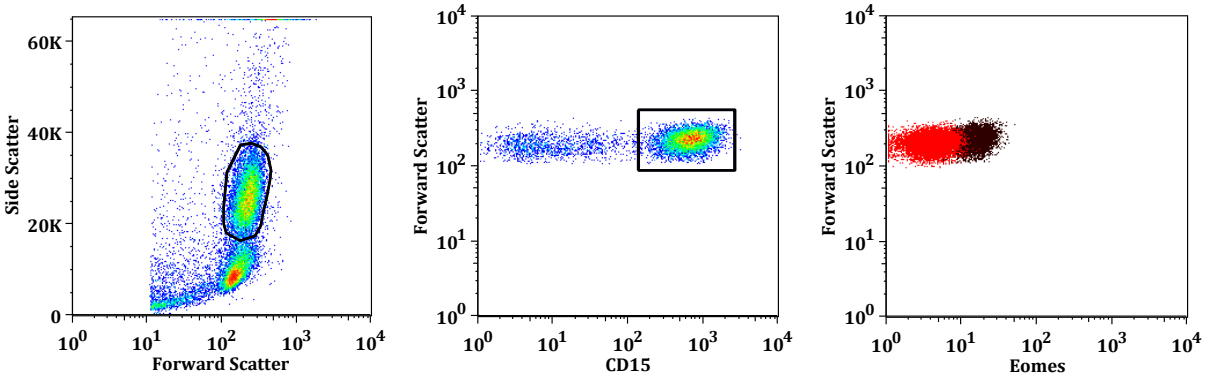


Fig 4.8. Flow cytometry gating of myeloid subsets: Neutrophils. Neutrophils were distinguished by their forward scatter and side scatter profile followed by gating for CD15 positivity.

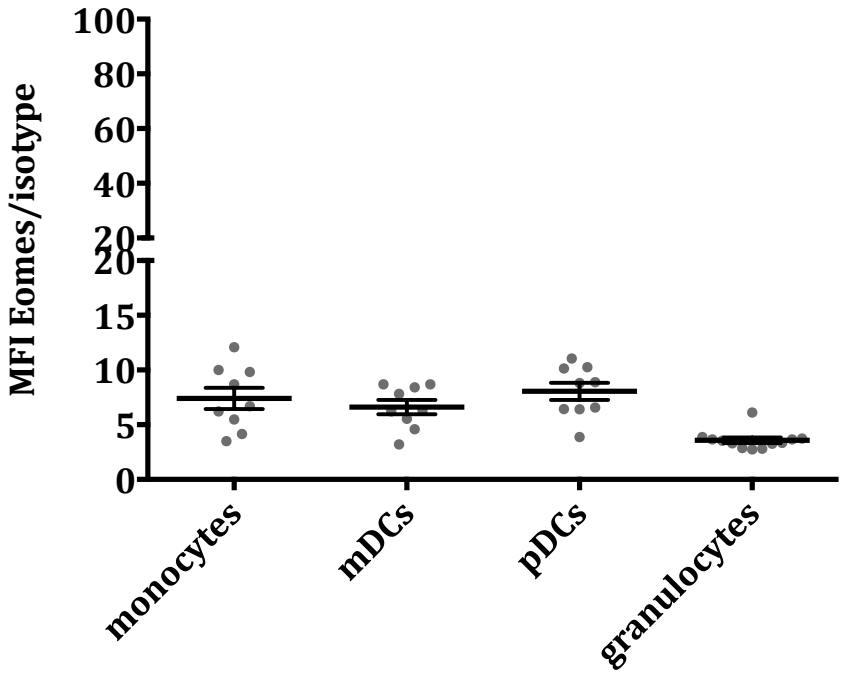


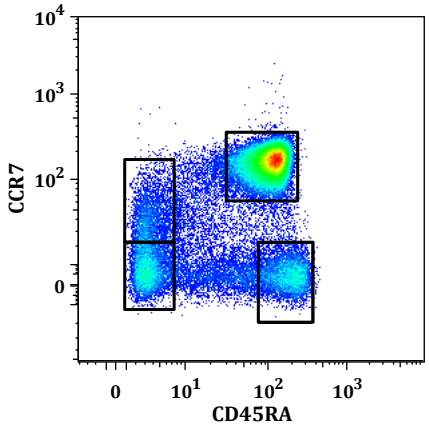
Fig 4.9 Eomes expression in myeloid immune subsets. Eomes expression was analysed by flow cytometry in the major peripheral blood myeloid populations and normalised to isotype control staining. Monocytes, n=9; DCs, n=9; granulocytes.

4.2.3 Eomes expression in CD8+ T cell subsets

In initial experiments performed on lymphocyte cones, four CD8+ T cell populations were identified according to CD45RA and CCR7 expression: naïve (CD45RA+CCR7+, Tn), central memory (CD45RA-, CCR7+, Tcm), effector memory (CD45RA-CCR7-, Tem) and terminal effector (CD45RA+CCR7-, Temra). Real time PCR on these populations showed that in accordance with previously published data (Gattinoni et al. 2011) a gradient of Eomes mRNA expression was observed, with increasing expression from Tn to Tcm and Tem. Temra cells showed the highest level of Eomes mRNA expression, a finding not previously described (Fig 4.10).

An alternative marker to CCR7 is CD62L, a lymph node homing molecule. Expression of these two markers is largely concordant (Sallusto et al. 2004) and CD62L is frequently used to identify the four populations mentioned above. Here, trials of antibodies were performed and better separation of the populations, particularly the Tcm/Tem populations, was achieved using the CD62L marker (Fig 4.11 A). Using this method showed an identical pattern of expression at the protein level to that observed at the mRNA level using CCR7, with highest Eomes expression in Temra cells (mean normalised MFI 36.4, mean positive 76%). Expression was slightly but significantly lower in Tem (30.4, 66.5%) and Tcm cells (18.3, 46%). All differentiated cells expressed higher levels than Tn cells (8.1, 16.8%) (Fig 4.11 B).

A.



B.

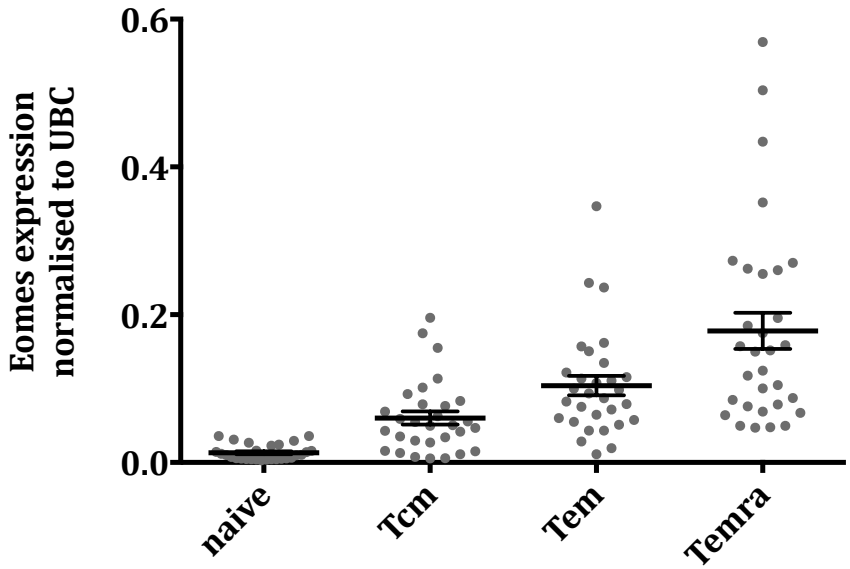


Fig 4.10. Eomes expression in CD8 subsets: mRNA. A. Gating of CD8+ T-cells on the basis of CCR7 and CD45RA for sorting by flow cytometry. B. Eomes mRNA expression in the subsets was assessed by real time PCR and measured as 2^{-dCT} vs UBC.

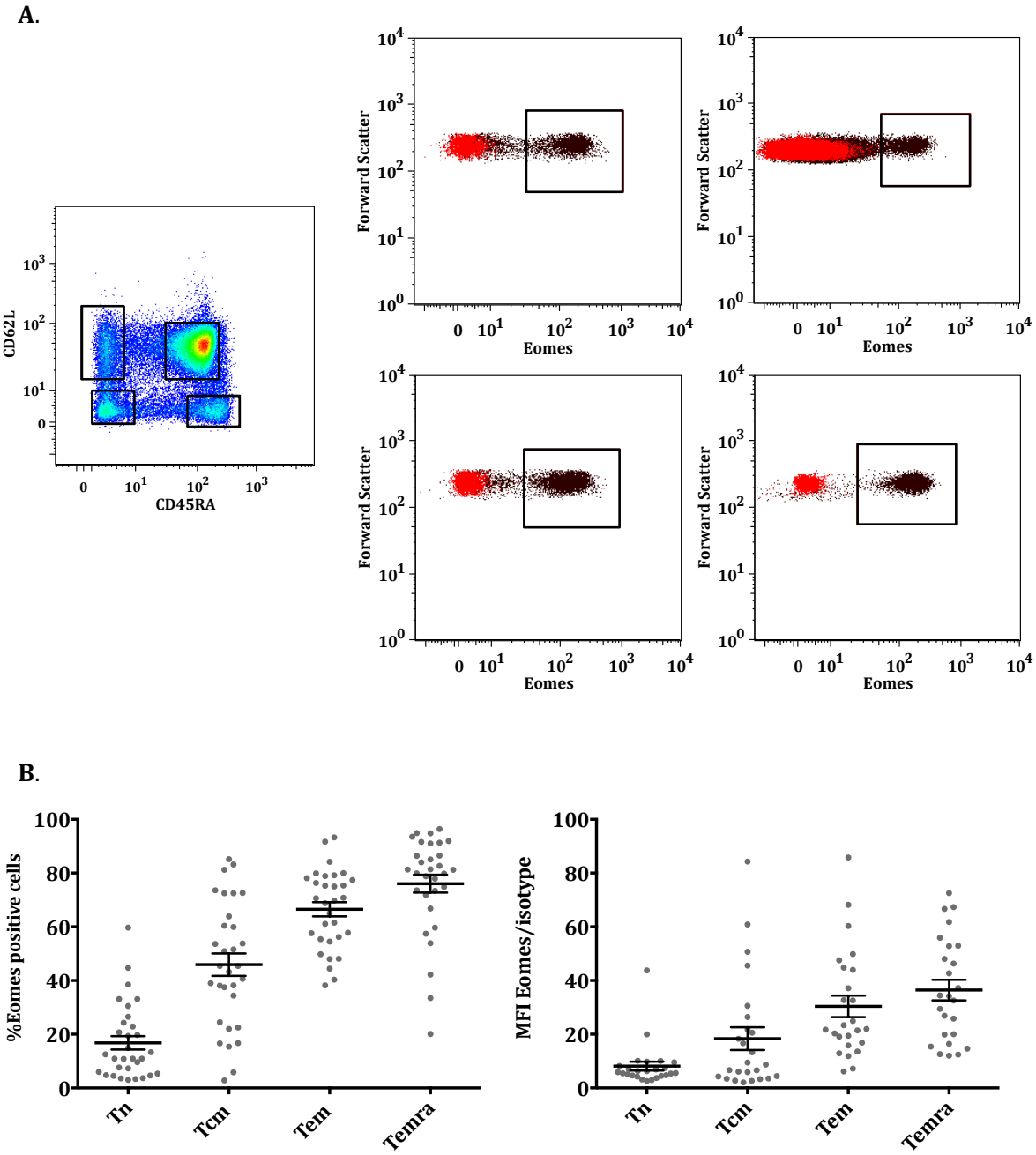


Fig 4.11. Eomes expression in CD8+ T cells: protein. A. CD8+ T cells were further subdivided into four effector/memory populations on the basis of CD45RA and CD62L expression. **B.** Eomes expression was measured as the proportion of positive cells and the normalised MFI.

4.2.4 Eomes expression in CD4+ T cell subsets

CD4+ T cells can be further defined by their cytokine expression or surface marker expression. In order to draw a parallel with CD8+ T cells, they were subdivided on the basis of CD45RA and CD62L into naïve (CD45RA+CD62L+), central memory (CD45RA-CD62L+) and effector memory (CD45RA-CD62L-) subsets. The fourth population observed in CD8+ T cells, CD45RA+CD62L-, was not observed consistently to any significant extent in CD4+ T cells and therefore was not included in analyses. Dot plots showed that all three populations contained Eomes expressing cells, although these constituted an extremely low proportion of naïve cells (mean 0.7%, range 0.2-3.3) and very few central memory cells (3%, 1.7-6.4) (Fig 4.12 A). However, up to a third of effector memory CD4+ T cells (mean 14%, range 7.5-32.7%) expressed Eomes, consistent with Eomes playing an effector role in CD4+ T cells. As the population was bimodally distributed and only a minority of cells was positive, the MFI values for all populations were low when the populations were taken as a whole (mean normalised MFI Tn 2.0; Tcm 2.0; Tem 2.3) (Fig 4.12 B).

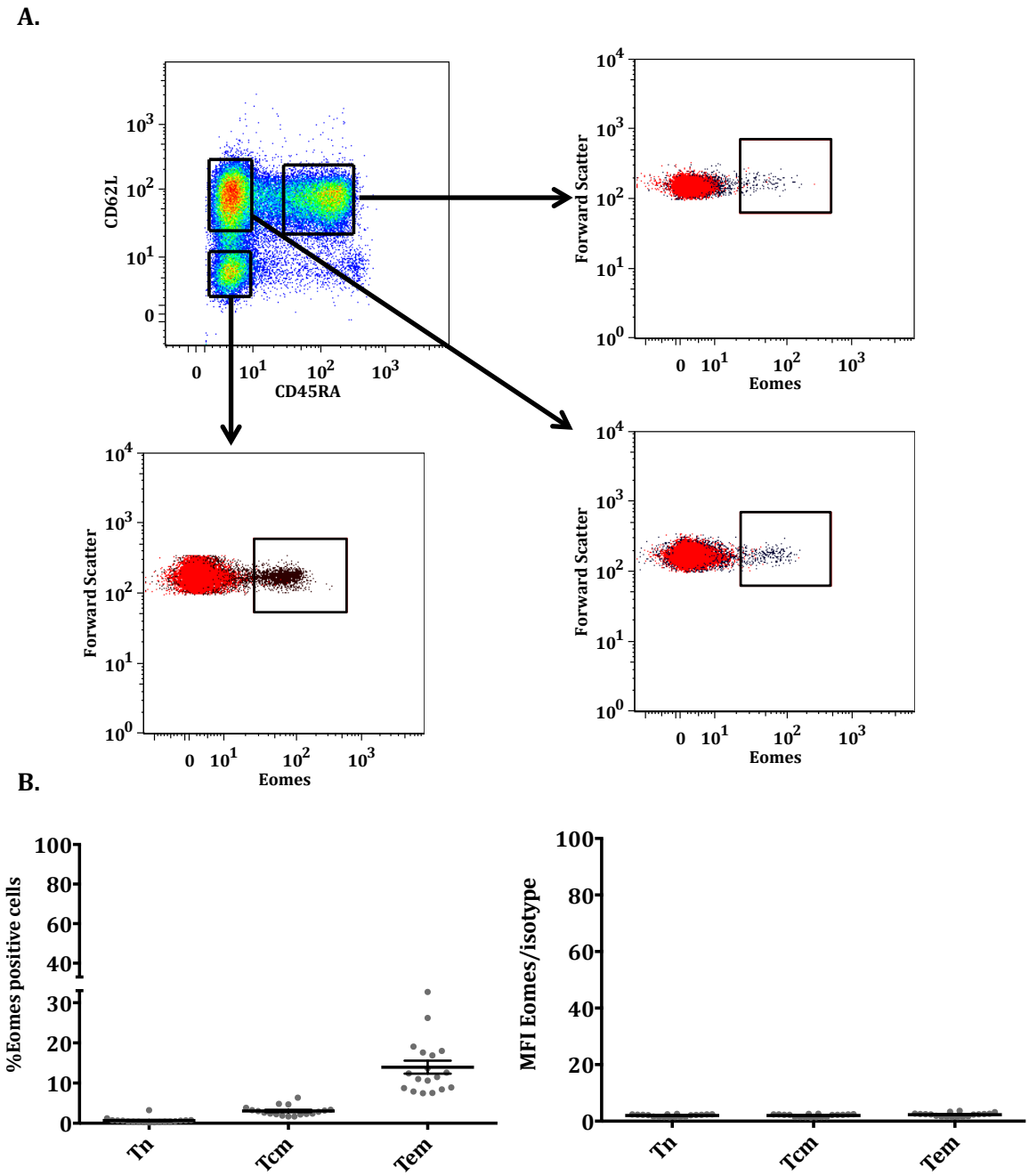


Fig 4.12. Eomes expression in CD4+ T cell subsets. A. CD4+ T cells were further subdivided into naïve, central memory and effector memory subsets on the basis of CD45RA and CD62L expression. **B**. Eomes expression was measured both as the proportion of positive cells and the normalised MFI.

4.2.5 Eomes expression in Natural Killer cell subsets.

CD3-CD56+ NK cells were further defined as CD56++CD16- and CD56+CD16+ (Fig 4.13 A). These two populations constitute the majority of NK cells present in peripheral blood and are thought to have differing properties, and may be at different points of lineage development, with the CD56++CD16- cells considered by some to give rise to the CD56+CD16+ population (Chan et al. 2007, Poli et al. 2009). The highest Eomes expression was observed in the smaller population of CD56++CD16- cells; this population was almost uniformly Eomes+ in all donors assessed (mean 96.3%, range 92.6-98.9%), and accordingly the MFI was highest in these cells (mean normalised MFI 51.2, range 26.6-89.6). The CD56+CD16+ population, by far the largest peripheral blood NK population, was largely Eomes+ in all donors (85.7%, 71-96.9%, mean normalised MFI 22.8, 11.1-43.3) (Fig 4.13 B).

NKT cells were defined as CD3+CD56+. This is an outdated definition of NKT cells, which are more accurately defined on their expression of restriction to the MHC-related molecule CD1 and their expression of a semi-invariant TCR. The CD3+CD56+ population is likely to encompass these cells and others, and may therefore be a somewhat heterogeneous population, which is sometimes termed “NKT-like” cells. This population expressed similar levels of Eomes to the CD56+CD16+ NK population both in terms of proportion of Eomes+ cells (76.5%, 39.8-88.3%) and MFI (26.9, 7.6-89.4) (Fig 4.13 B).

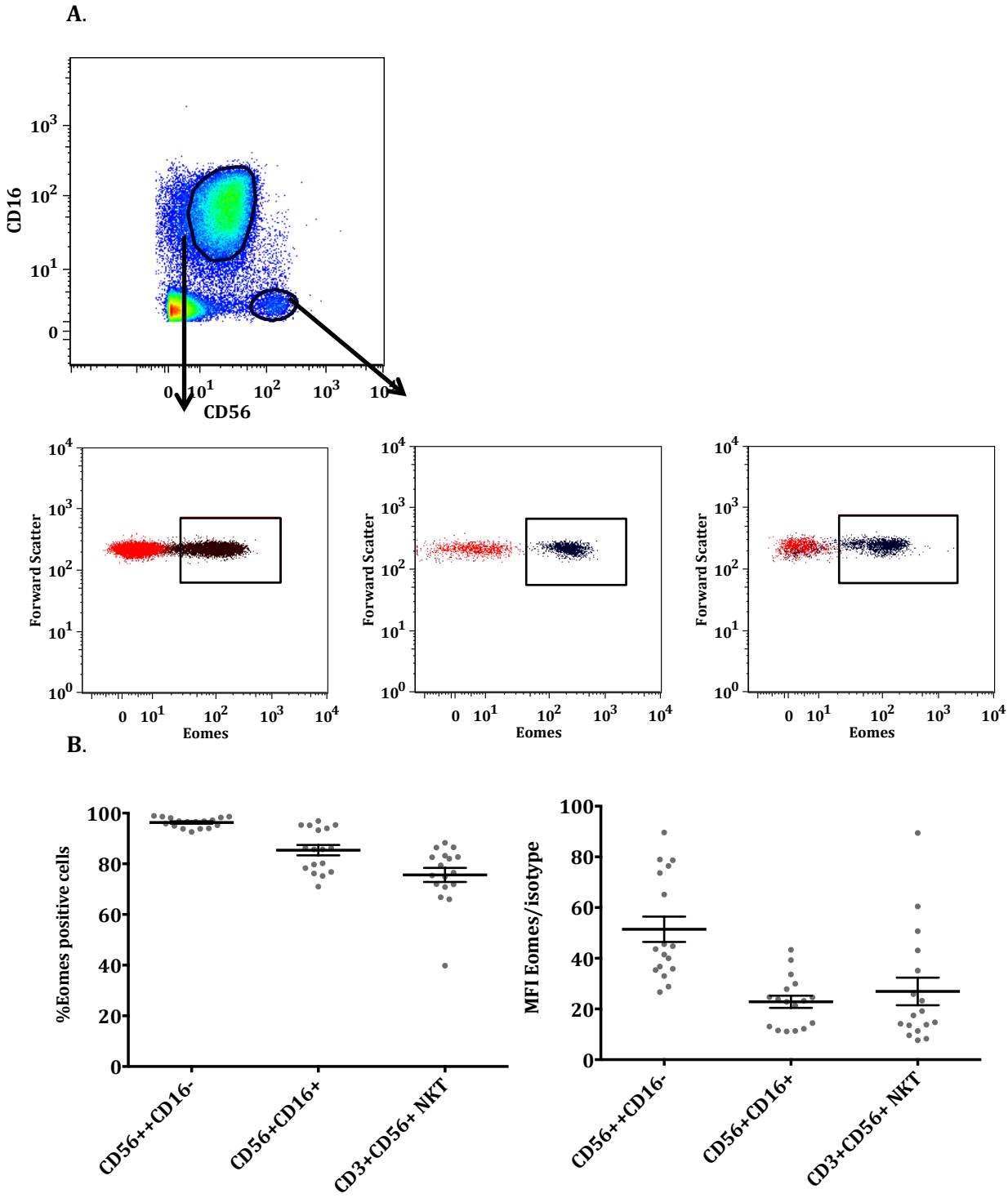


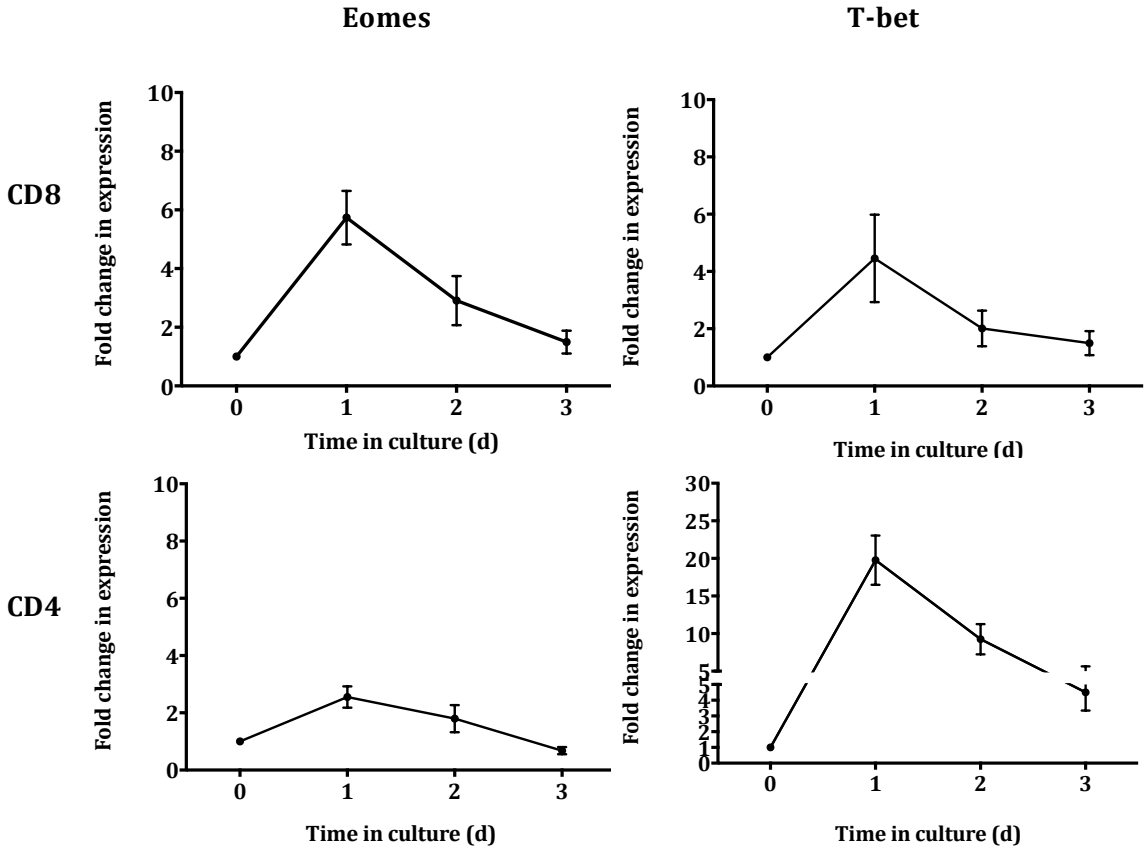
Fig 4.13 Eomes expression in NK cell subsets and NKT cells. A. NK cells, defined as CD3-CD56+, were further subdivided into two major populations on the basis of CD16 and CD56. NKT cells (right panel) were defined as CD3+CD56+ cells. **B.** Eomes expression was measured both by proportion of cells positive for Eomes and by normalised MFI.

4.2.6 Eomes expression in CD4+ and CD8+ T cells following TCR stimulus

Eomes expression was measured by real-time PCR in CD4+ and CD8+ T cells cultured with IL-2 for 72 h following a TCR stimulus with anti-CD3 and CD28 beads. The T-box paralogue of Eomes, T-bet, was also measured as a comparator and to examine the reciprocal nature of expression of these master transcription factors. At baseline, in comparison to Ubiquitin C (UBC, the housekeeping gene), Eomes expression was several-fold higher in CD8+ T cells than CD4+ T cells (Mean expression as 2^{-dCT}, CD8 0.10±0.02 v CD4 0.011±0.003). Following the stimulus, in CD8+ T cells both Eomes (6-fold) and T-bet (4-fold) were upregulated at d1 and subsequently decreased, returning to approximately baseline levels by d3. The overall pattern was similar in CD4 T cells, but the upregulation of Eomes was lower (3-fold), whereas T-bet expression rose 20-fold at d1 and despite subsequent decrease, remained upregulated over baseline at d3 (Fig 4.14 A).

CD8+ T cells were taken at each time point for FACS analysis. The proportion of Eomes+ cells rose slightly at d1 before decreasing, in keeping with the pattern of mRNA expression following stimulation (Fig 4.14 B).

A.



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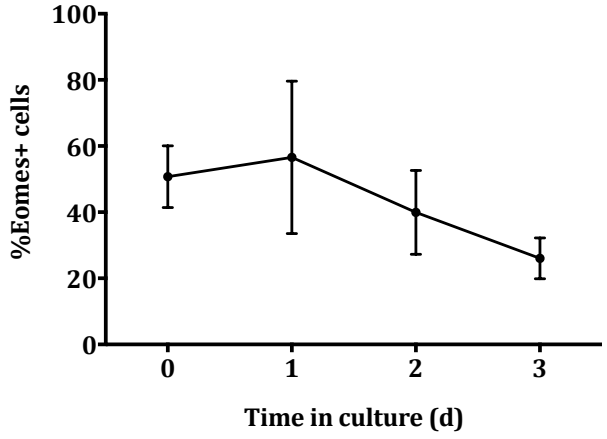


Figure 4.14. **Eomes and T-bet expression in CD4 and CD8+ T cells following stimulation**. CD4 and T cells isolated by MACS were stimulated with anti-CD3/anti-CD28 dynabeads in a bead:cell ratio of 1:2 and cultured for three days in the presence of IL-2. **A**. Cells were taken for mRNA isolation at baseline and days 1-3. Real-time PCR was performed for Eomes, T-bet and UBC (n=7). **B.** At each time point CD8+ T cells were stained for Eomes and the proportion of Eomes positive cells was measured (n=6).

4.2.7 Eomes expression in CD4 and CD8+ T cells following TCR stimulus and culture with IL-2 and IL-15

Eomes is known to directly target and induce expression of CD122, the IL2R/IL15R β-chain (Intlekofer et al. 2005). IL-15, as well as IL-2, is known to significantly affect memory function and cell proliferation (Alves et al. 2003, Castro et al. 2011). Therefore, the effects of this cytokine on Eomes expression in addition to IL-2 were investigated. Following MACS isolation, CD4+ and CD8+ T cells were stimulated with dynabeads as previously described and cultured for 72h in the presence of IL-2 with or without IL-15. At each time point cells were taken for mRNA isolation and analysis of Eomes expression by real-time PCR. Consistent with previous observations, Eomes was upregulated by both CD8+ and CD4+ T cells at d1, to a greater extent in CD8+ T cells, before subsequent reduction and return to baseline levels. The addition of IL-15 did not make a significant difference to Eomes expression (Fig 4.15).

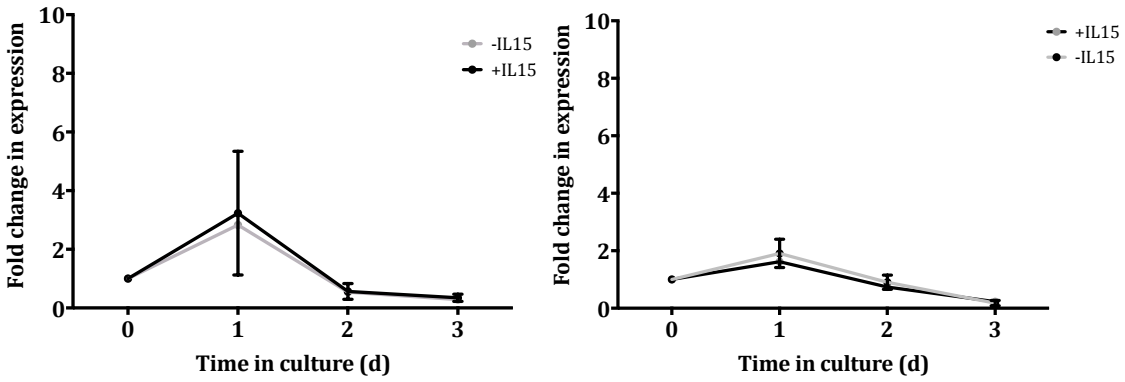


Figure 4.15. Eomes expression in CD4 and CD8+ T cells following stimulation and culture with IL-2 and IL-15. CD4+ and CD8+ T cells were isolated by MACS, stimulated with anti-CD3/CD28 dynabeads and cultured with IL-2 +/- IL-15 (n=2).

4.2.8 Eomes and T-bet expression in CD4 and CD8+ T cells in culture following various stimuli

Following the observation that Eomes is moderately and transiently increased following a TCR stimulus in the presence of IL-2, the effect of IL-2 alone was examined, given its known function in T cell survival and protein synthesis (Cornish et al. 2006), as well as the effect of lipopolysaccharide (LPS) and IFN-γ, two molecules with wide-ranging and profound effects on the immune system.

At baseline, both Eomes and T-bet expression was higher in CD8+ T cells compared with CD4+ T cells (2^{-dCT} v UBC: Eomes, 0.1 v 0.011; T-bet 0.37 v 0.06; Fig 4.16); this may be a reflection of higher baseline metabolic activity in CD8+ T cells, or that a higher proportion of circulating CD8+ T cells are memory cells, which are known to be more metabolically active than naïve cells (van der Windt et al. 2012).

In CD4 T cells, significant upregulation of Eomes expression was observed in all culture conditions by 4 h. In culture with anti-CD3/CD28 beads, this upregulation peaked at 4 h and was lower than other culture conditions at all other time points, returning to baseline levels by 48 h, whereas in all other conditions upregulation continued to the final time point at 72 h. The addition of LPS or IFN-γ did not make a significant difference compared to IL-2 alone (Fig 4.17 A). In contrast, T-bet was upregulated only in response to an anti-CD3/CD28 stimulus, where upregulation was rapid, peaking at 4 h and declining to baseline levels by 48 h (Fig 4.17 B).

In CD8+ T cells, a similar pattern was observed; in culture with anti-CD3/CD28 beads, Eomes expression increased to 24 h and subsequently declined. Even at peak levels, upregulation was lower than in all other conditions, in which Eomes upregulation continued to the final time point at 72 h (Fig 4.18 A). As was the case for CD4+ T cells, the addition of LPS or IFN-γ did not make a significant difference compared with IL-2 alone. In contrast, T-bet expression was only upregulated in response to anti-CD3CD28

beads, although this was not to the same extent as in CD4 T cells. After a peak of upregulation at 4h, T-bet expression returned to baseline levels by 48h (Fig 4.18 B).

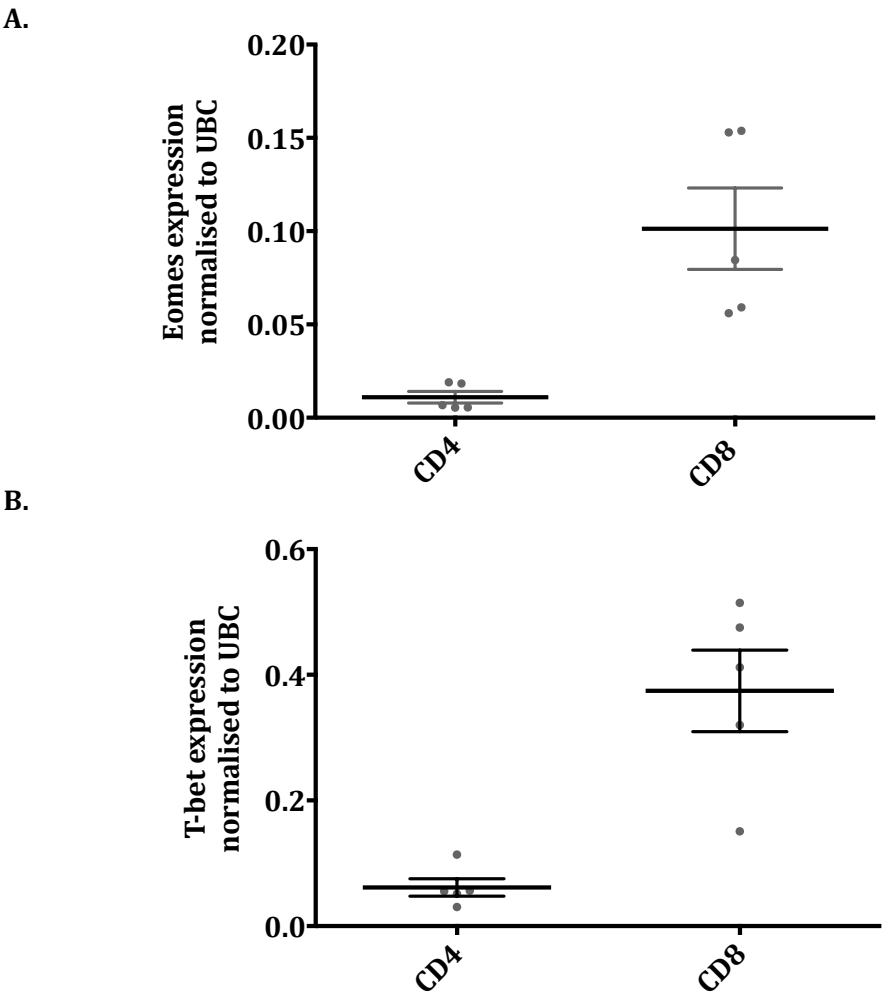
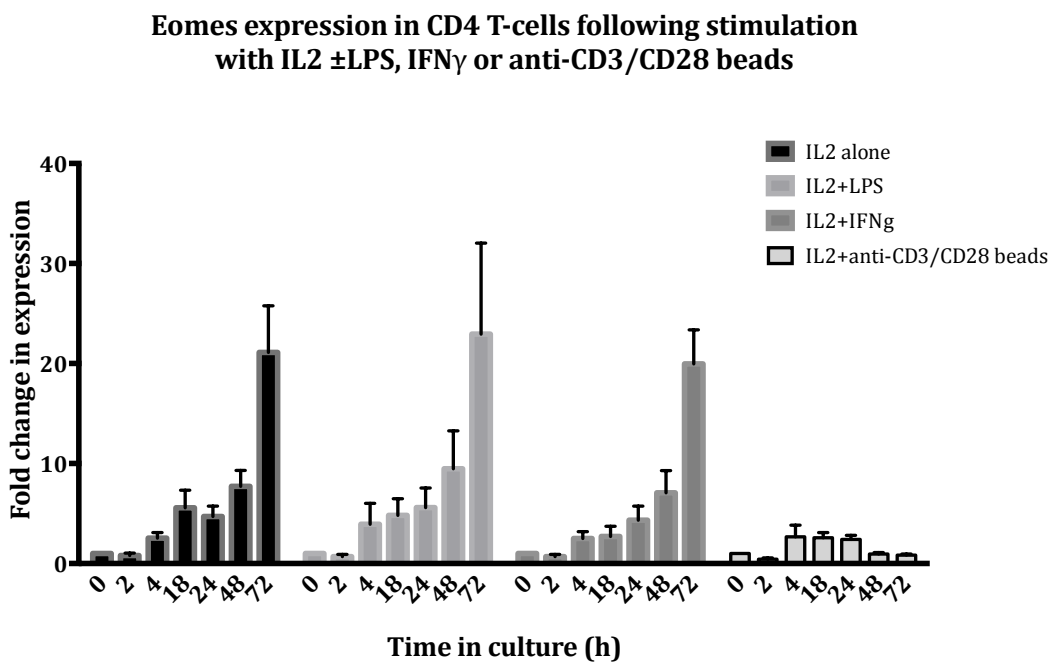


Fig 4.16. Expression of Eomes and T-bet mRNA in T cells. A. Eomes expression in CD4 and CD8+ T cells relative to UBC at baseline**. B.** T-bet expression in CD4 and CD8+ T cells relative to UBC at baseline (n=5).

A.



B.

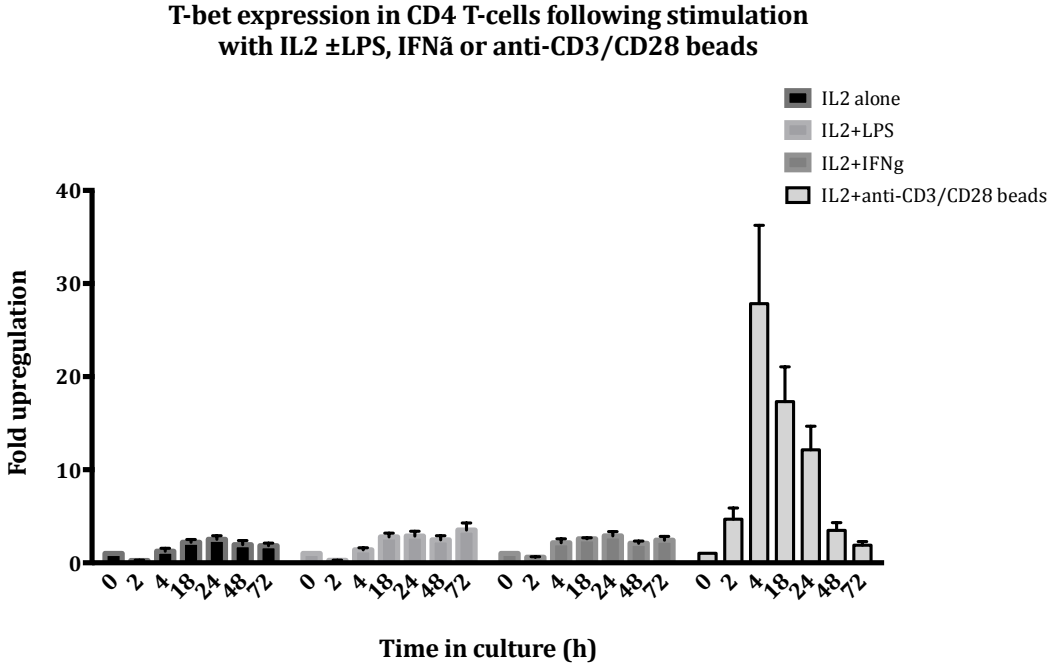
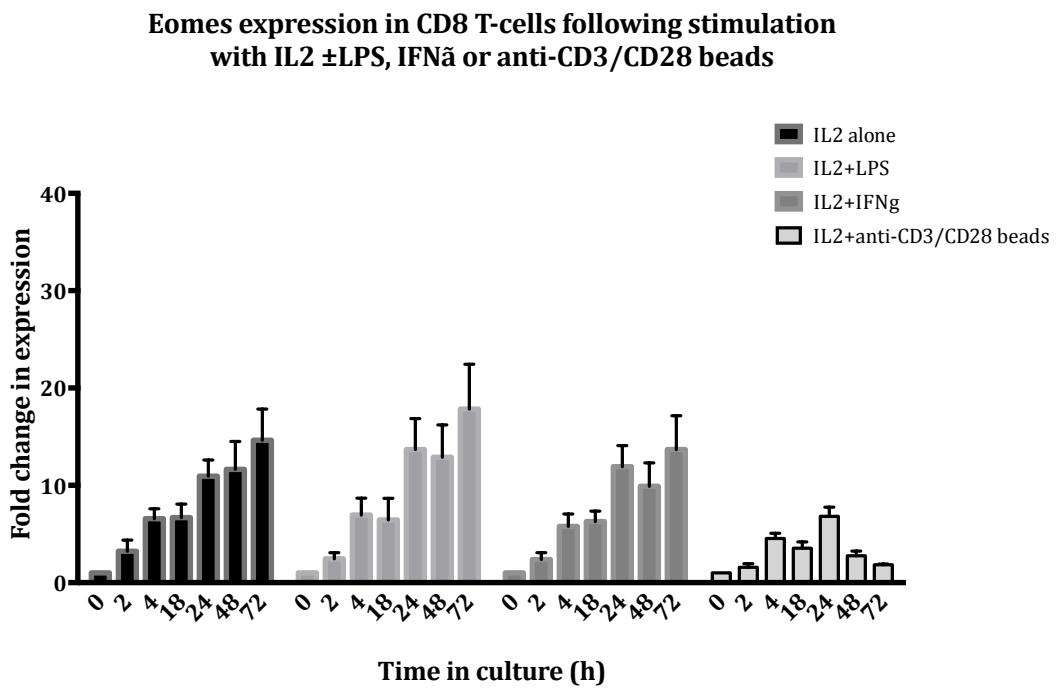


Figure 4.17. T-bet and Eomes expression in CD4+ T cells following stimulation and culture under different conditions. MACS-isolated CD4+ T cells were stimulated with either CD3/CD28 dynabeads, LPS or IFN-γ in the presence of IL-2, or IL-2 alone. Cells were taken at a number of time points and real-time PCR performed for expression of Eomes (upper panel) and T-bet (lower panel). Units are fold upregulation compared to baseline expression against UBC (n=3).

A.



B.

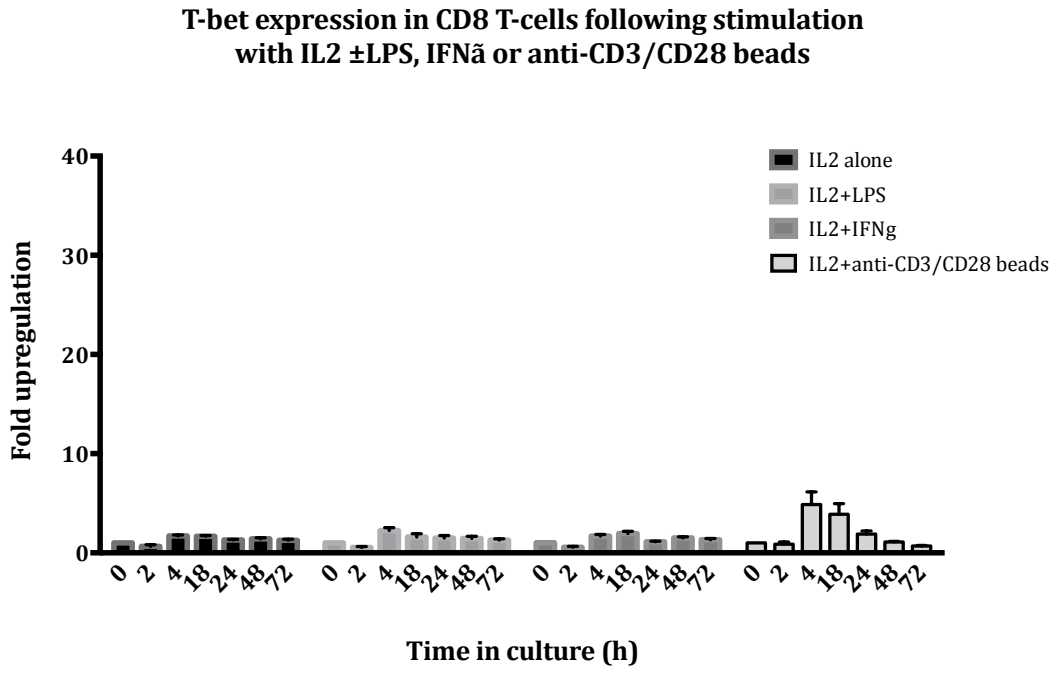


Figure 4.18. T-bet and Eomes expression in CD8+ T cells following stimulation and culture under different conditions. MACS-isolated CD8+ T cells were stimulated with either CD3/CD28 dynabeads, LPS or IFN-γ in the presence of IL-2, or IL-2 alone. Cells were taken at a number of time points and real-time PCR performed for expression of Eomes (upper panel) and T-bet (lower panel). Units are fold upregulation compared to baseline expression against UBC (n=3).

4.3 Discussion

This work determined that Eomes protein, detected by intracellular antibody staining and flow cytometry, was highly expressed by NK and CD8+ T cell populations, as well as a small proportion of CD4+ T cells. In the majority of these populations a bimodal distribution of positive and negative cells was observed. In contrast, little expression was detected in myeloid cells, where despite small apparent shifts in expression compared to isotype control there were no observable positive and negative populations. Concurrent with the experiments performed in this study, a survey of Eomes protein expression in peripheral blood immune cells in humans has been published by another group (Knox et al. 2014). The study investigated expression of Eomes and T-bet in 8 donors, and although different markers were used in certain cases – for instance to identify the four sub-populations of CD8+ T cells – the work shown here is consistent with their findings. In particular, the populations expressing the highest levels of Eomes expression and Eomes positivity, NK cells and CD8+ T cell memory/effector populations, were consistent between these two sets of data. At the mRNA level, a gradient of Eomes expression in CD8+ T cells was observed from naïve to terminal effector cells. This is consistent with microarray data previously published (Gattinoni et al. 2011) and suggests that at least in CD8+ T cells, Eomes mRNA and protein levels correlate despite the effects of post-transcriptional regulatory processes. It also implied that the assays used were sound, and that in further studies it might be possible to infer the mRNA level from protein level assessment in isolation.

From mouse studies there is considerable knowledge of Eomes function and its gene targets. In terms of memory function it is known to directly target the promoter of CD122, the IL-2/IL-15 receptor beta-chain, which is required for intermediate and high affinity IL-15 and IL-2 binding; these cytokines share some physiological roles, and are

particularly important in the development and maintenance of memory cells (Alves et al. 2003, Cornish et al. 2006, Castro et al. 2011). Eomes has also been demonstrated to bind to the loci of the effector molecules IFN-γ and perforin, and to regulate granzyme B expression, molecules associated with cytotoxicity and effector function (Intlekofer et al. 2005, Cruz-Guilloty et al. 2009). This is consistent with high levels of expression in CD8+ T cells and NK cells as observed here.

In CD8+ T-cells the highest levels of Eomes expression were observed in the effector subsets, with a gradient of increasing expression with differentiation from Tn to Tcm, Tem and Temra cells. This differs slightly from mouse studies, in which Eomes has been shown to be particularly important for generation and maintenance of the CD8+ central memory subset, as Eomes knockout mice show reduced ability to generate Tcm cells (Intlekofer et al. 2005, Banerjee et al. 2010, Knudson et al. 2013).

This may reflect species differences in the role of Eomes; in mice Eomes may have a greater role in the regulation of CD122, which appears to play a more significant role in murine memory cells and can be used as a marker of CD8+ T cell memory. In humans Eomes may have a greater role in effector cells. However, it is also possible that Eomes is required for human CD8+ Tcm development but is not highly expressed in mature Tcm cells in the peripheral blood. A possible source of the discrepancy is that mouse studies have access to all cells in both blood and secondary lymphoid organs; it remains a possibility that Eomes is upregulated in lymphoid tissue Tcm cells during their development compared with those in peripheral blood. As the frequency of Tcm cells in secondary lymphoid organs is likely to be higher than in peripheral blood (Sathaliyawala et al. 2013) a considerable degree of Eomes expression in humans may be undetected by sampling only the peripheral blood.

The importance of CD8 T cells in MS is underscored by their presence in MS lesions (Booss et al. 1983, Hauser et al. 1986) and the association of HLA class I alleles

with MS. The size of memory and effector subsets, which express the highest levels of Eomes, appear to be altered in patients with MS (Okuda et al. 2005). Furthermore, limiting the migration of at least one of these subsets is likely to be key to the disease modifying actions of fingolimod, a sphingosine receptor modulator (Johnson et al. 2010).

In CD4+ T cells expression of Eomes was observed in all populations, but only by a tiny proportion of naïve cells and a small proportion of Tcm cells. The Tem population demonstrated the highest Eomes expression with up to a third of these cells Eomes+, similar to the expression of Eomes in CD8+ T cells being highest in the effector populations. This is consistent with *Knox et al*, although that study also included a CD4+ Temra population. On gating with CD45RA and CD62L, this population was not consistently large enough for robust analysis and was therefore excluded. The role of Eomes in CD4+ T cells is unclear, as it has a number of overlapping functions with T-bet, which is a master regulator of Th1 cells. Cytotoxic potential of a small population CD4+ T cells is recognised in both mice and humans (Appay et al. 2002, van de Berg et al. 2008, Zaguia et al. 2013); forced overexpression of Eomes in mouse Th2 cells can induce cytotoxicity (Eshima et al. 2012), and Eomes upregulation is critical for the development of cytotoxicity in mouse CD4+ T cells after stimulation of CD134/CD137 (Qui et al. 2011). It is therefore possible that the small population of Eomes+ CD4+ Tem cells is a cytotoxic population, a thought that has occurred elsewhere due to the similarity of these cells to cytotoxic CD8+ T cells (Martirosyan et al. 2013).

CD4+ T cells are potent cytokine producers, and Eomes may be important in this regard particularly in the production of IFN-γ. Further possible roles of Eomes in CD4+ T cells are in lineage differentiation, where it has been shown to divert cells toward a Th1 phenotype and away from a Th17 phenotype in some contexts (Yang et al. 2008). Memory CD4 T cells from MS patients, but not healthy controls, have been shown to express IL-17 in response to myelin peptides (Venken et al. 2010) and Eomes may be

important in this regard; IL-17 is a potent proinflammatory cytokine required for most models of EAE and present in the lesions of MS (Tzartos et al. 2008).

Given its role in the production of IFN-γ, the archetypal Th1 cytokine, it could be hypothesised that Eomes is expressed by Th1 cells in the absence of T-bet, which is the master regulator of Th1 cells and shares several overlapping functions with Eomes. Lastly, in mice, expression of Eomes has been demonstrated in memory Th2 cells where it has a role in the regulation of IL5 production via interaction with GATA3 (Endo et al. 2011). The role of Eomes in CD4+ T cells has received little attention compared to CD8+ T cells, possibly due to initial studies of mRNA expression on the bulk CD4+ T cell population revealing low levels of expression. Further characterisation of its expression in this subset in humans, with regard to cytokine production and cytotoxic molecule expression, would shed further light on its importance. The role of CD4+ T cells in MS is clearly significant as evidenced by the association of HLA Class II, their presence in MS lesions and as the disease-causing population in EAE.

NK cells were almost uniformly positive for Eomes, consistent with the findings of Knox *et al*. Several downstream targets of Eomes are highly expressed by NK cells, including perforin, CD122 and IFN-γ. Eomes expression was highest in the CD56++CD16- subpopulation of cells which are thought to be cytokine producing, proliferating NK cells (Fehniger et al. 2003). In the peripheral blood this population is small compared to the CD56+CD16+ population, but in secondary lymphoid organs this trend is reversed (Poli et al. 2009). This population expresses a number of surface markers associated with cell migration and trafficking not expressed by other populations of NK cells (Campbell et al. 2001) and has previously been found to expand in the affected joints in inflammatory arthritis (Dalbeth et al. 2002), where they were found to produce IFN-γ in response to IL-15. However, an immunoregulatory role for this subset had already been postulated following the observation that they are able to

secrete significant quantities of IL-10 and IL-13, both cytokines with immunoregulatory properties (Cooper et al. 2001). More recently, this population has been noted to expand in the peripheral blood and CSF of MS patients treated with Daclizumab, an anti-CD25 monoclonal antibody, with further studies suggesting that this subset mediates the immunosuppressive effects of the molecule (Bielekova et al. 2006, Martin et al. 2010, Bielekova et al. 2011).

In mice, Eomes is thought to be necessary for NK cells to maintain a mature phenotype, with T-bet required to reach an earlier checkpoint of development (Gordon et al. 2012). However, this finding is debated; Eomes and T-bet may be responsible for distinct NK lineages developing in parallel (Daussy et al. 2014). In humans, several lines of evidence suggest that CD56++CD16- cells are precursors of CD56+CD16+ cells (Chan et al. 2007, Ouyang et al. 2007, Romagnani et al. 2007), although this remains to be definitively proven. T-bet is also highly expressed by NK cells, particularly by the larger CD56+CD16+ population, and it seems likely that both of these T-box transcription factors play an important role in the development and function of human NK cells.

Over 90% of CD3+CD56+ "NKT" cells were positive for Eomes. This is a greater proportion of NKT cells than in *Knox et al*, but this is likely to be due to the difference in identification; less stringent criteria were used here, and therefore this population is likely to contain effector memory T cells as well as NKT cells, increasing the proportion of Eomes+ cells. Another population of innate like T cells, γδ-T cells, was also reported to express Eomes by *Knox et al*, but was not assessed in this study.

For reasons of practicality, and the limitations of peripheral blood sampling, there were a number of other immune cell subsets in which Eomes expression could not be assessed. This includes tissue-resident memory (Trm) T cells, T-follicular helper (Tfh) cells, macrophages and microglia. It may be unlikely that the latter two express Eomes given the low expression in the rest of the myeloid compartment but this cannot be excluded. Tfh cells are important in shaping the immune response, and the

interaction between CD8+ T cells and CD4+ Tfh cells has been implicated in a mouse model of autoimmunity (Kim et al. 2011). Bcl-6 is thought to determine Tfh lineage (Yu et al. 2009) commitment but Eomes expression in this population is unknown. CD8+ Trm cells persist in specific tissues following infection or vaccination. In mice this population has been suggested to express lower levels of Eomes than other memory CD8+ T cells (Wakim et al. 2012) but in humans their expression signature remains unknown.

Eomes expression was assessed in myeloid cells. Myeloid cells express varying levels of Fc receptors including CD16, CD32 and CD64, causing increased background staining. In addition to blocking with mouse serum, an isotype control antibody used at the same concentration as the Eomes antibody was employed to normalise the data. In spite of this, most myeloid populations showed a low level of expression over isotype control. As this was an order of magnitude lower than the expression observed in lymphoid populations, this was thought most likely to represent either a low level of constitutive expression or a mismatch of fluorophore conjugation between the Eomes antibody and the control antibody.

Overall, little Eomes expression was observed in myeloid cells, consistent with available microarray data and the findings of mouse studies (Heng et al. 2008). Accordingly, studies of these cells were not taken forward to investigation of genotype dependent analyses. However, as mentioned above, it was not possible to analyse tissue macrophages and microglia, and Eomes expression in these populations cannot be ruled out. These populations play an important role in MS and are prominent in histopathological studies of the MS lesion in both RRMS and progressive MS.

Eomes expression in stimulated and cultured T cells was also analysed; the aim of performing cell stimulations was to approximately recreate in vivo conditions in

which Eomes expression might occur, including a range of stimuli from a direct TCR stimulus to "bystander" activation conditions, with the possibility of using these conditions in further studies. As the major subsets of cells thought to be involved in the adaptive immune activity of MS, CD4+ and CD8+ T cells were studied. Cells were cultured for 3 days, and IL-2 was included in all cultures to improve cell survival. We observed Eomes upregulation in all conditions, but surprisingly a TCR stimulus (anti-CD3/CD28 beads) significantly reduced Eomes expression when compared with IL-2 alone. Addition of IL-15, IFN-γ or LPS did not cause significantly increased expression compared with IL-2 alone. In contrast, T-bet expression was significantly increased by addition of a TCR stimulus.

These findings are consistent with known roles of these transcription factors; namely that T-bet plays a greater role in short-term effector function in response to antigen stimulation, and that Eomes expression is increased once the immediate stimulus has subsided, in response to cytokines necessary for survival and memory function such as IL-2 (Cruz-Guilloty et al. 2009, Pipkin et al. 2010). A recent study, published since the work in this chapter was performed, has shown that culture of naïve mouse CD8+ T cells with IL-2 in TCR-independent conditions results in differentiation into memory cells associated with the expression of Eomes (Cho et al. 2013). A limitation of this study is that IL-2 was ubiquitously included as a survival factor; in light of its effects on Eomes expression, it would have been useful to culture cells in the absence of IL-2 to explore the specific effect of this cytokine on Eomes expression. This could be included as part of future work.

IL-2 signals through the IL-2 receptor, which has three components, CD25 (IL2Rα), CD122 (IL2Rβ) and the common gamma chain γc; all three components are required for the high-affinity receptor, but signaling also occurs through the intermediate and low affinity receptors, which are homodimers of the β or α chains complexed with γc. Two of the components, CD122 and γc, are shared by the

intermediate affinity IL-15 receptor. However, IL-15 can also signal through the high affinity IL15Rα (Chae et al. 1996, Castro et al. 2011). In this study IL-15 had no effect on Eomes expression in addition to IL-2. This may have been due to saturation of receptor subunits by the levels of IL-2 used. Furthermore, IL-15 may have a significant effect on cell proliferation, which was not assessed here, and less effect than IL-2 on transcription and protein synthesis (Cornish et al. 2006).

IFN-γ is recognised to have autocrine effects and to regulate a number of CD8+ and CD4+ T cell functions, such as proliferation, increased expression of cytolytic molecules and further expression of IFN-γ (Schroder et al. 2004, Reed et al. 2008, Curtsinger et al. 2012). LPS is more more commonly studied in the context of the innate immune system, where it is known to activate toll-like receptor 4 (TLR4) on macrophages and DCs, but recent studies have shown that it can act directly on CD8+ T cells, inducing granzyme B and perforin expression and altering behavior such as chemotaxis in inflammatory states (Zanin-Zhorov et al. 2007, Komai-Koma et al. 2009). Despite these widespread effects, neither molecule increased the expression of Eomes over IL-2 alone. These molecules signal via distinct pathways from IL-2, and the effect of LPS has been noted to synergise with IL-12, which is a repressor of Eomes expression (Takemoto et al. 2006, Komai-Koma et al. 2009). It is possible therefore that they increase the production of effector molecules in the short term, which in vivo may be via T-bet (Kim et al. 2012), but do not have an effect on memory function and the expression of Eomes.

The kinetics of Eomes expression are evidently complex, and considering the results of the work shown above a decision was made that cell stimulations would not be performed in genotype-dependent analyses in this phase of the study. This was partly for reasons of practicality and the need to focus the project on assays that were felt to provide the greatest chance of detecting a genotype dependent difference in Eomes expression. The variation between donors introduced by prolonged cell culture would

have required greater numbers of donors to be recalled on a given day to optimize study standardization, causing conflicts in study design, as the wide range of ex-vivo analyses could only be performed for a limited number of donors at any one time. Furthermore, it was felt that given the possible small effect of the SNP, the focus of the work should be on unmanipulated cells studied directly ex-vivo. This included those populations that to some extent have already undergone activation such as effector and memory T cells, which might recapitulate some of the effects of in-vitro stimulation.

However, there is little doubt that, at least at the mRNA level, a proportion of expression quantitative trait loci (eQTL) only become apparent on stimulation (Fairfax et al. 2014). This would need to be be explored in future work, which before embarking on studies of genotype dependent expression would necessarily involve further identification of the conditions in which Eomes is maximally upregulated. This would involve different titrations of the TCR stimulus, for different lengths of time, as well as different combinations of cytokines including IL-7, IL-2 and IL-15. As T cell subsets respond differently to various cytokines (Lugli et al. 2013), this might need to be performed on a subset-specific basis following cell sorting.

Chapter 5

Genotype to phenotype correlation: analysis of Eomes expression and downstream molecules in primary immune cell subsets by genotype at rs2371108 and rs669607

5.1 Introduction

In Chapter 3, analysis of the relevant genomic regions provided evidence of an association between the MS-associated SNPs and altered expression of Eomes. Examination of available eQTL datasets indicated that the effect was isolated to Eomes rather than on other genes in the region. This prompted a focus on Eomes for the remainder of the study. The characterisation of Eomes expression in peripheral immune cell subsets, described in Chapter 4, identified the cell populations in which Eomes was most highly expressed and thus likely to play an active role; those subsets were investigated further in a genotype dependent manner. The hypothesis was that in Eomes-expressing primary immune cell subsets, change of genotype at MS-associated SNPs, rs669607 and rs2371108, would be associated with altered levels of expression of Eomes protein. These two SNPs were used due to the GWAS findings at the time and represent the primary and secondary association signals respectively. eQTL studies suggest that risk genotype at the primary association signal correlates with reduced Eomes expression, whereas risk genotype at the secondary association signal correlates with increased expression. Therefore it was hypothesized that these patterns of correlation would be observed at the protein level, and that the cell types in which these observations were made would be different for each association signal.

Given the wide-ranging functions of Eomes in immune cells, a further hypothesis was that a change in Eomes expression would lead to detectable downstream effects, including potential changes in the proportion of certain cell populations and the production of cytolytic molecules. It was also possible that although an Eomes protein eQTL might prove difficult to observe, these downstream effects of genotype might be more easily detected due to amplification of the signaling pathway.

As the proportion of healthy individuals homozygous for the minor (risk) allele at rs669607 and rs2371108 is 14.4% and 22% respectively, the Oxford Biobank was utilised to obtain a genotype-specific cohort of donors; recalling individuals on the basis of genotype enabled enrichment of the cohort for homozygous donors and increased the statistical power of the study (see Section 2.3.2). A pilot study on retrospectively genotyped donors included a heterozygote group due to abundance of heterozygotes in the general population. For the prospectively genotyped main cohort, only homozygotes were recalled to maximize the chance of detecting a difference in functional readout by genotype at either of the SNP loci.

Detecting a genotype dependent change in Eomes expression would yield another link between single nucleotide polymorphisms and disease susceptibility, further validating the hypothesis-generating approach of GWAS, and enabling the map of "susceptibility pathways" to be expanded. This might demonstrate overlap with previously elucidated pathways and other susceptibility genes, building the knowledge of key pathways in the susceptibility of multiple sclerosis that might eventually lead to the identification of drug targets or important environmental factors in the aetiology of MS.

An association with expression would also provide proof of concept for SNPs in non-coding regions affecting protein level expression and determining susceptibility. As previously mentioned, the vast majority of variants detected in GWAS are in non-coding regions. While some of these are located close to the genes they influence, and may

affect the gene promoter or a splice site, many are located in distant regions whose precise role is unknown, as is the mechanism by which they affect disease susceptibility. For this reason, although there are several previously published functional genomics studies investigating the effects of polymorphisms, few of these address variants in regions more than a few hundred bases from the coding regions. Non-synonymous coding variants are infrequently found in GWAS of common diseases compared with non-coding variants, but their effects have been identified in certain cases (Couturier et al. 2011). There has also been some success investigating intronic polymorphisms at splice sites: an intronic SNP in TNFRSF1A resulted in splicing out of exon 6 and the creation of a soluble TNF receptor (Gregory et al. 2012) and similarly, an MS associated polymorphism in IL7R results in splicing out of an exon (Gregory et al. 2007) leading to altered levels of soluble IL7R (Lundstrom et al. 2013). Investigations of genotype dependent changes in expression level have mostly measured mRNA levels (De Jager et al. 2009, Leikfoss et al. 2013). A study of type 1 Diabetes (T1D)-associated polymorphisms using a similar approach to this study detected expression level changes in IL2RA on CD4+ T cells, as well as the number of CD25+ CD4+ T cells; the SNP with an associated dosage effect was intronic (Dendrou et al. 2009). This has subsequently been found to correlate with the number of granulocyte macrophage colony stimulating factor (GM-CSF) –producing Th cells (Hartmann et al. 2014).

This project therefore aimed to demonstrate that distant variants exert a specific effect on gene/protein expression with effects in primary immune cells that are quantifiable by commonly used lab methods.

5.2 Results

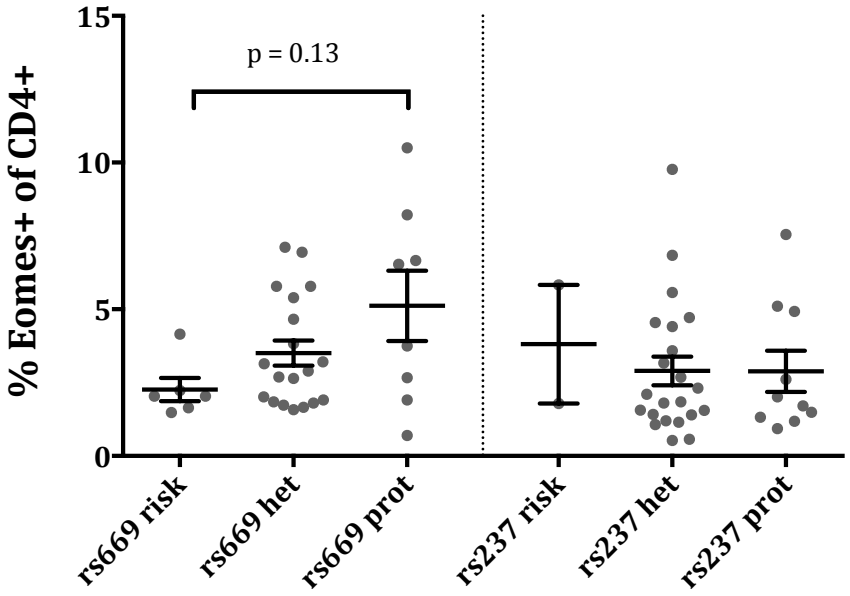
5.2.1 Eomes expression in CD4+ and CD8+ T cells by genotype; results of pilot study

The study was divided into a primary pilot study and a subsequent main cohort study. The pilot study included analyses of Eomes expression in CD4+ and CD8+ T-cells and their sub-populations. It was better powered to test expression differences by genotype at the primary signal than at the secondary signal, with more than ten donors per homozygote group for rs669607. Although apparent differences in expression in the total CD8+ (risk 37.3% v prot 49.5%, p=0.10) and CD4+ (2.26% v 5.1%, p=0.13) populations were observed (Fig 5.1) these did not reach significance. However, analysis of sub-populations revealed a significant difference in Eomes expression in CD8+ central memory cells (Tcm) by genotype at rs669607 (Fig 5.2); higher proportions of Eomes+ cells correlated with the protective allele (47.8±3.7% v 67.6±4.6%; p=0.004). No difference was observed by genotype at rs2371108 (Fig 5.3).

A similar trend was observed in analysis of CD4+ T cells, with higher proportions of Eomes+ CD4+ Tem (11.3±3.0% v 18.7±3.3%; p=0.10), Tn (0.95±0.2% v 1.73±0.4%, p=0.10) and total CD4+ (2.26±0.4 v 5.12±1.2, p=0.13) cells correlating with the protective allele at rs669607 (Fig 5.2). Fewer donors were included in these analyses (n=5 v n=8) and the difference did not reach significance.

These findings prompted expansion of the study to a larger cohort (referred to as the main cohort) to ensure that the eQTL was replicable with greater numbers, to determine associations in other cell populations and to explore downstream effects. The numbers for this cohort were generated using power calculations based on the pilot study. Data from the pilot study was incorporated into the results for some measures of Eomes expression in CD8+ and CD4+ T cells as detailed below.

A.



B.

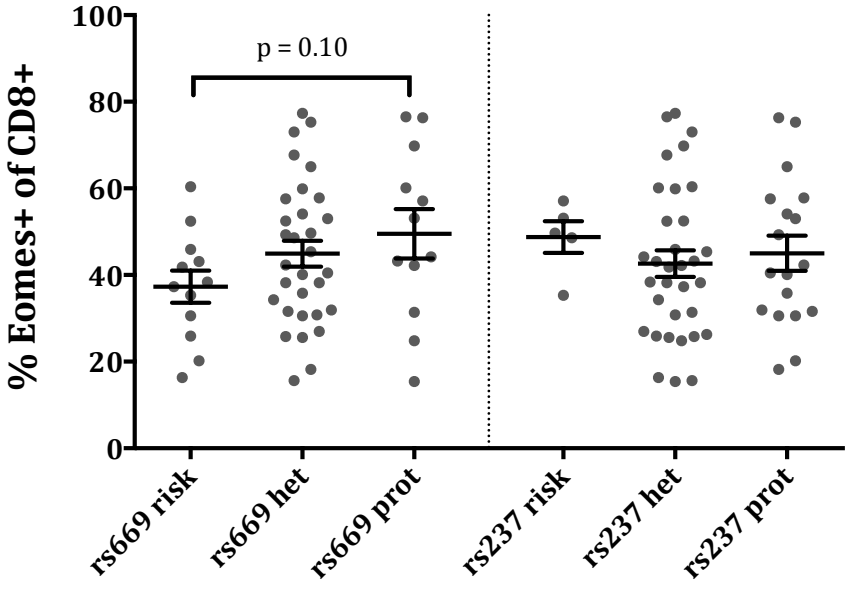
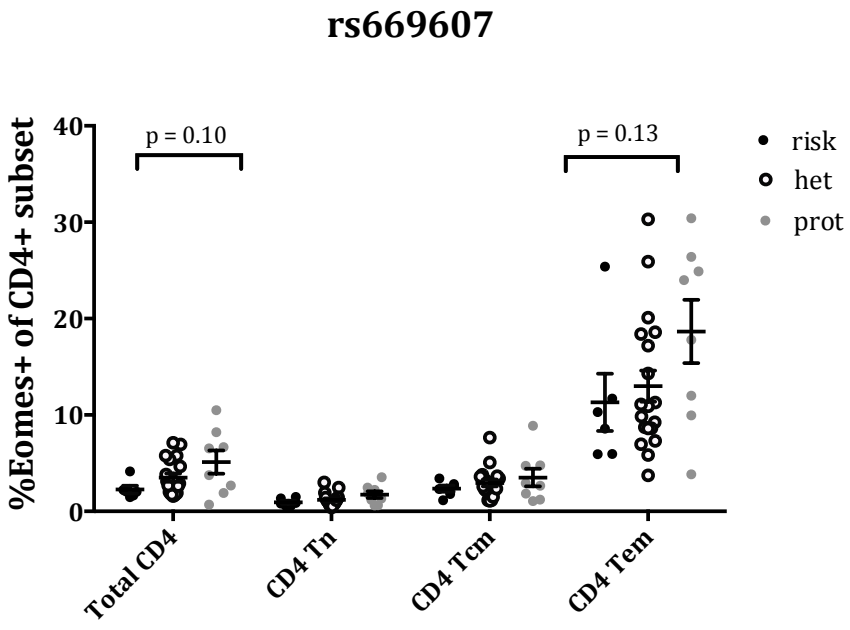


Fig 5.1. Pilot study of Eomes expression in CD4+ and CD8+ T cells by genotype at rs2371108 and rs669607. **A**. The proportion of Eomes-expressing cells was quantified by flow cytometry in bulk CD4+ (A) and CD8+ (B) T cells by genotype at rs669607 and rs2371108. P-values were calculated using the general linear model in SPSS and are uncorrected for multiple analyses. Graphs show mean±SEM.

A.



B.

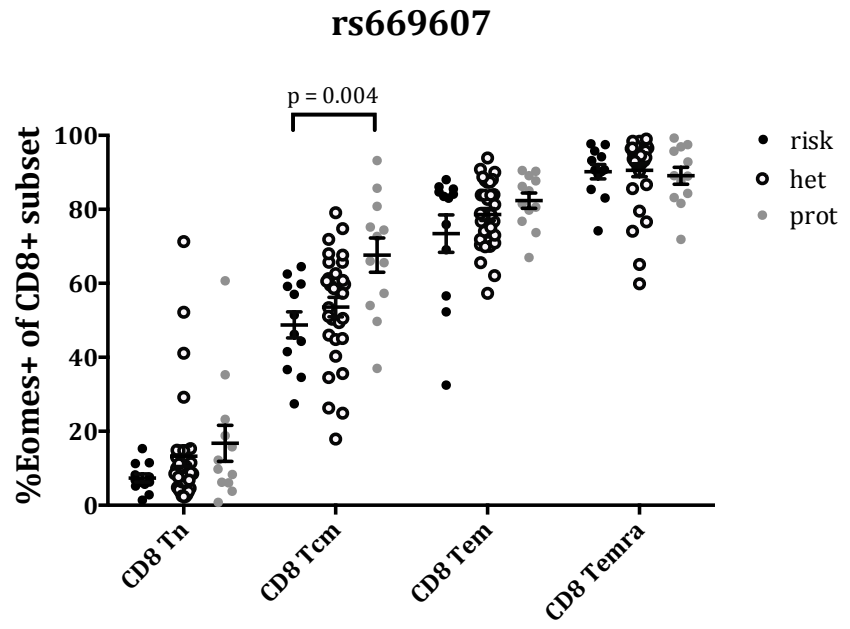
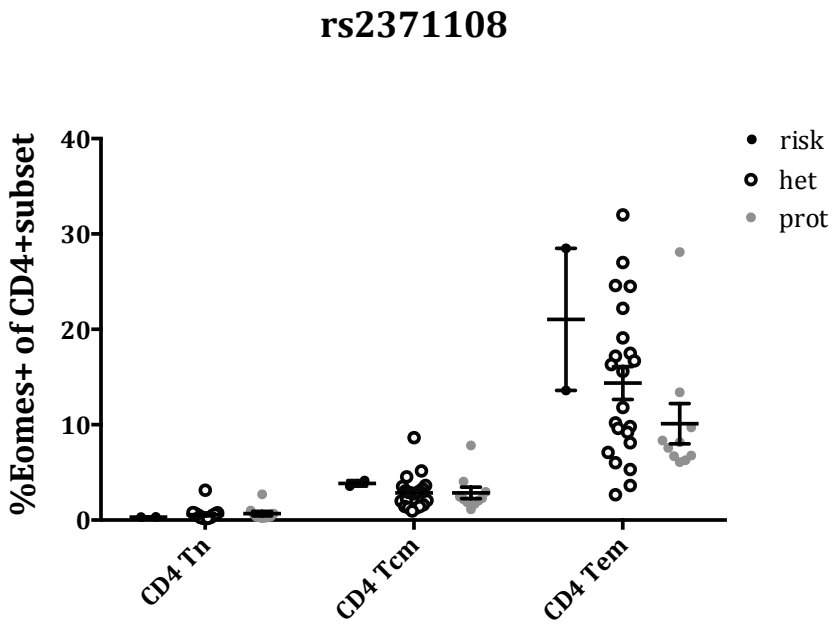


Fig. 5.2. Pilot study of Eomes expression in CD4+ and CD8+ T cells by genotype at rs2371108 and rs669607. The proportion of Eomes-expressing cells was quantified by flow cytometry in CD4+ (A) and CD8+ (B) T cell subsets by genotype at rs669607. CD4+: n=6 (risk) v n=8 (prot); CD8+: n=12 (risk) v n=12 (prot). Graphs show mean±SEM.

A.



B.

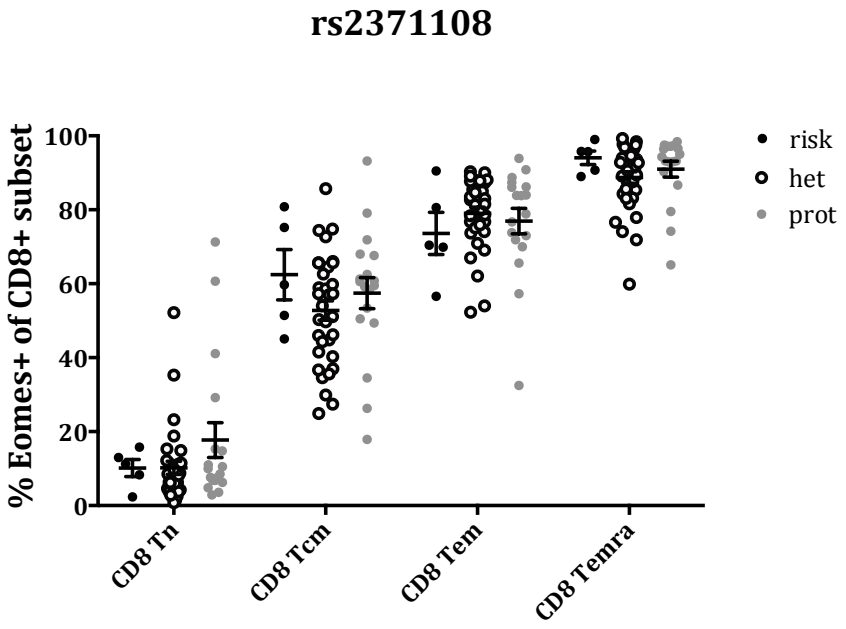


Fig. 5.3. Pilot study of Eomes expression in CD4+ and CD8+ T cell subsets by genotype at rs2371108. The proportion of Eomes-expressing cells was quantified by flow cytometry in CD4+ (A) and CD8+ (B) T cell subsets by genotype at rs2371108. CD4+: n=2 (risk) v n=10 (prot); CD8: n=5 (risk) v n=18 (prot). Graphs show mean±SEM

5.2.2 CD4+ T-cell polarisation by genotype at rs669607 and rs2371108: results of pilot study.

As Eomes has been reported to influence the polarisation of CD4+ T cells into Th1 or Th17 phenotypes (Yang et al. 2008, Ichiyama et al. 2011), a pilot study was undertaken to investigate whether CD4+ T cells from individuals with different genotype at the two SNP loci underwent differentiation more readily into Th17 cells. Magnetic bead sorted CD4+ T cells were cultured for 8 days in medium containing IL-2, IL-6, IL-23, TGF-β, IL-1β and a low ratio (1:50) of anti-CD3/CD28 beads. Stimulation and staining for IL-17 and IFN-γ was performed at day 1 and day 8. The absolute increase in percentage of cells expressing IL-17 was measured (Fig 5.4).

No significant difference was observed in the polarisation of CD4+ T cells by genotype at either SNP (Fig 5.5). Due to concerns about the high inter-individual variability, polarisation was plotted against day of experiment; clustering by day of experiment was observed (Fig 5.6). This suggested that variability due to additional genetic or environmental factors also contributed to CD4+ T cell polarisation. Therefore, this pilot study was not expanded to the main cohort due to the projected difficulty achieving statistical power with the number of donor samples available.

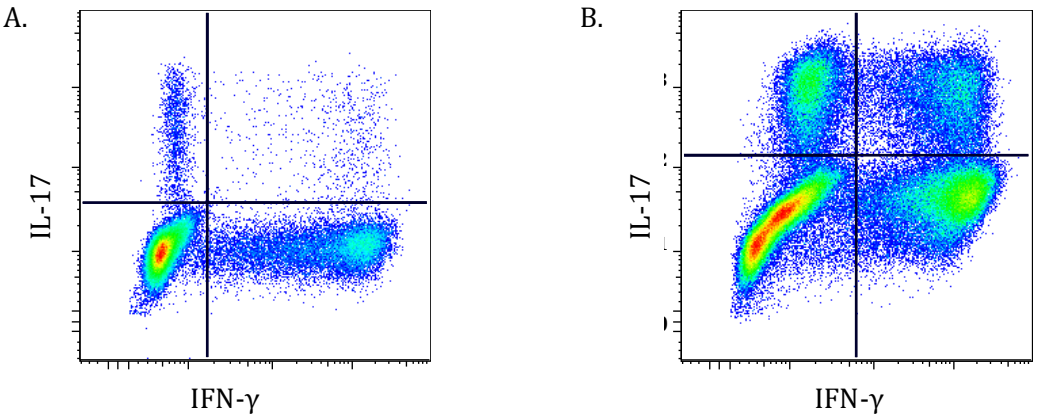


Fig 5.4. CD4+ T-cell polarisation. MACS-sorted CD4+ T cells were cultured in the presence of IL-2, IL-6, IL-23, TGF-β, IL-1β and anti-CD3/CD28 beads in a 1:50 bead to cell ratio. Stimulation with PMA/Ionomycin was performed at day 1 (A) and day 8 (B).

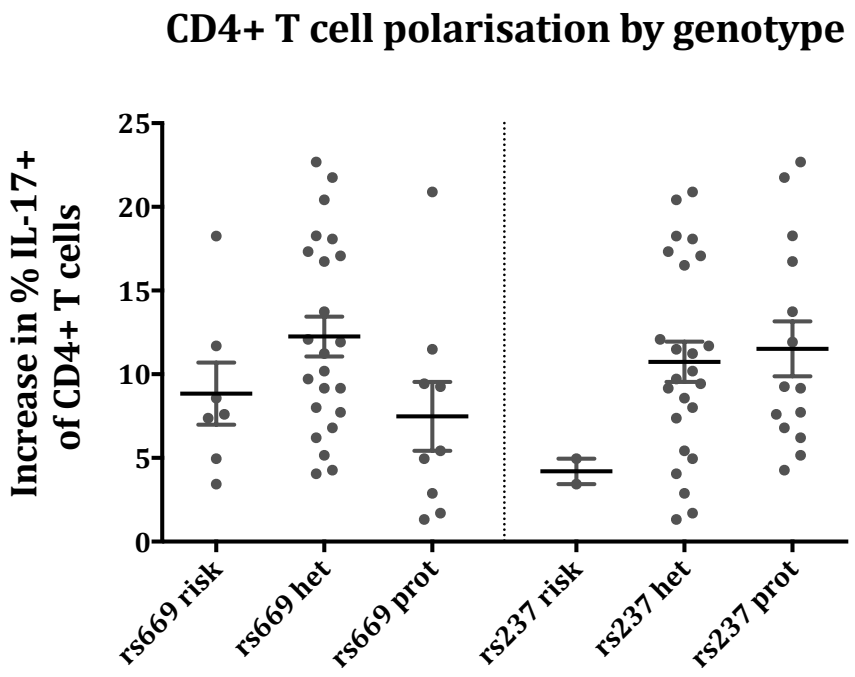


Fig 5.5. CD4+ T-cell polarisation by genotype: pilot study. The increase in %IL-17+ cells after 8 days in culture was analysed by genotype at rs2371108 and rs669607.

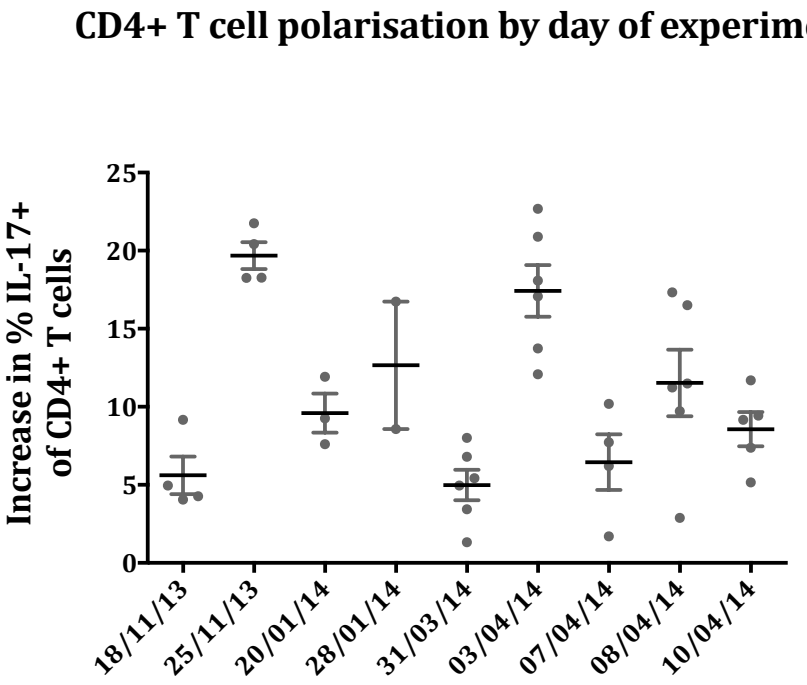


Fig 5.6. CD4+ T-cell polarisation by genotype: pilot study. The increase in %IL-17+ cells after 8 days in culture was analysed by day of experiment.

5.2.3 Eomes expression in CD8+ T cells by genotype at rs669607 and rs2371108

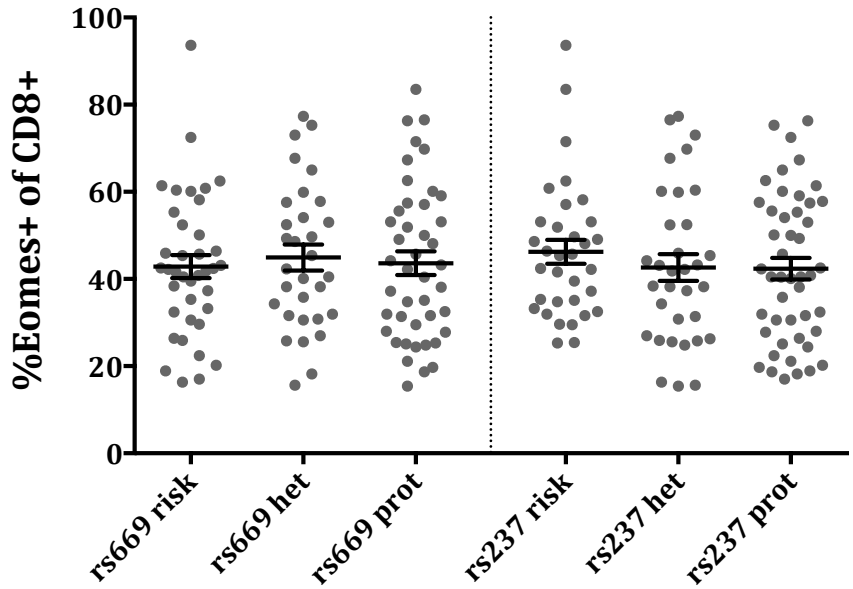
CD8+ T cells were gated as per Fig 4.7; first on the basis of CD8 expression, and then on the basis of CD45RA and CD62L expression, to define 4 populations. On expansion of the study to the main cohort, the antibody panel for CD8+ T cell assays was altered to include a CCR7 antibody and a CD127 antibody. Due to changes in the flow cytometry panel, data from the pilot and main cohorts was combined for analyses of "%Eomes+" CD8+ T cells, as due to the bimodal population these cells could still be easily distinguished, but not for analyses of "Eomes MFI" in these populations (see section 2.3.2 for further details). Therefore, where a heterozygote group is included this indicates that data from both the pilot and main cohorts has been used.

Initially, total CD8+ T cells were analysed and the level of Eomes expression between homozygous risk and protective donors at rs669607 and rs2371108 was compared, in terms of Eomes+ cells and MFI relative to isotype (Fig 5.7). The mean percentage of Eomes+ cells across all genotypes was 43.6±1.6%, and mean expression was 45.3±2.5. However, analysis by genotype did not show any significant difference between genotype groups either in the percentage of cells positive for Eomes (rs669607 risk 42.9±2.6 v prot 43.6±2.7; rs2371108 46.22±2.7 v 42.2 ±2.5), or the level of expression in positive cells (Fig 5.7, rs669607 risk 43.90±3.0 v prot 46.45±3.8; rs2371108 49.25±3.2 v 41.35±3.6).

Subsequently, analyses of the CD8+ T cell subsets were performed by genotype. As expected, higher Eomes expression was observed in increasingly differentiated cells, consistent with the characterisation analyses: expression was highest in Temra cells, which were almost universally Eomes+ (90.3%), followed by Tem (78%) and Tcm (56%) populations, both of which had significantly higher numbers of Eomes+ cells than Tn cells (12.6%) (Figs 5.8, 5.9). However, contrary to the findings of the pilot study,

analysis in a larger cohort did not reveal any significant difference between genotype groups at either SNP in either the proportion of positive cells or Eomes MFI. In particular, the difference in Eomes expression in CD8+ Tcm cells by genotype at rs669607 was not replicated in the main cohort (Fig 5.8). Apparently higher Eomes expression in the Tem population in rs237-risk donors (53.55±3.24 v 45.3±2.46, p=0.038, Fig 5.9), did not reach significance after correction for multiple testing, which had an adjusted p-value significance threshold of 0.005 (section 2.6.1).

A



B.

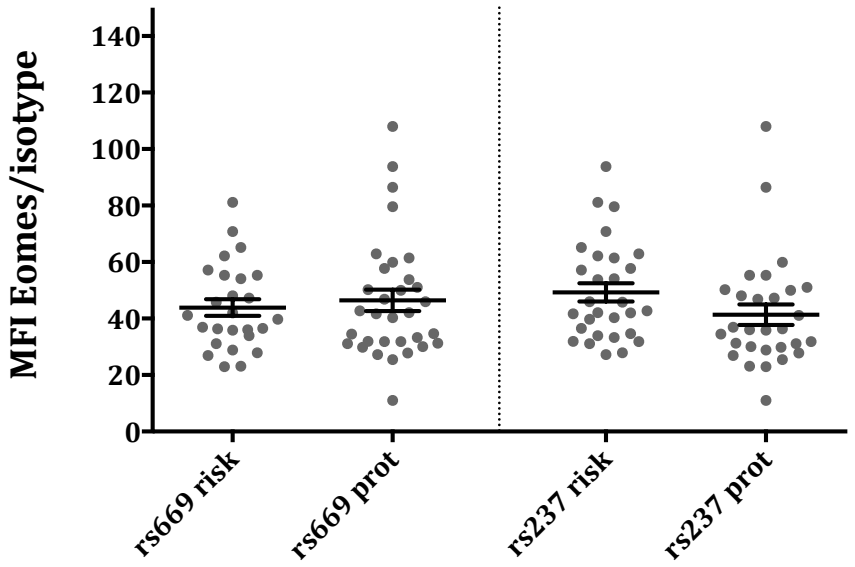
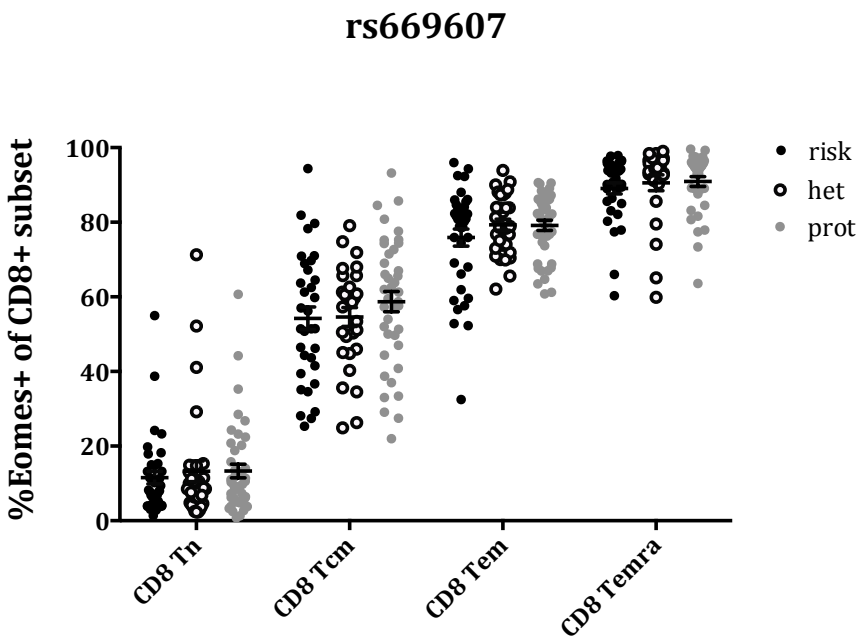


Fig 5.7. Eomes expression in total CD8+ T cells by genotype. Eomes expression by genotype at rs2371108 and rs669607 was analysed in total CD8+ T cells, both as the proportion of Eomes expressing CD8+ T cells (A) and the MFI of Eomes-expressing cells normalised to isotype control (B). Graphs show mean±SEM.

A.



B.

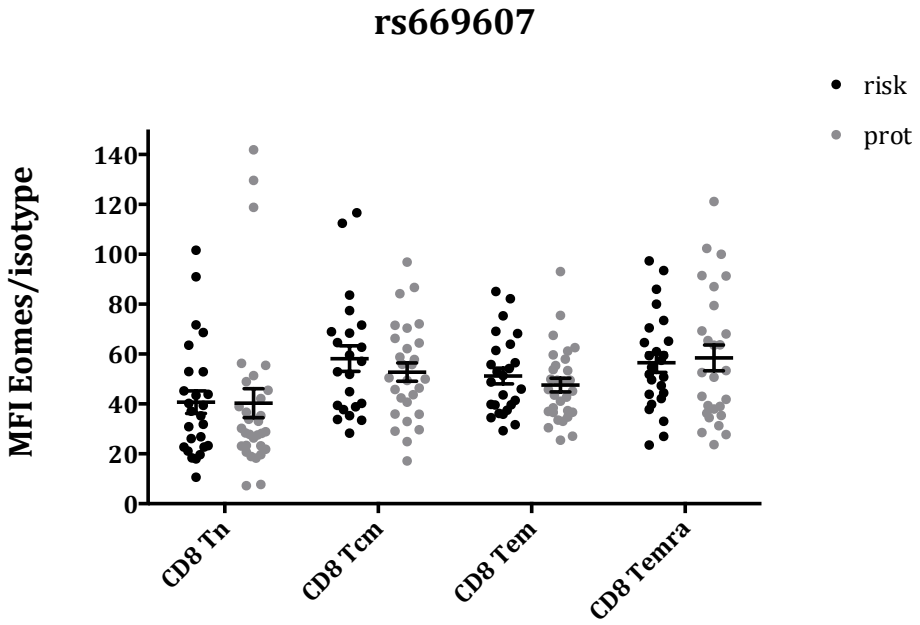
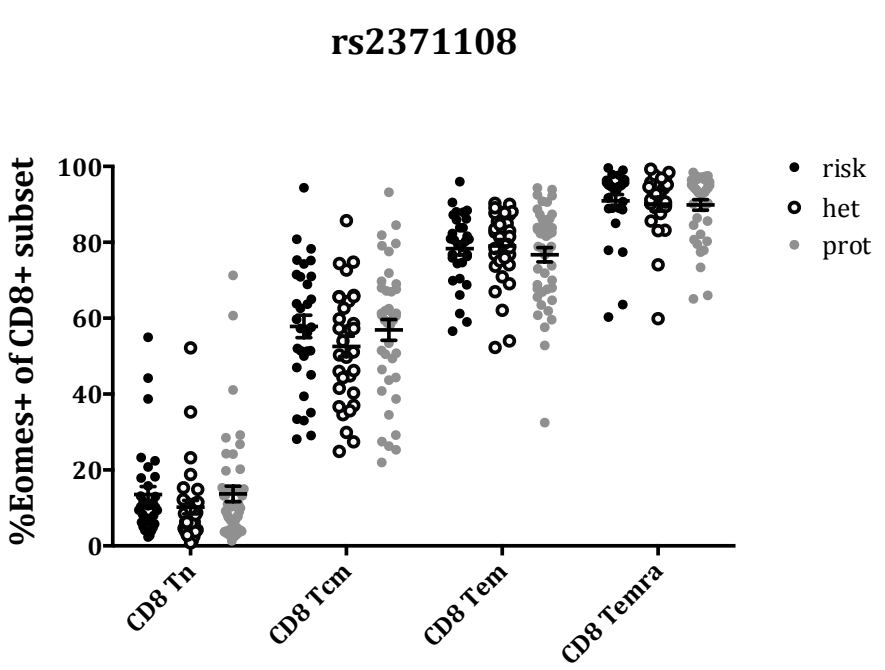


Fig 5.8. Eomes expression in CD8+ T cell subsets by genotype. Eomes expression by genotype at rs669607 was analysed in CD8+ T cell subsets as both the proportion of Eomes expressing cells (A) and Eomes MFI relative to isotype control (B). Graphs show mean±SEM.

A.



B.

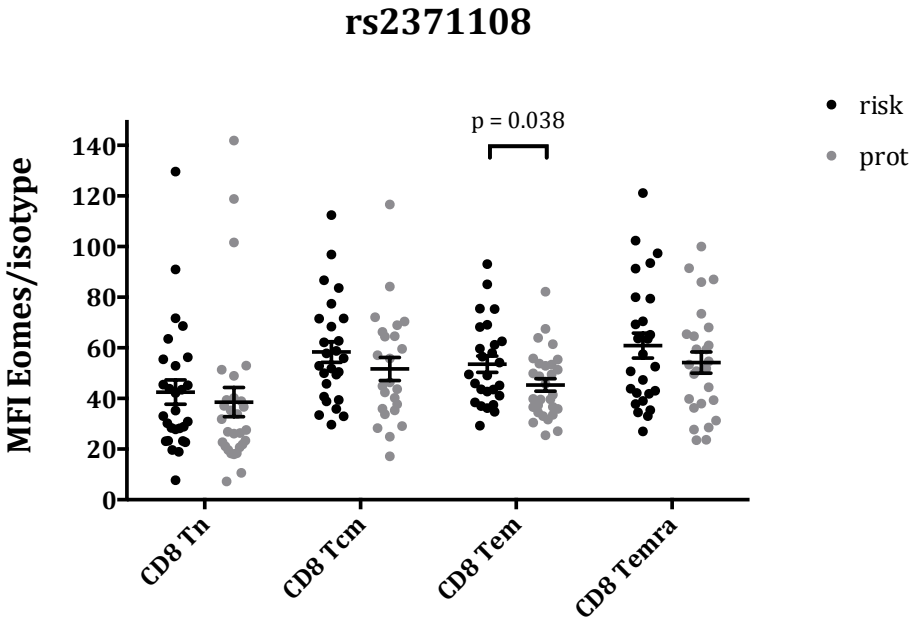


Fig 5.9. Eomes expression in CD8+ T cell subsets by genotype. Eomes expression by genotype at rs2371108 was analysed in CD8+ T cell subsets as both the proportion of Eomes expressing cells (A) and Eomes MFI relative to isotype control (B). P-values were calculated using the general linear model in SPSS, and where shown are uncorrected for multiple analyses. Graphs show mean±SEM.

5.2.4 Eomes expression in CD4+ T cells by genotype at rs669607 and rs2371108

Gating for CD4+ effector and memory populations was performed as per Fig 4.8, into Tn, Tcm and Tem populations on the basis of CD45RA and CD62L expression. In addition, in the main cohort, three populations were identified by gating for CD45RA and CXCR3, a chemokine receptor expressed on a proportion of effector cells, and which can be found on T cells and astrocytes in MS lesions (Balashov et al. 1999, Simpson et al. 2000, Groom et al. 2011). Almost all the Eomes+ CD4+ cells arose from the CXCR3+ population, with very few found in the CXCR3- populations (Fig 5.10 A).

In all of the defined CD4+ subsets, the proportion of Eomes+ CD4+ T cells was compared by genotype at rs2371108 and rs669607. This was initially performed in the total CD4+ population, where no significant differences between genotypes at either SNP locus was observed (Fig 5.10 B). Subsequently, differences in the Eomes+ proportion of CD4+ subsets, defined by CD45RA and either CD62L or CXCR3, were performed. These analyses did not reveal any significant difference between genotype groups for Eomes+ cells in these subsets whether defined by CD62L or CXCR3 at rs6689607 (Fig 5.11) or rs2371108 (Fig. 5.12); despite the larger cohort the data did not continue the trend observed in the pilot study (CD4+ Tn $p=0.856$; CD4+ Tcm $p=0.689$).

It should be noted that as "%Eomes+" cells was the only measure used in CD4+ T cell popoulations due to the low levels of Eomes expression, data from both pilot and main cohorts was combined, and therefore a heterozygote group is included in each figure in this section.

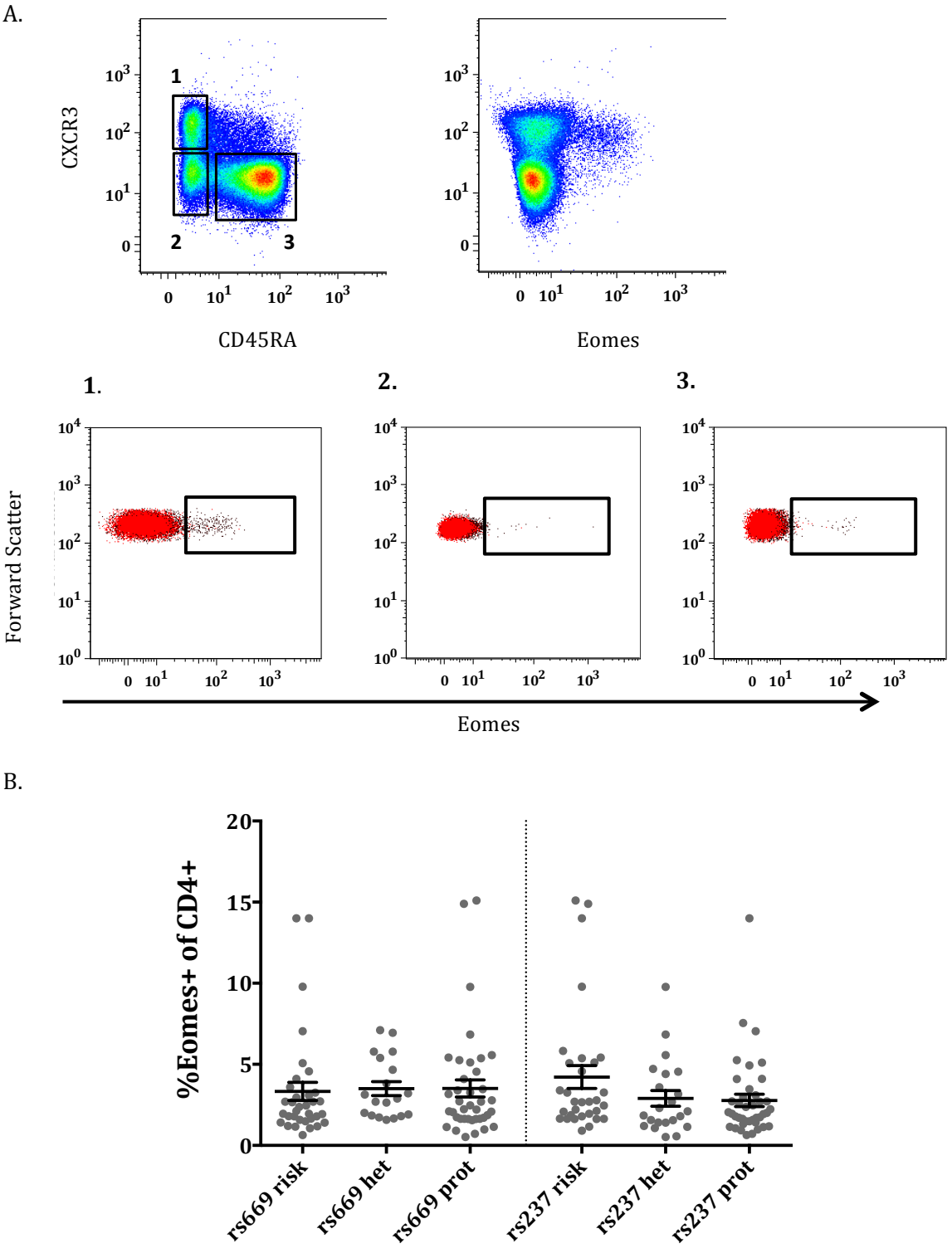
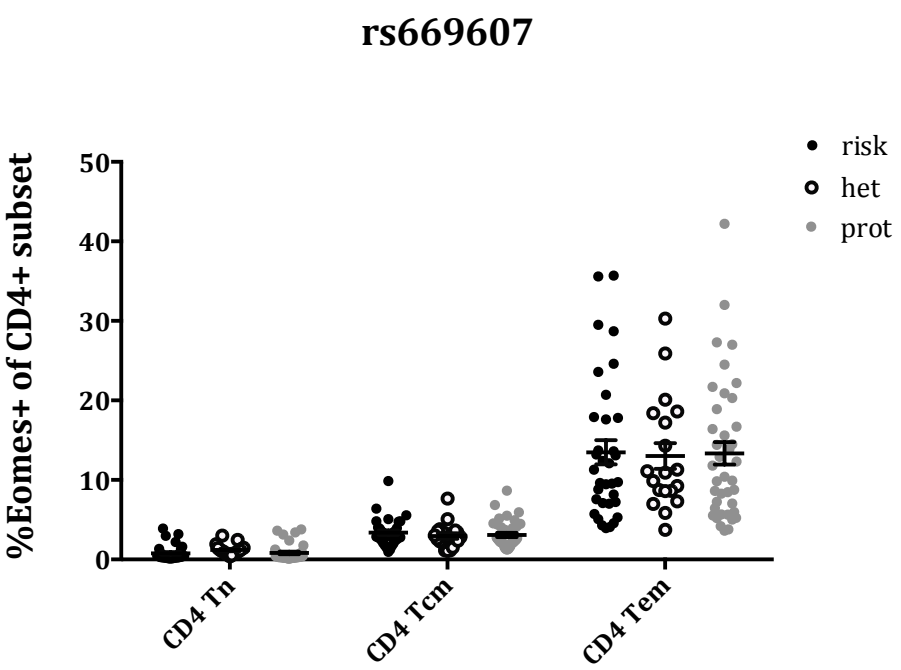


Fig 5.10. Analysis of Eomes expression in CD4+ T cells by genotype at rs2371108 and rs669607. A. Gating on lymphocytes, singlets and CD4+ cells was performed as demonstrated previously. In addition to previously defined CD4+ T cell subsets on the basis of CD62L and CD45RA, three subpopulations of CD4+ T cells were defined by CD45RA and CXCR3 expression. Eomes expressing cells were defined according to expression over isotype control (red dot plots). **B.** Eomes expression as the Eomes+ proportion of total CD4+ T cells by genotype at rs2371108 and rs669607. Graphs show mean±SEM

A.



B.

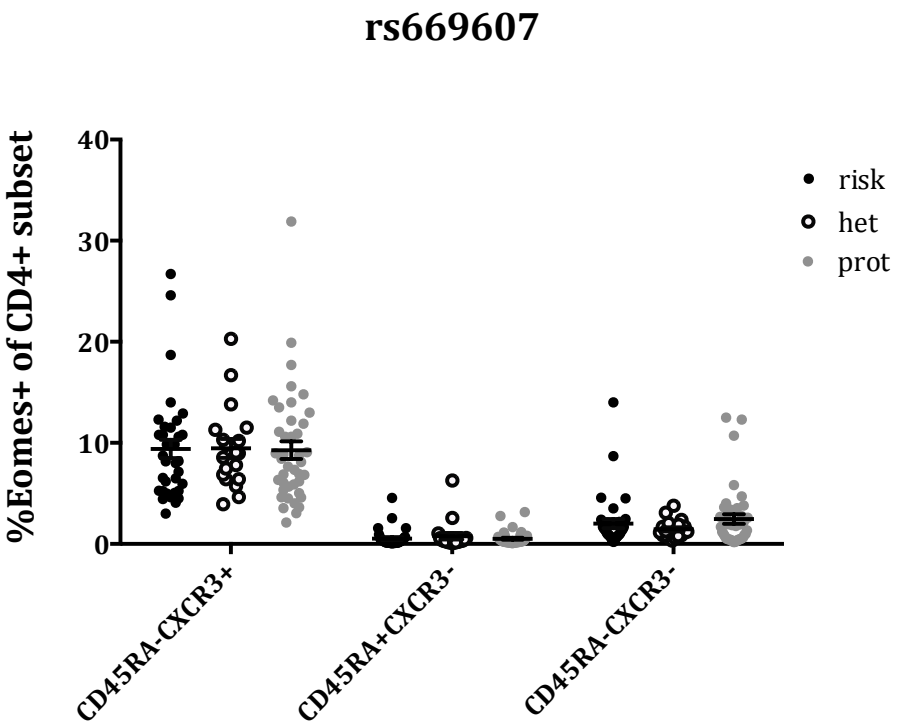
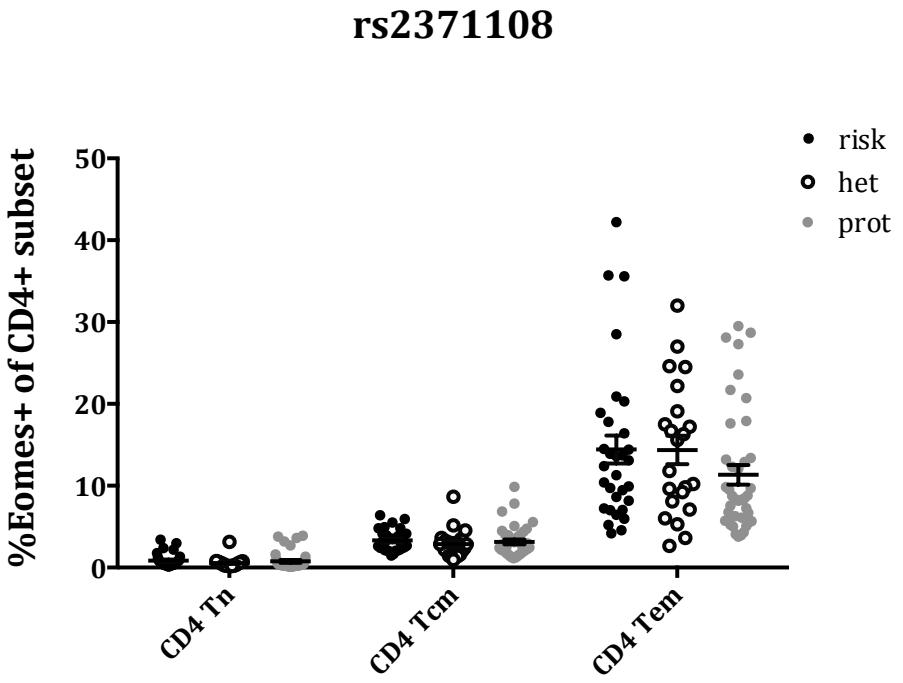


Fig 5.11 Analysis of Eomes expression in CD4+ T cells by genotype at rs669607. Eomes expression as the Eomes+ proportion of CD4+ subsets defined by CD45RA/CD62L (top) and CD45RA/CXCR3 (bottom) was analysed by genotype at rs669607.

A.



B.

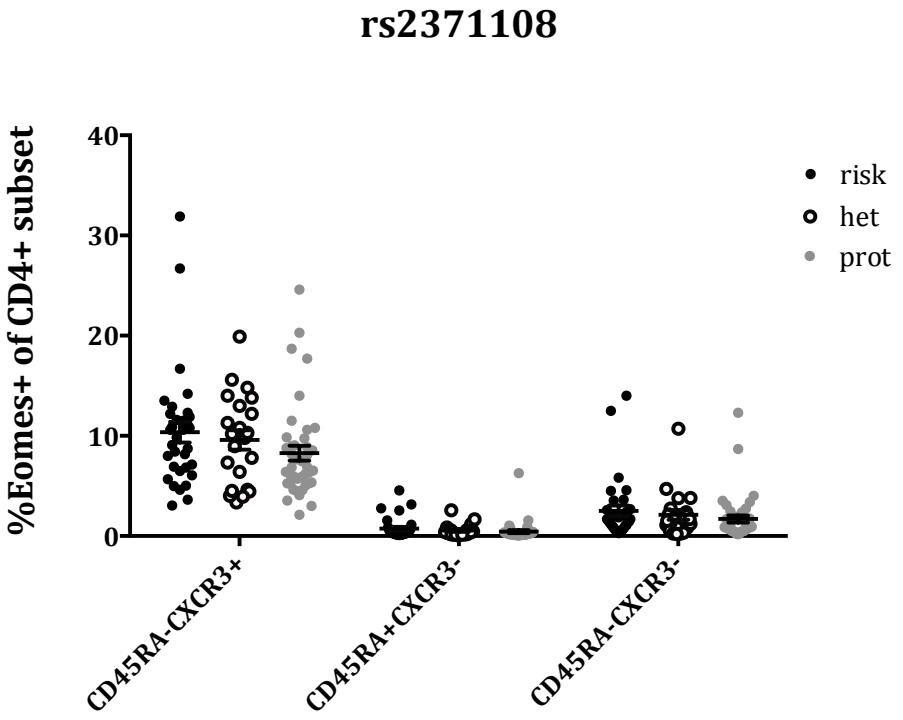


Fig 5.12 Analysis of Eomes expression in CD4+ T cells by genotype at rs2371108. Eomes expression as the Eomes+ proportion of CD4+ subsets defined by CD45RA/CD62L (top) and CD45RA/CXCR3 (bottom) was analysed by genotype at rs2371108.

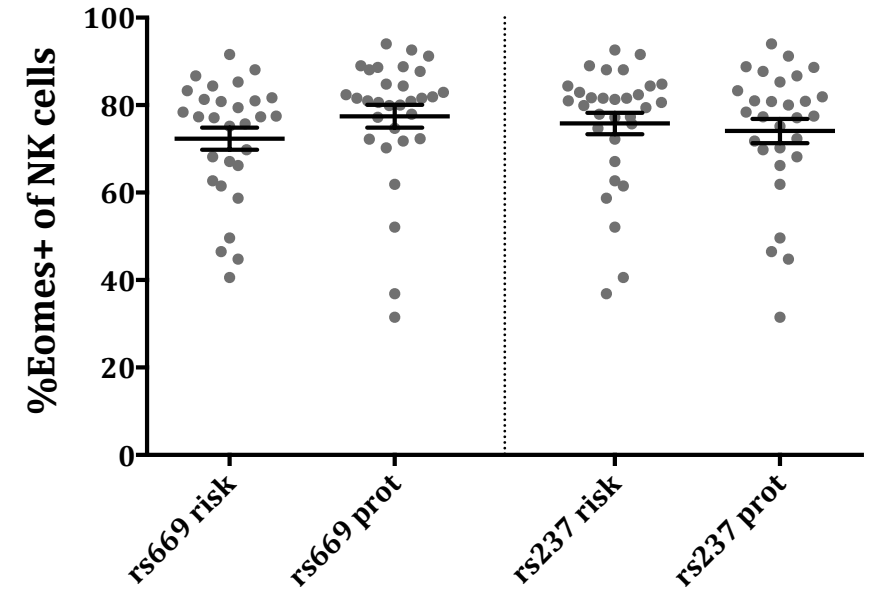
5.2.5 Analysis of NK cells by genotype at rs669607 and rs2371108

Natural Killer (NK) cells are known to express high levels of Eomes and its target genes CD122, perforin and granzyme B (Lettau et al. 2007, Montaldo et al. 2013). Furthermore, Eomes is crucial for the development of NK cells and the maintenance of NK cell maturity (Gordon et al. 2012). The study therefore went on to assess Eomes expression in total NK cells as well as the two most prominent NK cell subsets. These subsets are thought to represent two stages in NK cell development (Chan et al. 2007, Romagnani et al. 2007); therefore, given the role of Eomes in this regard, the relative size of these populations in peripheral blood was also analysed by genotype. Total NK cells and the CD56+CD16+/CD56++CD16- subsets were gated first on the basis of CD3 and CD56, and then on the basis of CD56 and CD16 (Fig 4.13). Initially the total NK cell population was analysed by genotype in terms of Eomes+ cells and Eomes expression. In total NK cells a high proportion of the cells (75±1.8%) was observed to express Eomes, as expected. No significant difference was observed by genotype in the bulk population in either the proportion of Eomes positive cells (rs237, risk 75.81 v prot 74.09%; rs669, 72.3 v 77.4%) or Eomes MFI (rs237, risk 27.2 v prot 21.7; rs669, 23.2 v 25.7)(Fig 5.13).

On analysing the NK cell subsets, in both populations (CD56+CD16+ and CD56++CD16-) the vast majority of cells expressed Eomes (84±1.9% and 95±0.4% respectively, Fig 5.11). As there was no consistently clear bimodal distribution, Eomes expression was analysed for the whole subset rather than the Eomes+ fraction. Comparison of Eomes expression in NK cell subsets by genotype at rs669607 (Fig 5.14) and rs2371108 (Fig 5.15) did not reveal any significant differences. An apparent trend toward higher MFI in the CD56++CD16- subset in rs2371108-risk donors (36.5 v 27.2) was observed, but this difference was diminished after correcting for gender (p=0.28) (Fig 5.15).

Analysis of the relative sizes of the two NK cell subsets by genotype at both SNP loci was then carried out (Fig 5.16). This revealed a trend toward higher numbers of CD56++CD16- cells in rs669607 risk donors (4.5±0.5% v 3.35±0.2%; p=0.028), which also failed to reach significance after correction for multiple analyses (Bonferroni adjusted significance threshold 0.0083).

A.



B.

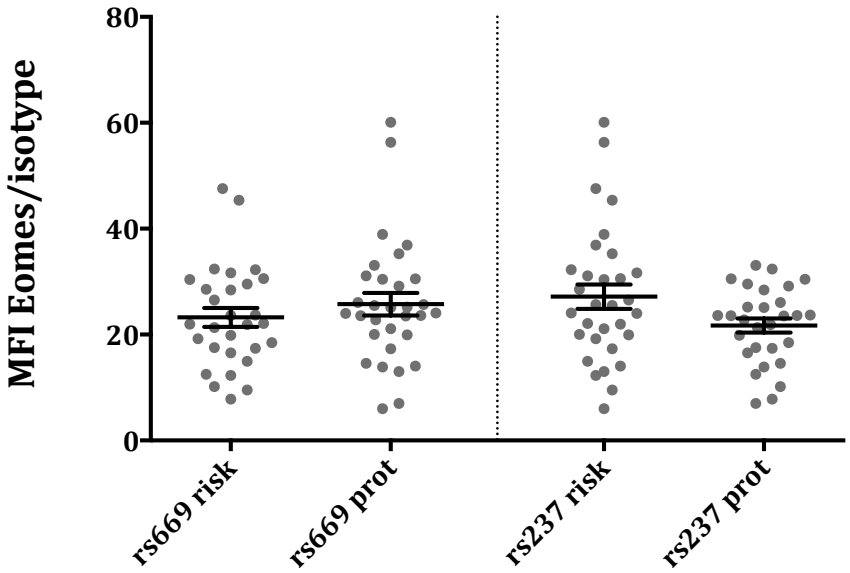
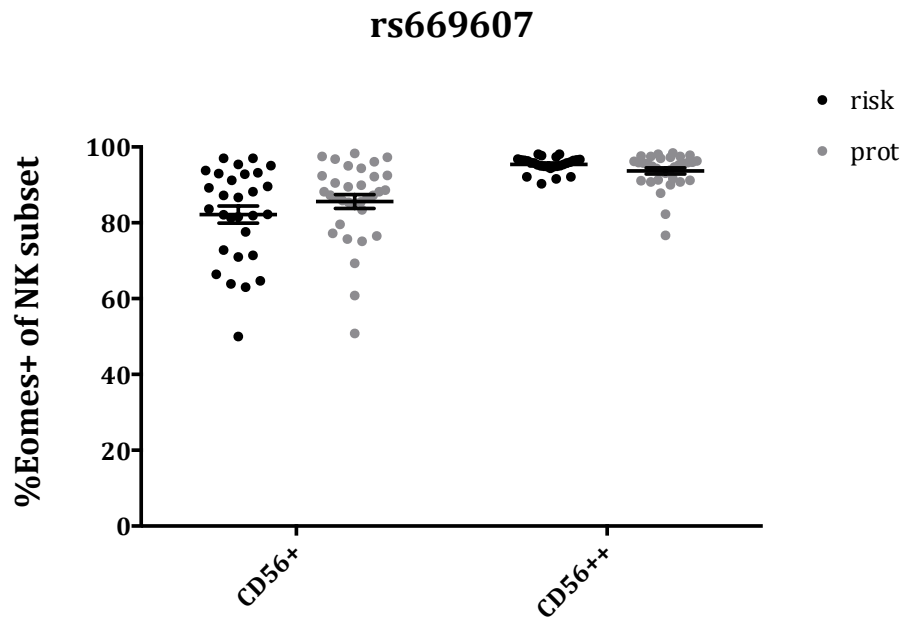


Fig 5.13. Analysis of Eomes expression in NK cells by genotype. Eomes expression by genotype at rs2371108 and rs669607 was analysed in total NK cells, both as the Eomes-expressing proportion of NK cells (A) and the MFI of NK cells normalised to isotype control (B). Graphs show mean±SEM.

A.



B.

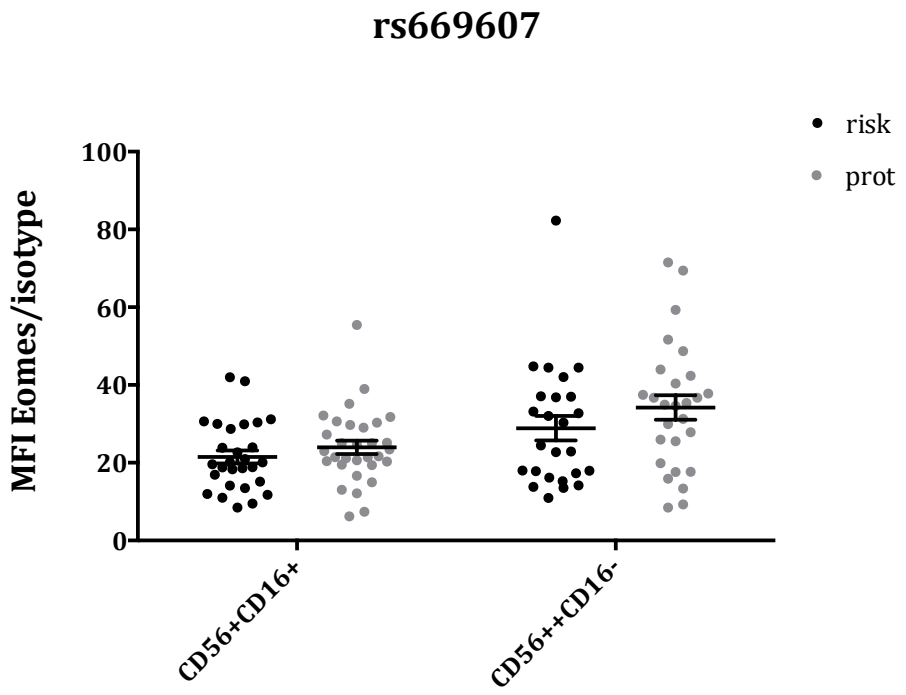
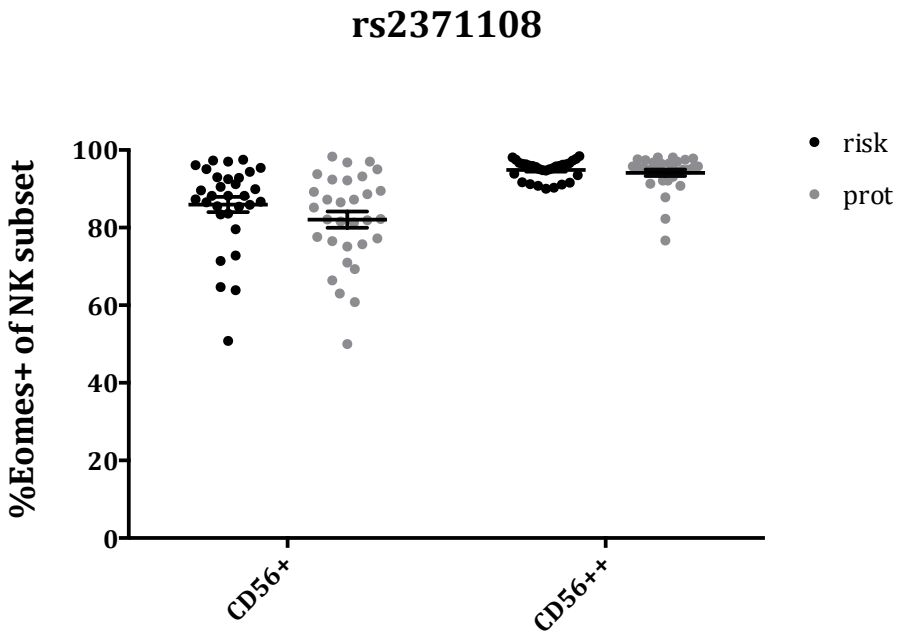


Fig 5.14. Eomes expression in NK cell subsets by genotype at rs669607. Eomes expression by genotype at rs669607 was analysed by flow cytometry in NK cell subsets as both the Eomes expressing proportion of subsets (A) and Eomes MFI of subsets relative to isotype control (B). Graphs show mean±SEM.

A.



B.

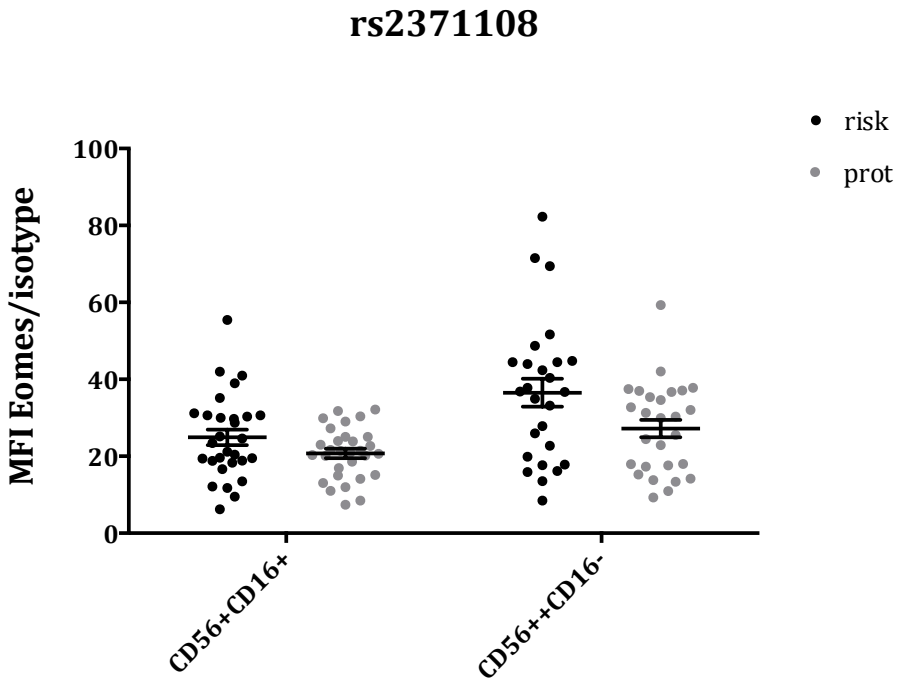
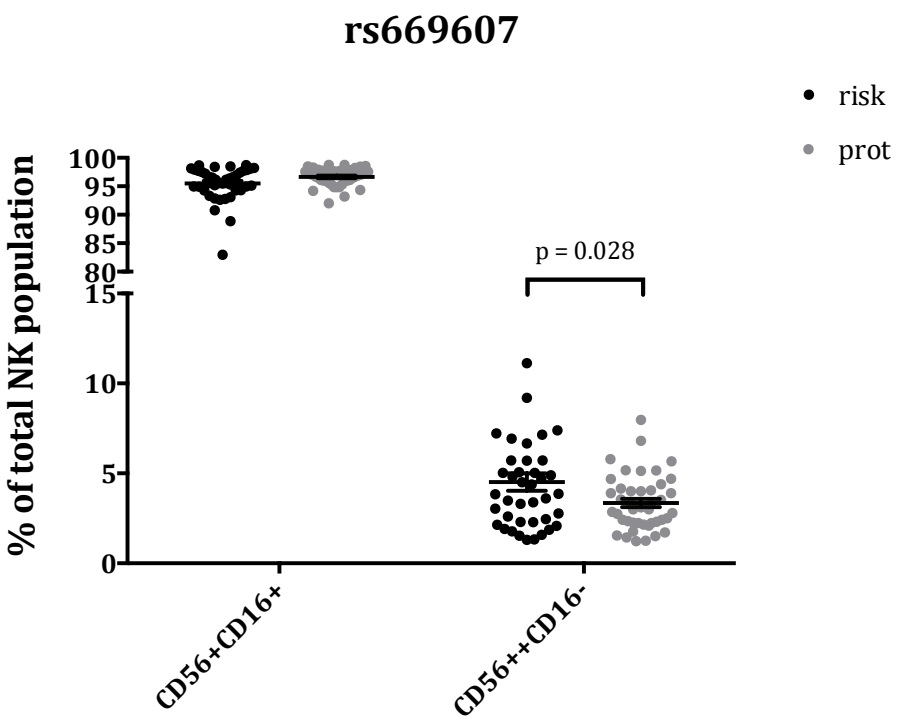


Fig 5.15. Eomes expression in NK cell subsets by genotype at rs2371108. Eomes expression by genotype at rs2371108 was analysed by flow cytometry in NK cell subsets as both the Eomes expressing proportion of subsets (A) and Eomes MFI of subsets relative to isotype control (B). Graphs show mean±SEM.

A.



B.

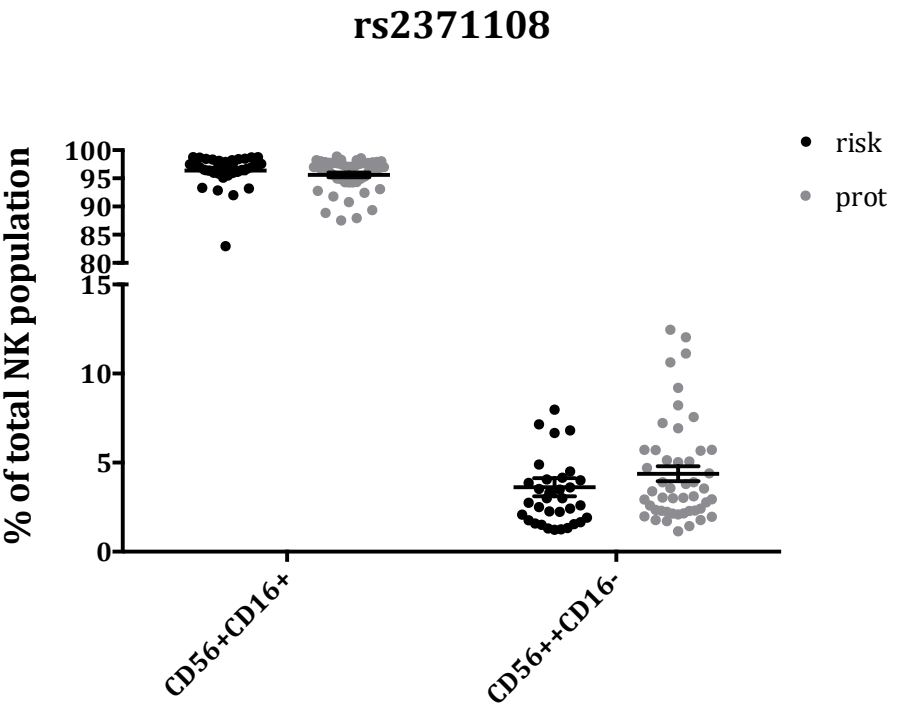


Fig 5.16. Analysis of relative NK subset size by genotype. The two major NK cell subsets were defined by CD56 and CD16 expression using flow cytometry. The relative size of these subsets was calculated and analysed by genotype at rs669607 (A) and rs2371108 (B). Graphs show mean±SEM.

5.2.6 Analysis of exhausted CD8+ T cells by genotype at rs669607 and rs2371108

"Exhausted" T cells are observed in the context of chronic viral infection and, although thought of as a state of T cell dysfunction, may represent a further state of differentiation distinct from effector or memory cells (Wherry 2011). Exhaustion has been linked to Eomes upregulation and PD-1 expression in mRNA expression microarray studies (Wherry et al. 2007, Doering et al. 2012, Schietinger et al. 2014). The nature of the association is unclear – whether high Eomes expression predisposes to exhaustion or whether exhaustion represents a distinct functional phenotype in which Eomes is upregulated remains to be elucidated. Hypothesising that altered Eomes expression might lead to altered numbers of exhausted T cells, we analysed the number of exhausted CD8+ T cells, defined as PD-1 expressing cells, as well as the level of Eomes expression in the exhausted cells by genotype.

The 4 effector/memory CD8+ T cell populations were stratified on the basis of CD45RA and CD62L as described previously. Exhausted CD8+ T cells were then gated on the basis of PD-1 expression above isotype control. The Tem population contained the highest proportion of PD-1+ cells (28.9±1.6%); very few PD-1+ cells were observed in the Tn (0.70±0.1%) or Temra populations (5.2±0.6%)(Fig 5.17). However, genotype dependent differences were not observed for PD-1+ population size (Fig 5.18 A, 5.19 A). Consistent with the literature, PD-1+ cells expressed uniformly high levels of Eomes relative to isotype control (PD-1+Tcm 55.3±3.4; PD-1+Tem 46.4±1.5) but no difference in level of expression was detected between risk and protective homozygotes at rs669607 (Tcm 51.9 v 58.8, p=0.352; Tem 45.0 v 47.9, p=0.624, Fig 5.18 B) or rs2371108 (Tcm 60.4 v 50.7, p=0.184; Tem 50.0 v 43.0, p=0.231, Fig 5.19 B).

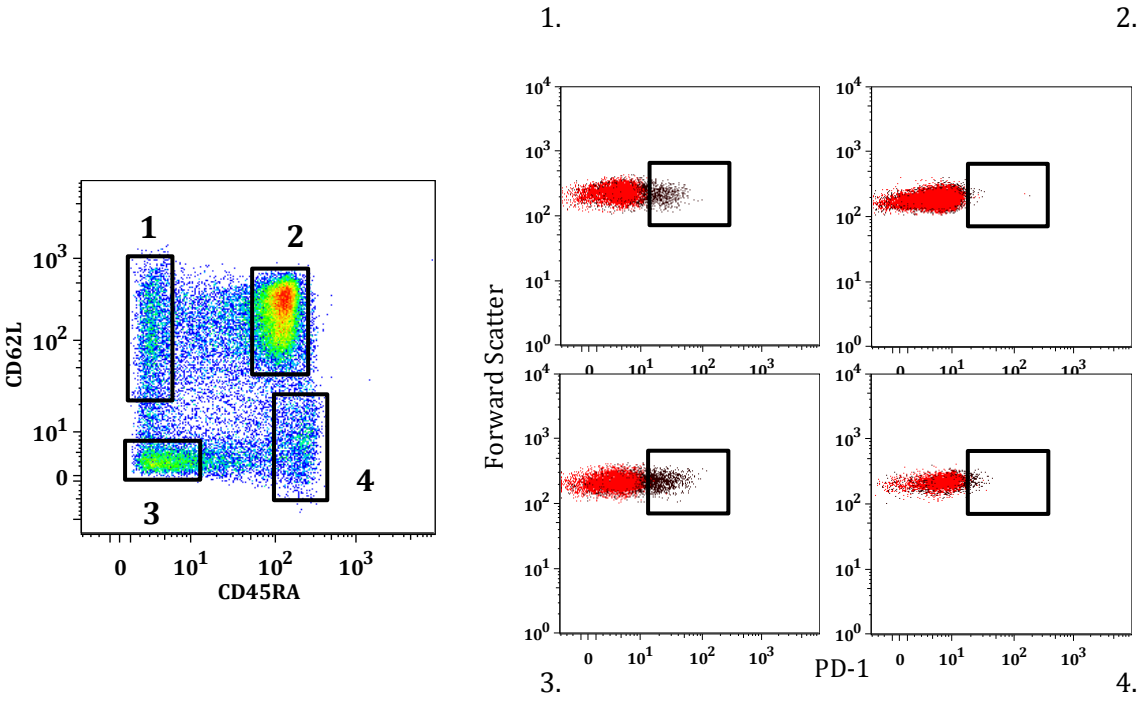
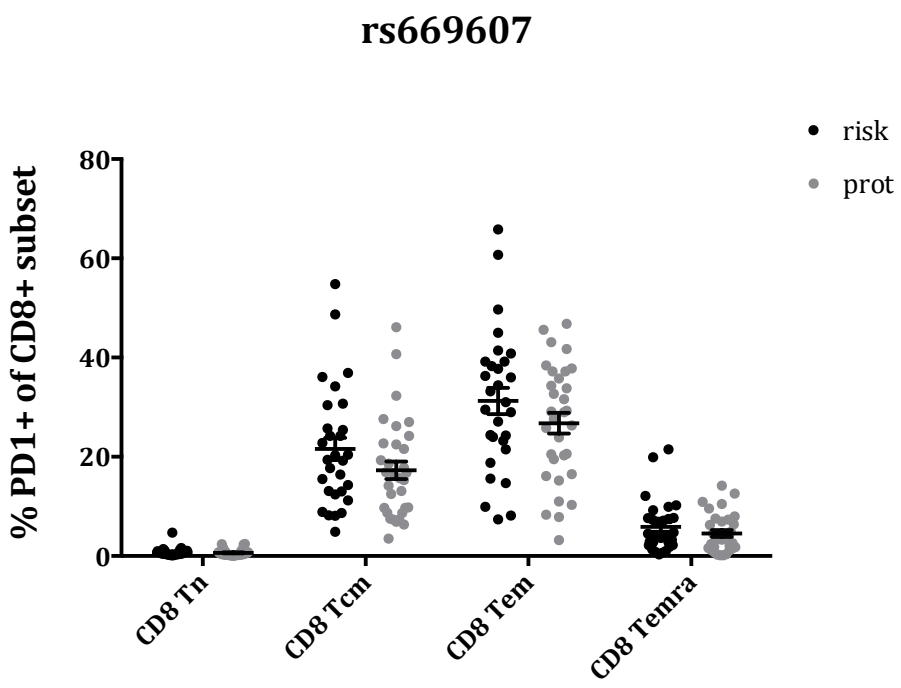


Fig 5.17. Gating of PD-1+ exhausted CD8+ T cells. CD8+ T cells were gated on the basis of CD8 expression as shown previously. Four populations were defined by CD45RA and CD62L. Exhausted cells in each population were defined by PD-1 expression (black dot plots) over isotype control (red dot plots).

A.



B.

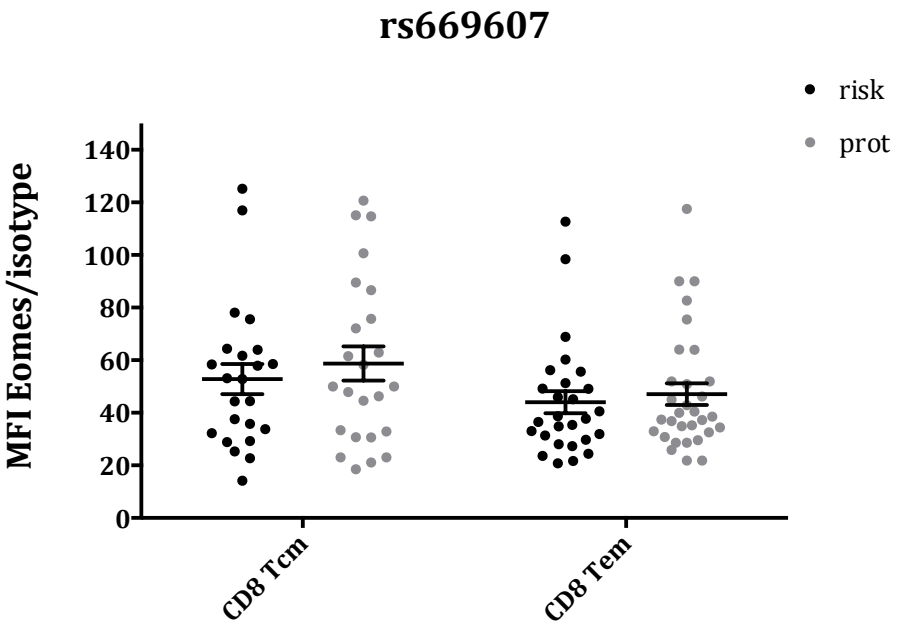
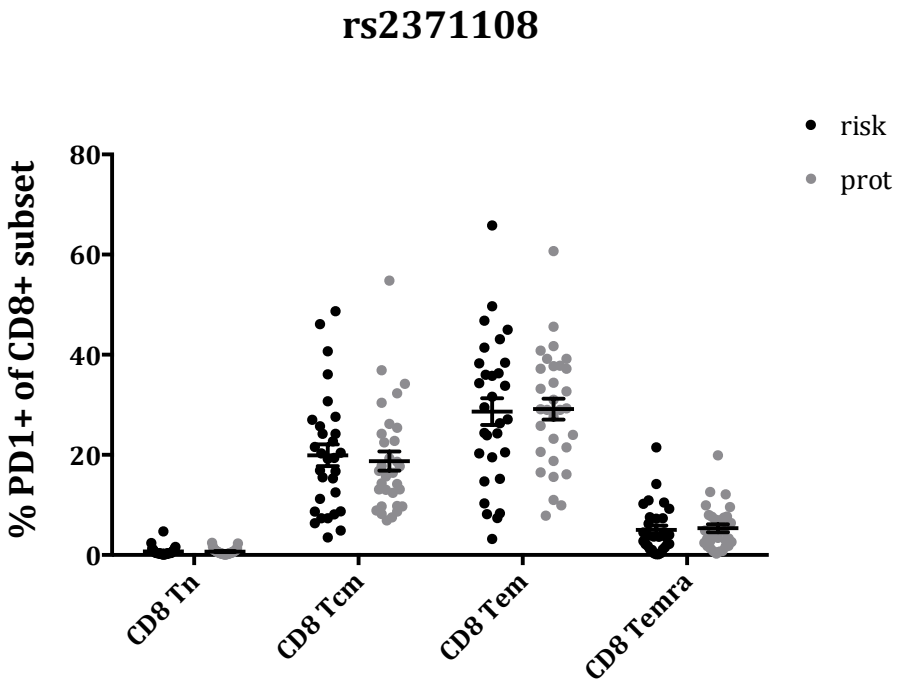


Fig 5.18. Analysis of PD-1+ exhausted CD8+ T cells by genotype at rs669607. The effect of genotype at rs669607 on exhausted CD8+ T cells was analysed by both the PD-1 expressing proportion of CD8+ T cell subsets (A) and Eomes MFI in PD-1 expressing cells (B). Graphs show mean ±SEM.

A.



B.

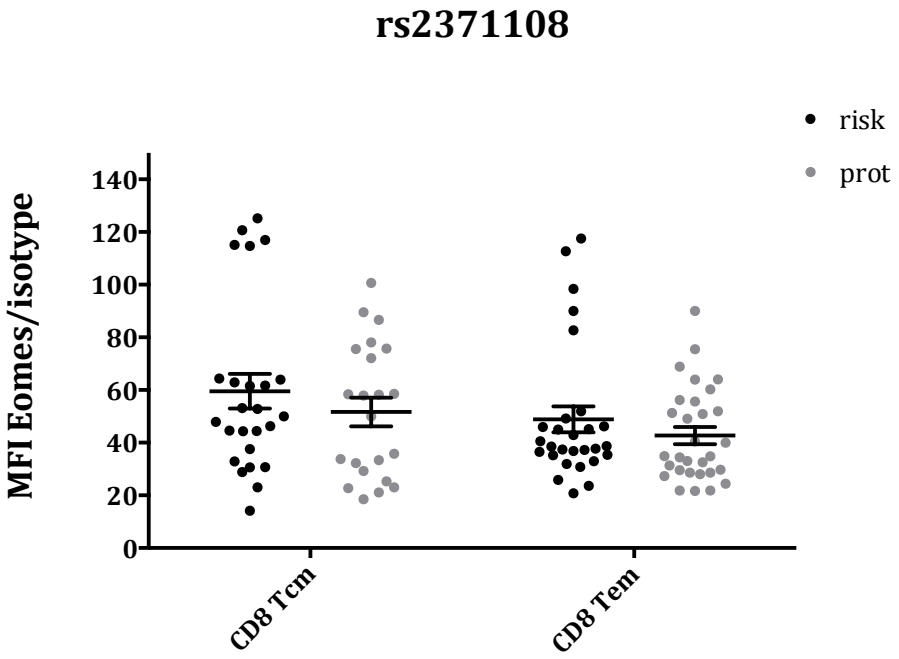


Fig 5.19. Analysis of PD-1+ exhausted CD8+ T cells by genotype at rs2371108. The effect of genotype at rs2371108 on exhausted CD8+ T cells was analysed by both the PD-1 expressing proportion of CD8+ T cell subsets (A) and Eomes MFI in PD-1 expressing cells normalised to isotype (B). Graphs show mean ±SEM.

5.2.7 Analysis of CD122 expression in CD8+ T cells by genotype at rs669607 and rs2371108

Having been unable to detect a significant change in Eomes expression associated to genotype, the study turned to investigating downstream pathways as these may be more likely to show changes due to amplification of a genotype effect. CD122 (IL2R/IL15R β-chain) has been identified as a direct downstream target of Eomes (Intlekofer et al. 2005), and through its role as part of the receptor complex for IL-2 and IL-15 is important in the proliferation and maturation of T cells (Cornish, Sinclair et al. 2006). The expression of CD122 on T cell subsets was analysed. Gating of CD8+ T cell subsets was performed as previously described. CD122+ cells were observed in all CD8+ T cell subsets (Tn, 6.0±0.3%; Tcm 43.3±3.6%; Tem, 51.3±1.3%; Temra, 43.4±2.1%), but relatively few arose from the naïve population, consistent with the role of CD122 in memory T cells (Fig 5.20). A large degree of inter-individual variation was observed in terms of the percentage of CD122+ cells, which was most marked in the Temra population (mean 43.57, range 5.9-85.6%; Fig 5.21 A, 5.22 A). No difference was observed either in the proportion of CD122+cells or the level of CD122 expression by genotype at rs669607 (Fig 5.21) or rs2371108 (Fig 5.22)

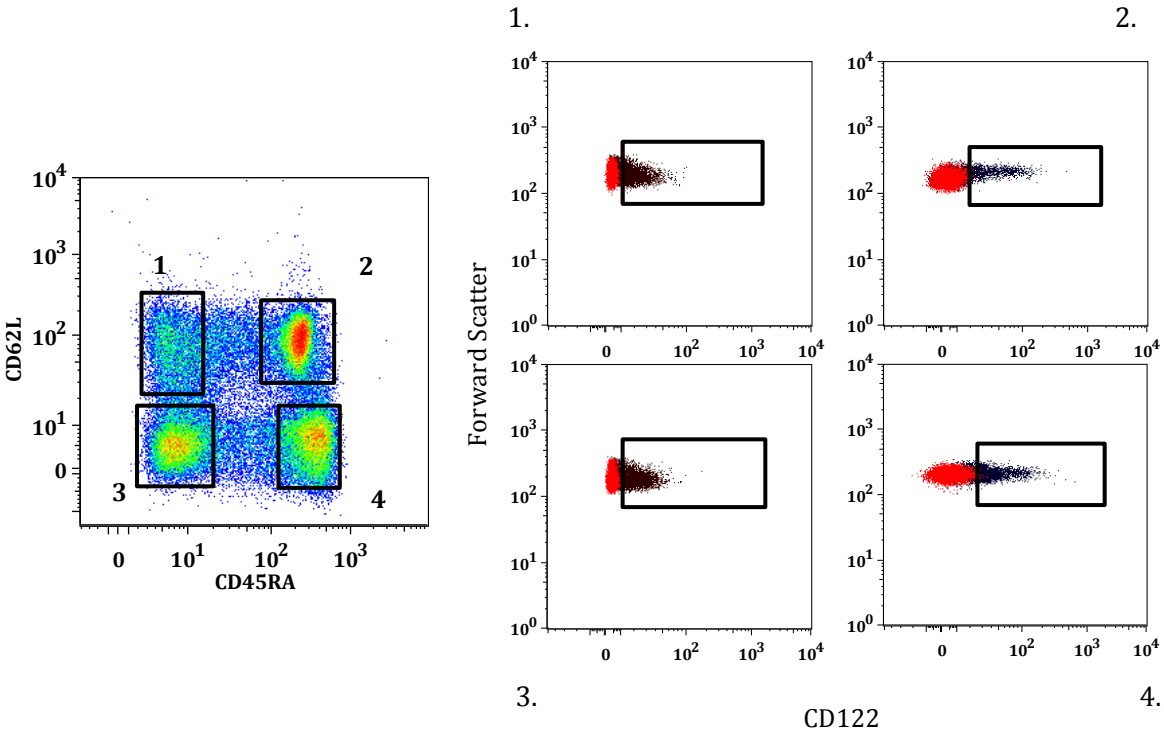
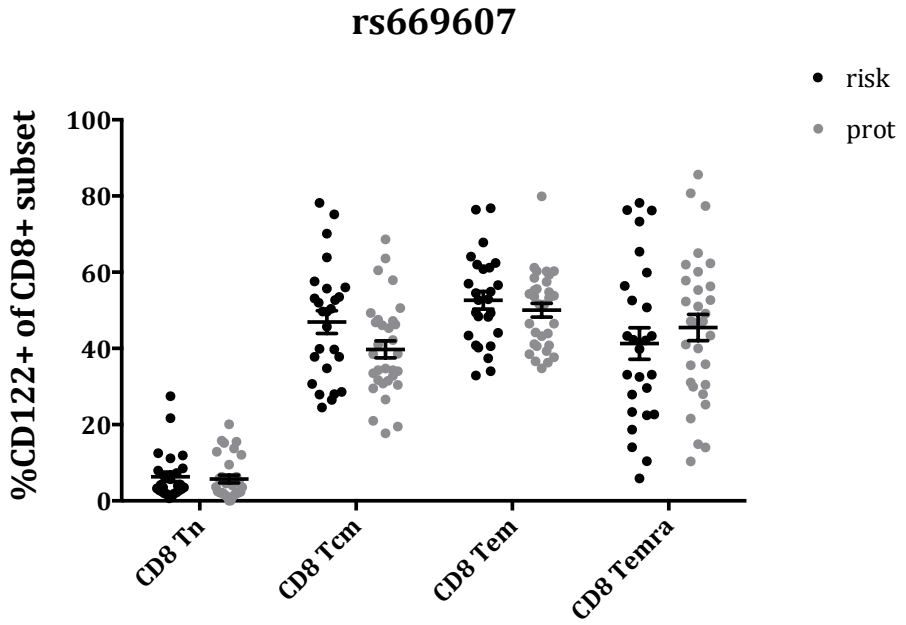


Fig 5.20. Gating of CD122-expressing CD8+ T cells. CD8+ T cells were gated as shown previously and four populations were defined on the basis of CD45RA and CD62L. CD122 expressing cells in each population were defined by CD122 expression (black dot plots) over isotype control (red dot plots).

A.



B.

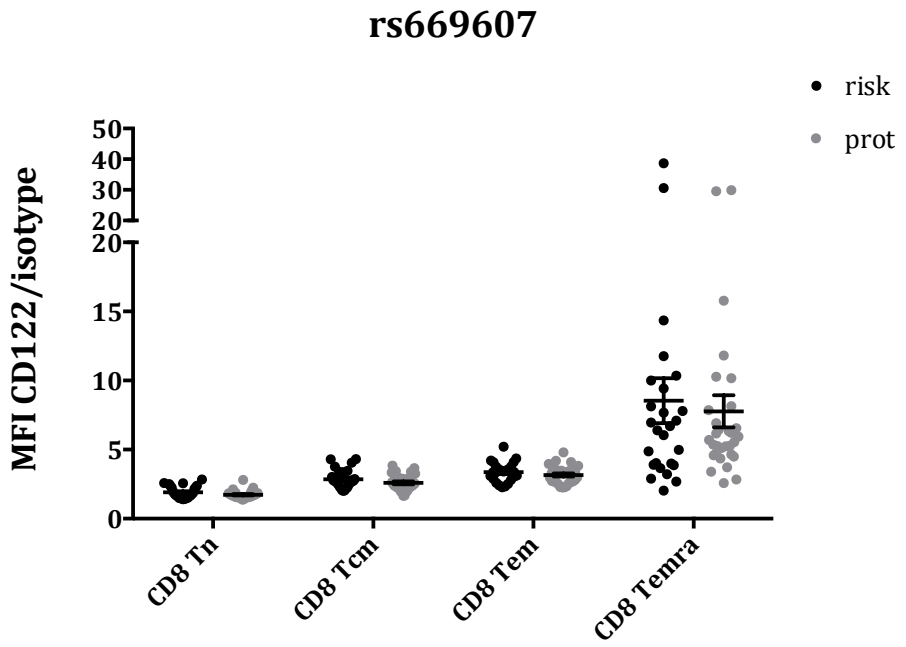
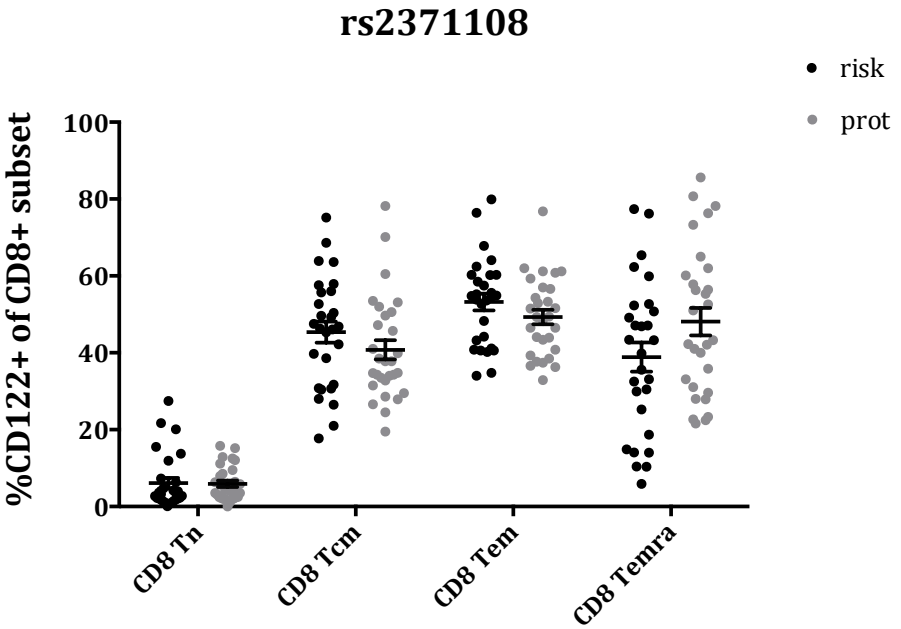


Fig 5.21. Analysis of CD122 expression in CD8+ T cell subsets by genotype at rs669607. Differences in CD122 expression by genotype at rs669607 were assessed by the CD122-positive proportion of CD8+ T cell subsets (A) and CD122 MFI normalised to isotype control. Graphs show mean±SEM.

A.



B.

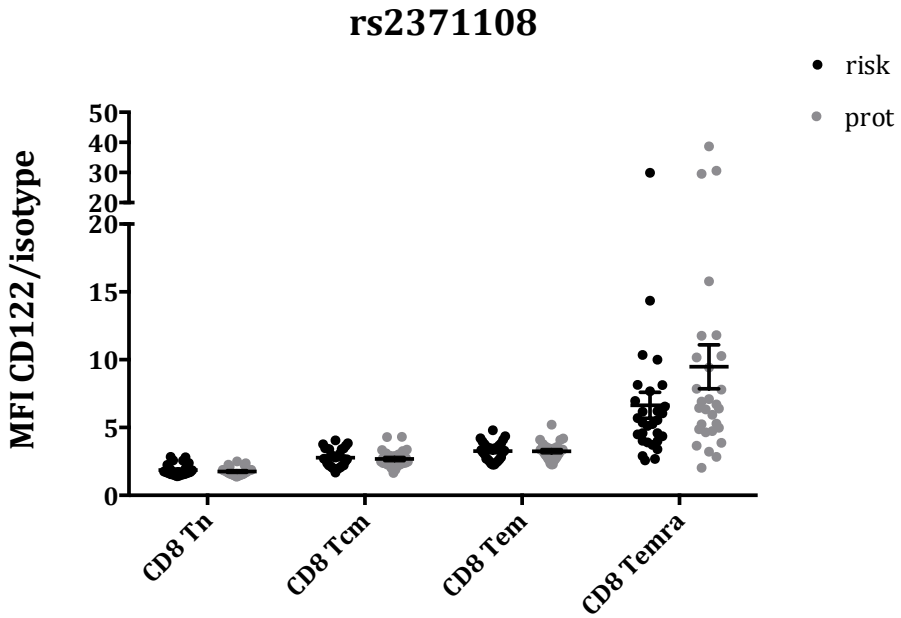


Fig 5.22. Analysis of CD122 expression in CD8+ T cell subsets by genotype at rs2371108. Differences in CD122 expression by genotype at rs2371108 were assessed by the CD122-positive proportion of CD8+ T cell subsets (A) and CD122 MFI normalised to isotype control. Graphs show mean±SEM.

5.2.8 Analysis of perforin and granzyme expression by genotype at rs669607 and rs2371108

Perforin and granzyme B are cytolytic molecules expressed by effector cells, mainly CD8+ T cells and NK cells (Konjevic et al. 1995). Eomes expression is associated with upregulation of both perforin and granzyme B (Pearce et al. 2003, Intlekofer et al. 2005, Pipkin et al. 2010, Eshima et al. 2012) and perforin has been identified as a direct target of Eomes (Cruz-Guilloty et al. 2009). Both granzyme B and perforin expression were analysed in CD8+ T cell subsets by genotype at both SNP loci. The proportion of double-positive cells in each population was also analysed.

For both molecules, and in all populations, there was a negative population with increased staining above isotype control, which may represent a degree of non-specific staining due to the intracellular staining protocol involving fixation and permeabilisation. CD4+ T cells, which do not significantly express cytolytic molecules, also demonstrated this high background staining (Fig 5.23 B). The positive gate was set just above this population, using the Tn population to set the gate as this was a more suitable threshold than isotype control. A gradient of increasing effector molecule expression with increasing differentiation was revealed (mean %DP cells of Tn 7.1±1%; Tcm 15.6±1.8%; Tem 33.7±2.0%; Temra 80.7±1.7%; Fig 5.24, 5.25). However, no difference was observed in proportion of granzyme B+, perforin+ or double positive CD8+ T cells by genotype at either rs669607 (Fig 5.24) or rs2371108 (5.25).

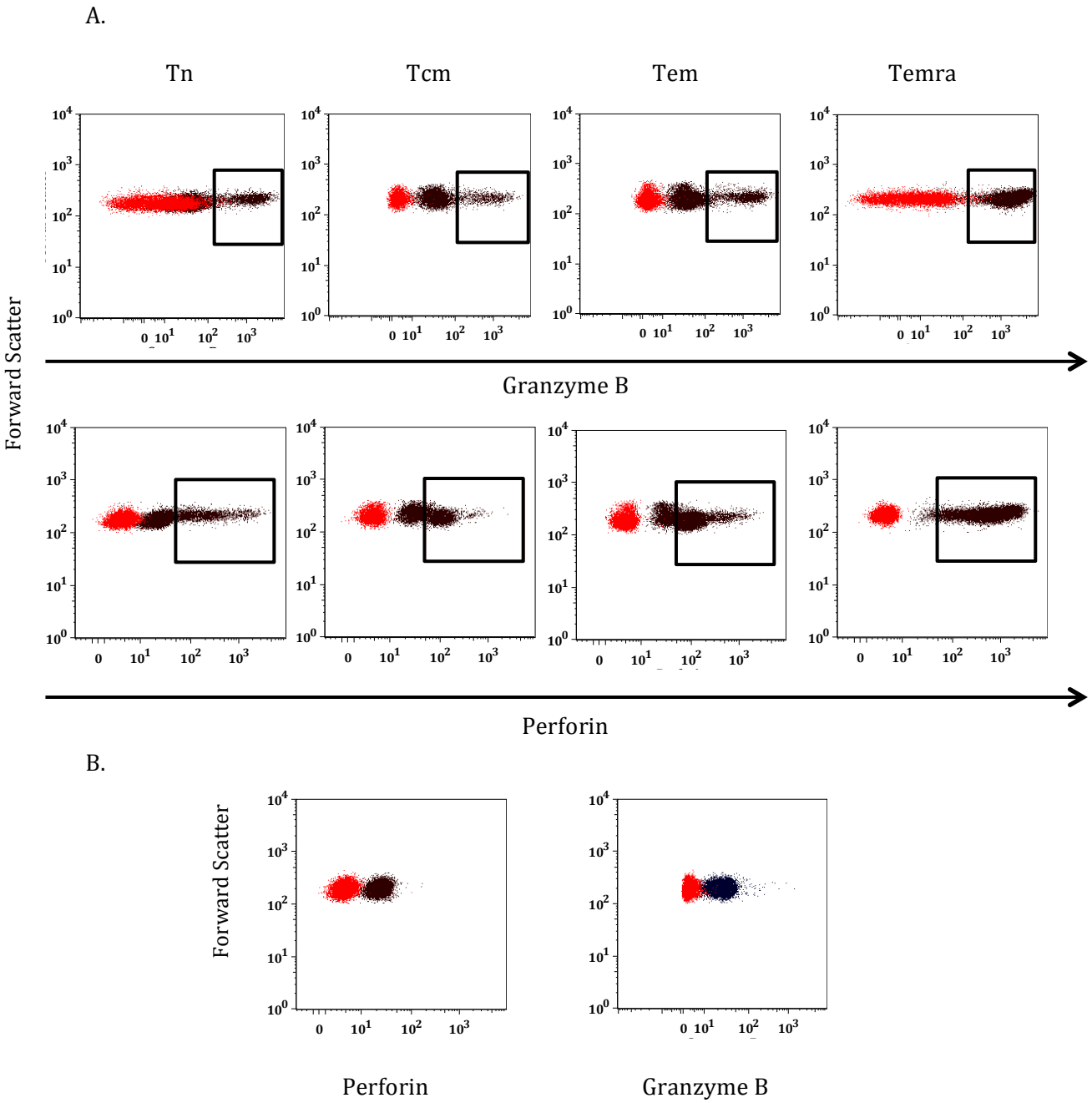
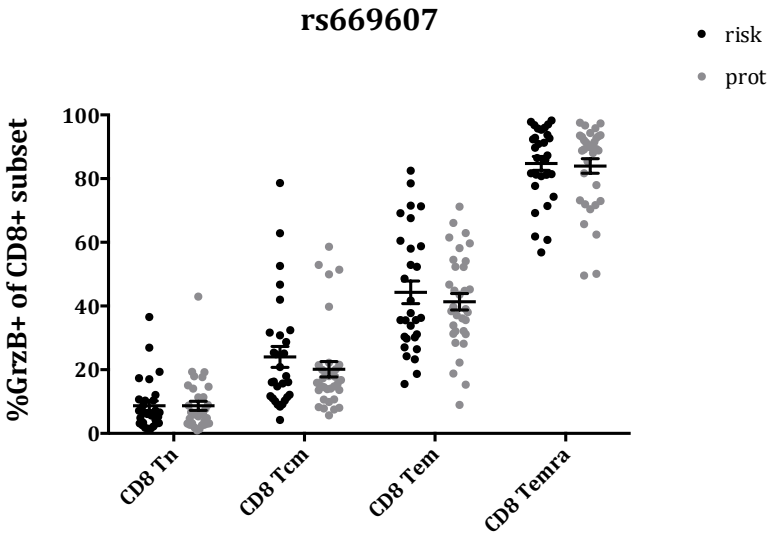
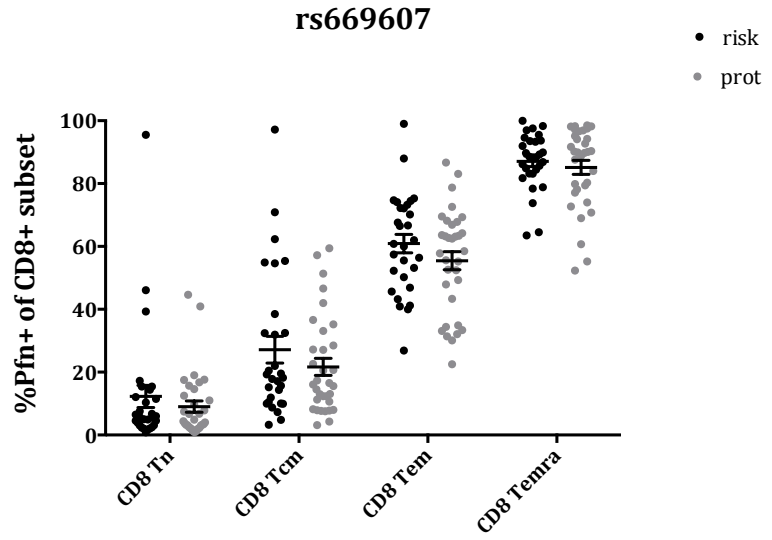


Fig 5.23. Gating of perforin and granzyme B positive CD8+ T cells. A. Lymphocytes, singlets, CD8+ cells and effector/memory subsets were gated as previously shown. Gates for granzyme B expressing cells (upper panels) and perforin expressing cells (lower panels) were set using the Tn population (far left panel). **B**. Rationale for gating strategy was reinforced by perforin and granzyme B staining in CD4+ cells demonstrating negative population with non-specific background staining (black dot plots) above isotype control (red

A.



B.



C.

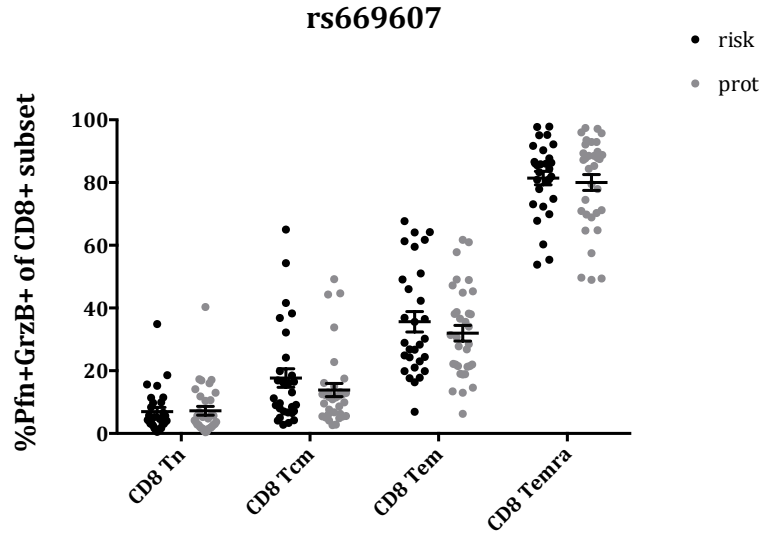
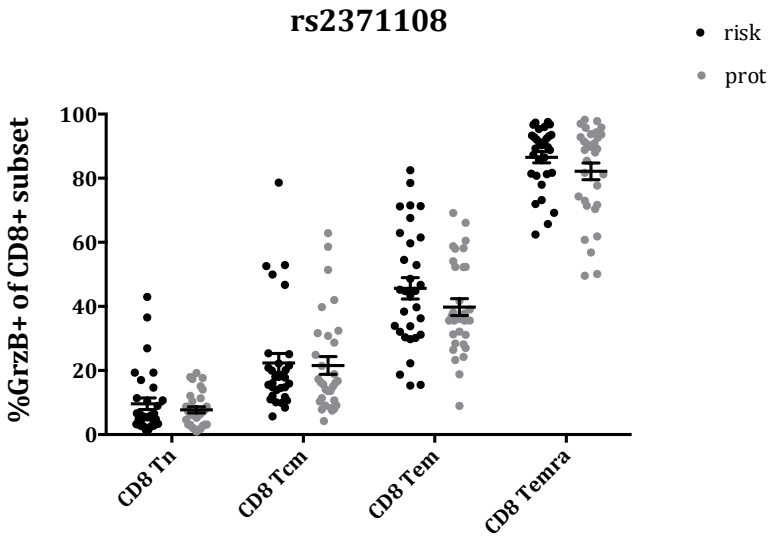
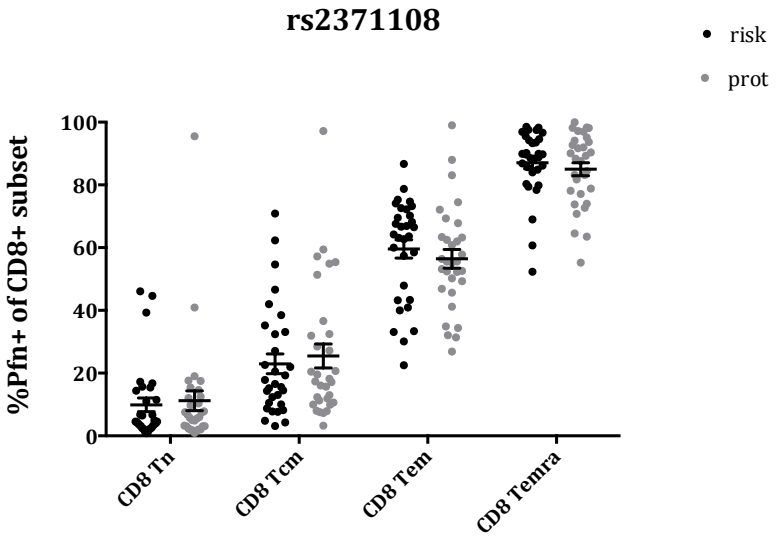


Fig 5.24. Analysis of perforin and granzyme B expression by genotype at rs669607. Differences in cytolytic molecule expression in CD8+ T cells subsets by genotype at rs669607 were assessed by proportion of cells expressing granzyme B (A) perforin (B) and Pfn/GrzB DP cells (C). Graphs show mean ±SEM.
dot plots).

A.



B.



C.

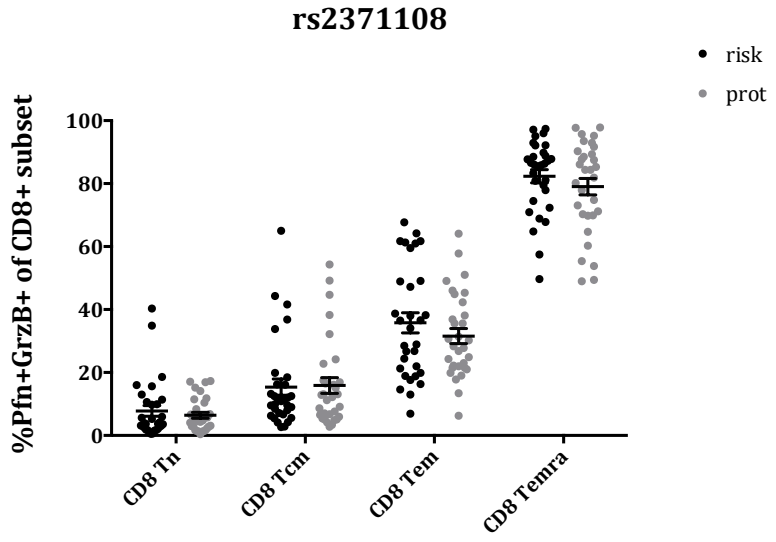


Fig 5.25. Analysis of perforin and granzyme B expression by genotype at rs2371108. **A**. Differences in cytolytic molecule expression in CD8+ T cells subsets by genotype at rs2371108 were assessed by proportion of cells expressing granzyme B (A) perforin (B) and Pfn/GrzB DP cells (C). Graphs show mean ±SEM.

5.2.9 Analysis of FoxP3 and T-bet expression by genotype at rs669607 and rs2371108

Eomes plays a role in CD4+ T cell lineage determination, particularly in diversion toward a Th1 rather than Th17 phenotype in certain conditions (Ichiyama et al. 2011, Qui et al. 2011). Therefore, the study sought to analyse the different peripheral populations, identified either through cytokine production (see below) or transcription factor expression. T-bet is a T-box transcription factor, most commonly associated with Th1 cell function, and is closely related to Eomes with overlapping functions in several cell populations (Intlekofer et al. 2005, Intlekofer et al. 2008, Gordon et al. 2012). FoxP3 is the master transcription factor of regulatory T cells (Tregs). It was hypothesised that alteration of Eomes expression by genotype might result in corresponding changes in T-bet expression, in a compensatory mechanism, or FoxP3 expression.

T-bet expression was observed as a shift in the population, most marked in Temra cells, and was therefore measured as MFI relative to isotype (Fig 5.26). No difference by genotype was observed in T-bet expression in either CD4+ or CD8+ T cell subsets (Figs 5.27, 5.28).

FoxP3+ cells were gated as cells expressing FoxP3 over isotype control; clear positive and negative populations were observed (Fig 5.29 A). The majority of FoxP3 positive cells arose from the Tcm population (Tn 0.98±0.1%; Tcm 9.60±0.1%; Tem 2.83±0.1%). No difference was observed between risk and protective genotype at either SNP (Tcm cells: rs669607 9.7% v 9.5%; rs2371108 10.0% v 9.2%, Figs 5.29 B, C).

A.

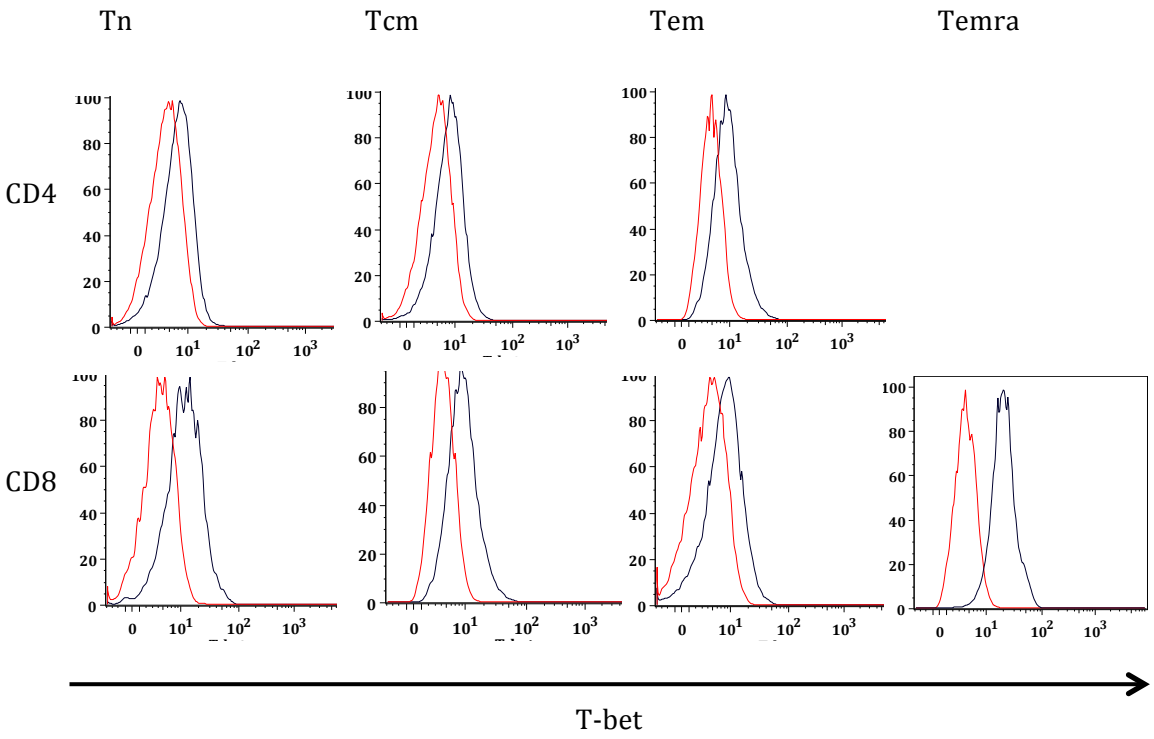
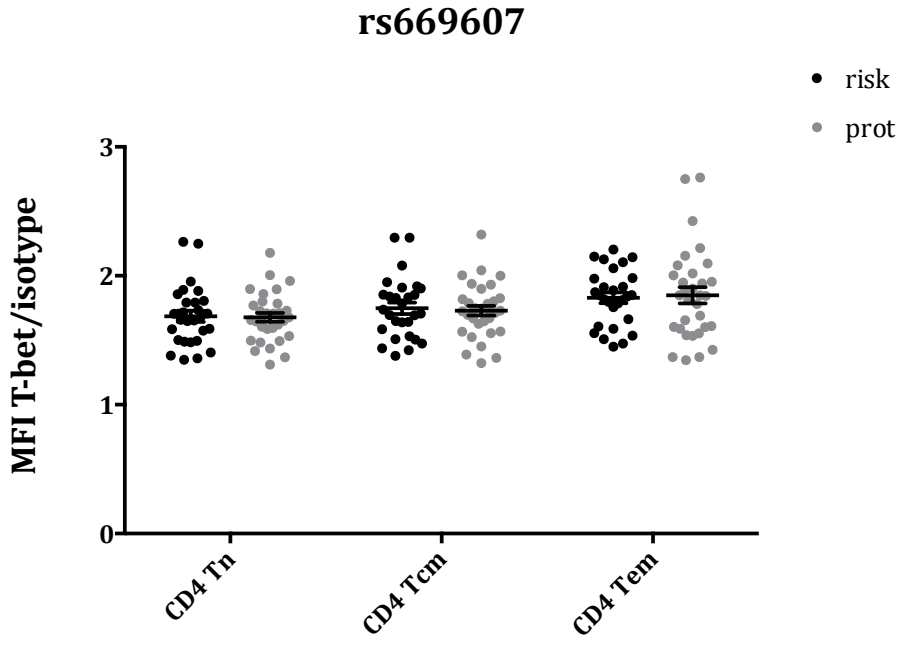


Fig 5.26. T-bet expression in CD4+ and CD8+ T cell subsets. CD4+ and CD8+ T cells memory and effector subsets were gated on the basis of CD62L and CD45RA expression as shown previously (Figs 4.11, 4.12) Histograms show T-bet expression (black lines) versus isotype control (red lines) in CD4+ (upper panels) and CD8+ (lower panels) subsets.

A.



B.

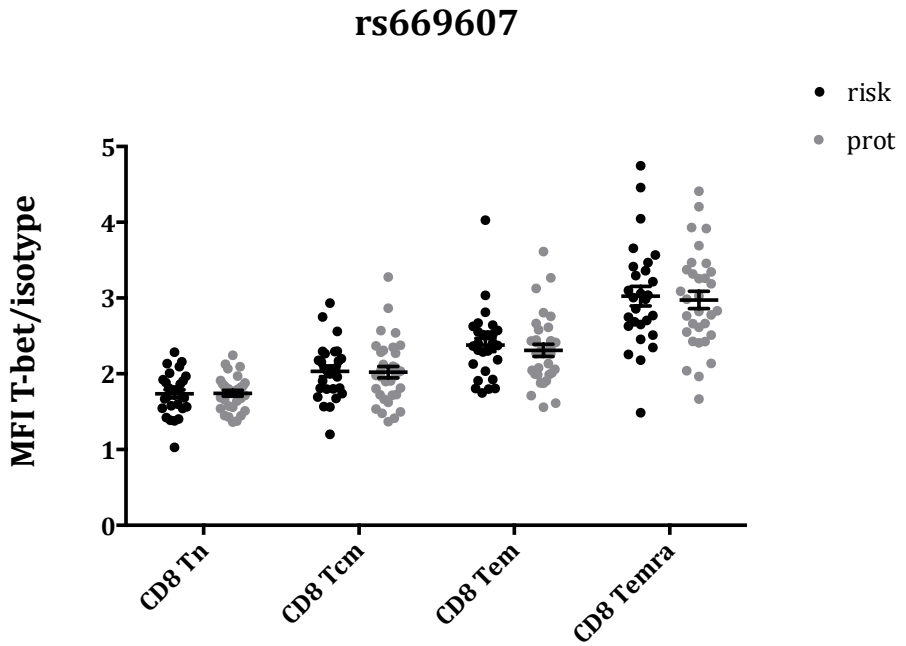
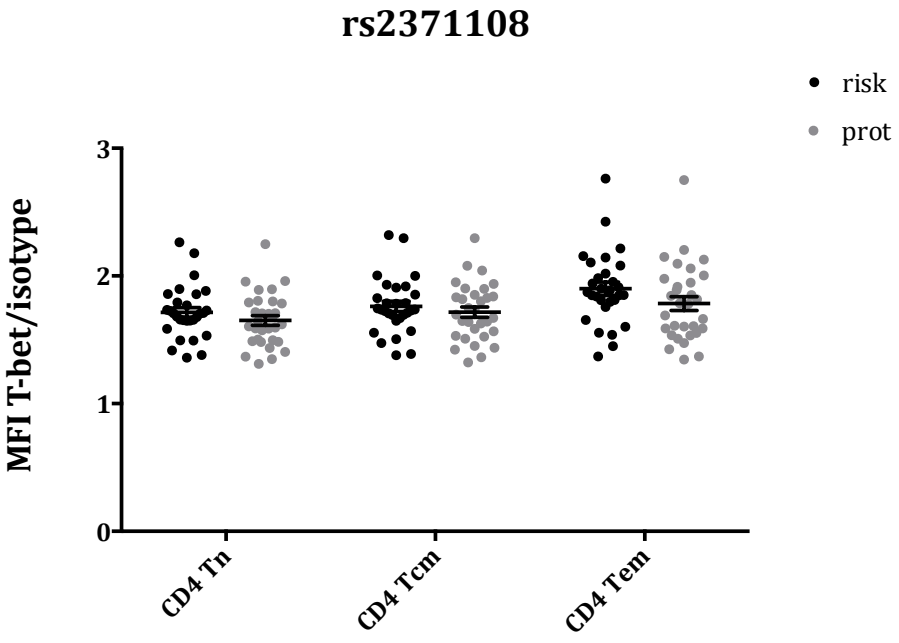


Fig 5.27. Analysis of T-bet expression in CD4+ and CD8+ T cells by genotype at rs669607. T-bet expression in T cells was analysed by MFI normalised to isotype control and assessed in CD4+ (A) and CD8+ (B) T cell subsets by genotype at rs669607.

A.



B.

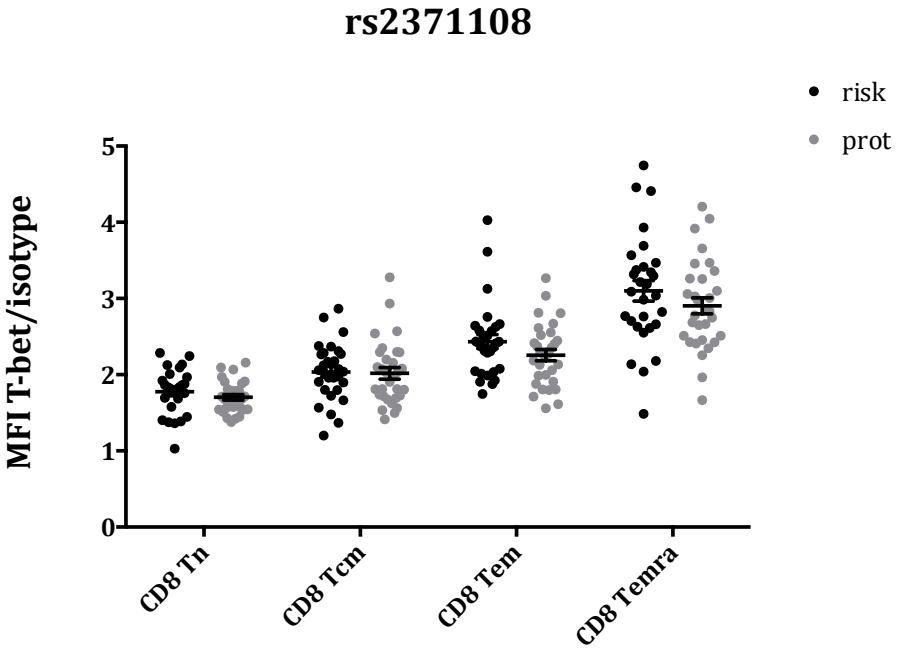


Fig 5.28. Analysis of T-bet expression in CD4+ and CD8+ T cells by genotype at rs2371108. T-bet expression in T cells was analysed by MFI normalised to isotype control and assessed in CD4+ (A) and CD8+ (B) T cell subsets by genotype at rs2371108.

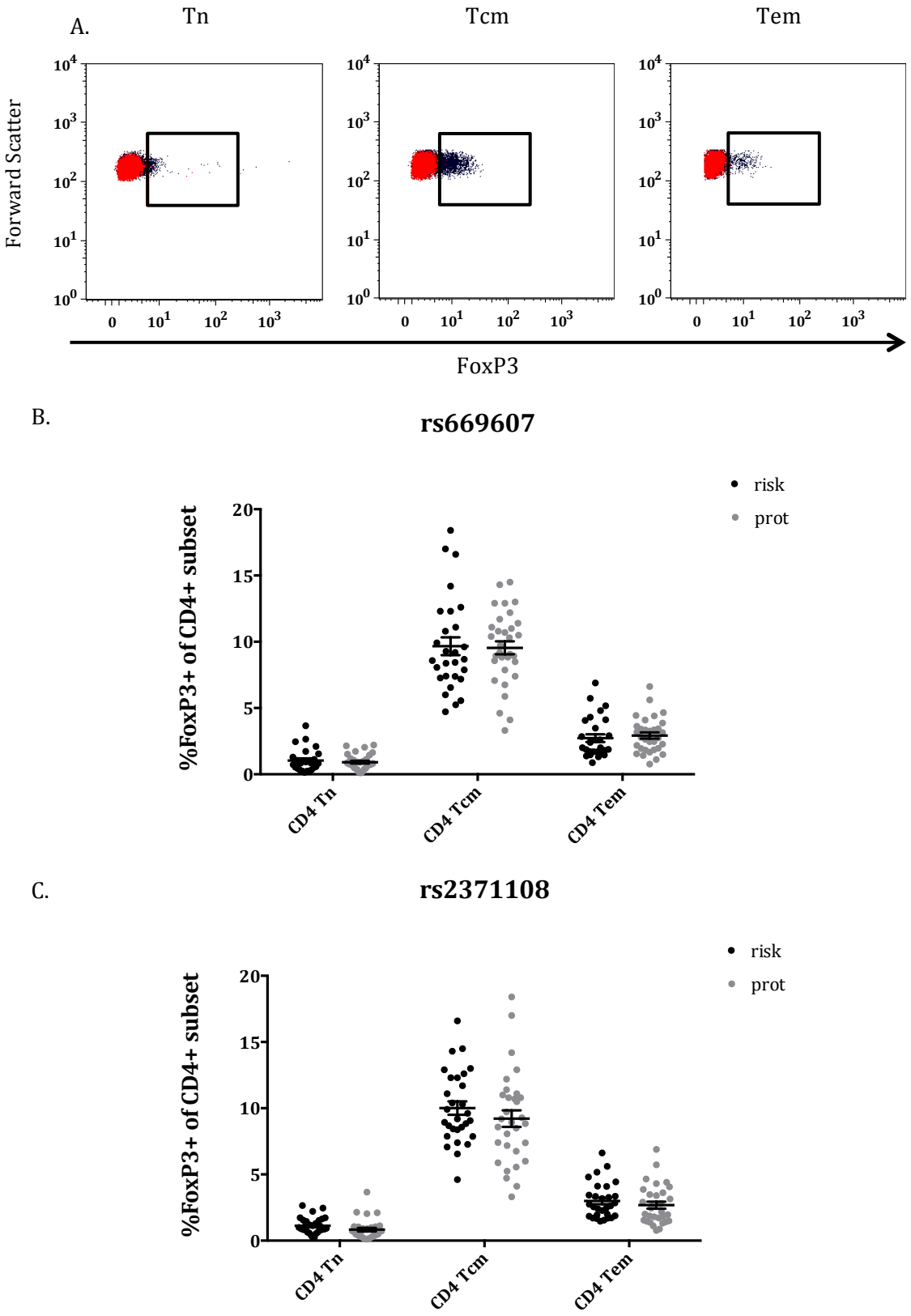


Fig 5.29. Analysis of FoxP3 expression by genotype. A. CD4+ T cell subsets were gated as previously shown. Gates were set according to FoxP3 expression (black dot plots) over isotype control (red dot plots). B. Differences in Fox P3 expression were assessed by proportion of FoxP3 expressing cells in CD4+ T cell subsets by genotype at rs669607 (B) and rs2371108 (C).

5.2.10 Analysis of cytokine-expressing populations by genotype at rs669607 and rs2371108

As mentioned above, Eomes has a role in the differentiation of T cells into memory cells and also in determination of effector phenotype, skewing CD4+ T cells toward a Th1 phenotype and away from Th17 (Intlekofer et al. 2008). Furthermore, Eomes has been shown to bind to the IFN-γ locus to increase expression (Cruz-Guilloty et al. 2009). IL-2 and TNF-α are important pro-inflammatory cytokines with wide ranging effects on the immune system, produced by a spectrum of different cells. Cellular Eomes expression may correlate with inflammatory cytokine production in the context of T cell exhaustion (Buggert et al. 2014), and expression of T-box transcription factors, both T-bet and Eomes, may be associated with functional plasticity of CD4+ T cells and the differential production of cytokines including TNF-α, IFN-γ and IL-2 (Edwards, Smith et al. 2014).

Based on this, the proportion of T cells expressing IFN-γ, IL-17, IL-2 and TNF-α was assessed in both the CD4+ and CD8+ T cell populations by genotype at rs669607 and rs2371108, to test the hypothesis that genotype-dependent effects on Eomes expression during T cell differentiation and activation would affect the proportions of cytokine-producing cells in these populations. Cells were stimulated directly ex-vivo for 3 h and then stained intracellularly for IFN-γ, IL-17, TNF-α and IL-2.

T cell activation by stimulating cells with PMA and ionomycin causes CD62L to be shed from the cell membrane (Yang et al. 2011). Therefore, CCR7 was used to define the four memory and effector populations. CCR7 and CD62L expression broadly correlate, although there is some overlap of Tcm and Tem populations (Sallusto et al. 2004). Presuming that any changes in cytokine expression between the two genotype groups would occur downstream of changes in Eomes expression, it was necessary to ensure that these populations were consistent in terms of Eomes expression. In a pilot

experiment, both markers were used to define the four subsets and Eomes expression was compared between the CD62L and CCR7-defined populations. CCR7-defined populations displayed the same gradient of Eomes expression, with no significant differences between CD62L and CCR7-defined cells (Fig 5.30).

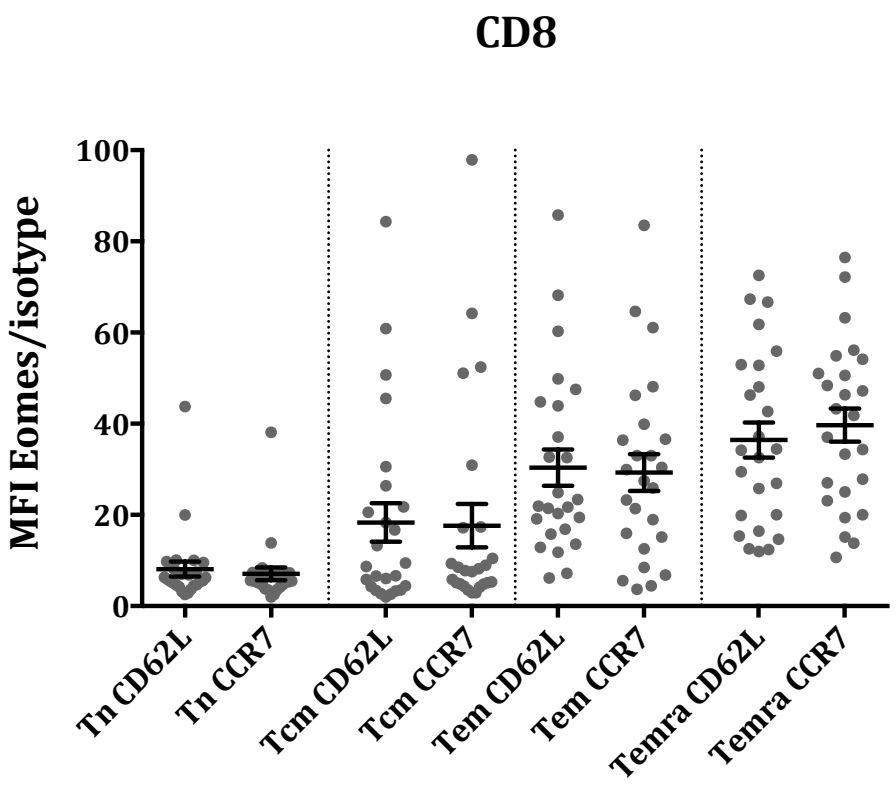


Fig 5.30. Comparison of Eomes expression relative to isotype control in CD8+ effector/memory cells. CD8+ T cell subsets were defined by CD45RA/CD62L or CD45RA/CCR7 and differences in Eomes expression between alternatively defined subsets were analysed.

5.2.10.1 IFN-γ expression in CD4+ and CD8+ T cells by genotype at rs669607 and rs2371108

Consistent with expectations, analysis of IFN-γ expression in the subsets revealed that only a small proportion of naïve cells expressed IFN-γ (CD4+ Tn, 0.68±0.06%; CD8+ Tn cells, 2.6±0.6%), with greater numbers of Tcm (CD4+ Tcm

12.45±0.8%; CD8+ Tcm 52±0.7%), Tem (CD4+ Tem 34.5±1.7%; CD8+ Tem 66.6±2.0%) and Temra (CD8+ Temra 67.4±3.1%) cells IFN-γ+ (Fig 5.31).

Comparison of IFN-γ expression by genotype revealed an apparent difference in the proportion of IFN-γ+ CD8+ Temra cells after correction for gender, with higher expression in rs2371108 risk homozygotes (70.4±3.5% v 64.3±3.3%) although this did not reach the adjusted significance threshold (p=0.007; significance threshold p<0.0018) (Fig 5.34). There were also trends toward higher expression in rs2371108 risk homozygotes in CD8+ Tem (68.6±2.8% v 64.7± 2.6%, p=0.055), CD4+ Tcm (13.2±1.1 v 11.6±1.0%, p=0.046) and CD4+ Tem cells (36.2±2.4 v 32.9±2.3%, p=0.077, Fig 5.34). In total CD4+ T cells the difference by genotype at rs2371108 was of greater statistical significance than in the subsets (10.2±1.1% v 7.0±0.6%, p=0.002) implying that the effect was present across all CD4+ subsets, including cells excluded by the gating strategy (Fig 5.32). However, in total CD8+ T cells the difference by genotype at rs2371108 was of lower statistical significance (p=0.25) implying that the effect of genotype was greatest in the CD8+ Temra subset. There were no differences in expression observed by genotype at rs669607.

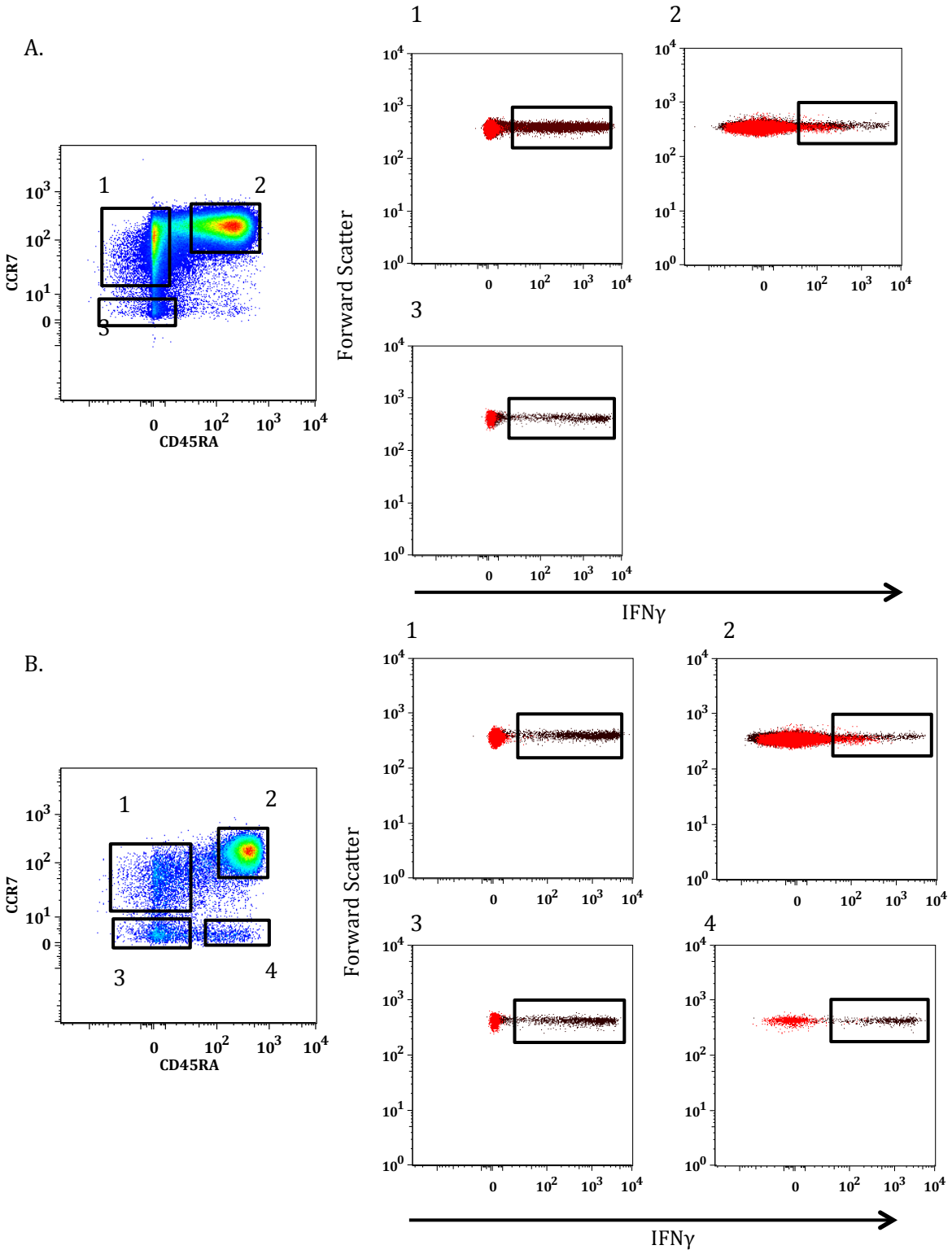
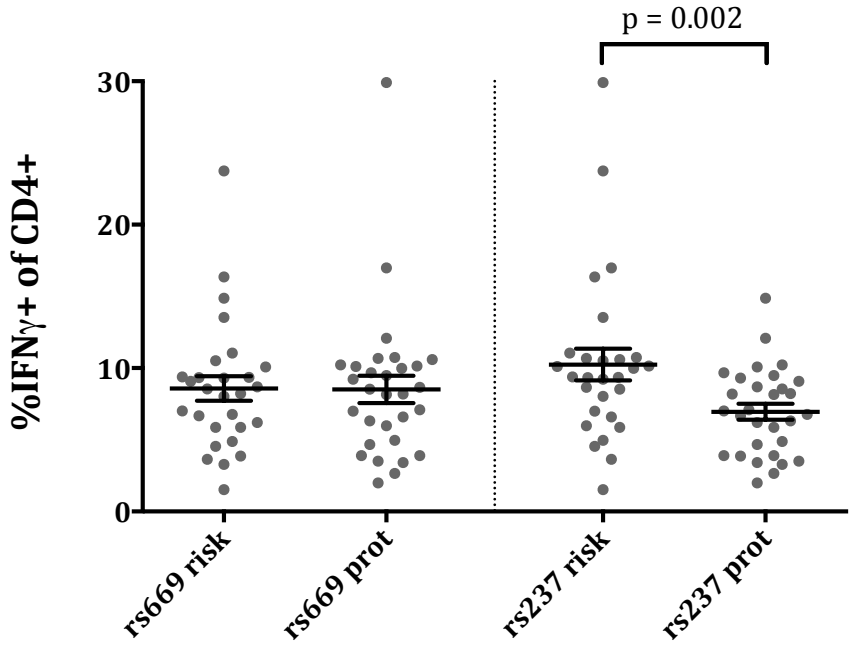


Fig 5.31. Flow cytometry analysis of IFN-γ expression in T cell subsets. PBMCs were stimulated for 3h with PMA/ionomycin followed by staining for cell subset markers and intracellular IFN-γ. Effector/memory CD4+ (A) and CD8+ (B) subsets were gated by CD45RA and CCR7. IFN-γ-expressing cells were identified by expression above the negative population and a fluorescence-minus-one (FMO) control (red dot plots).

A.



B.

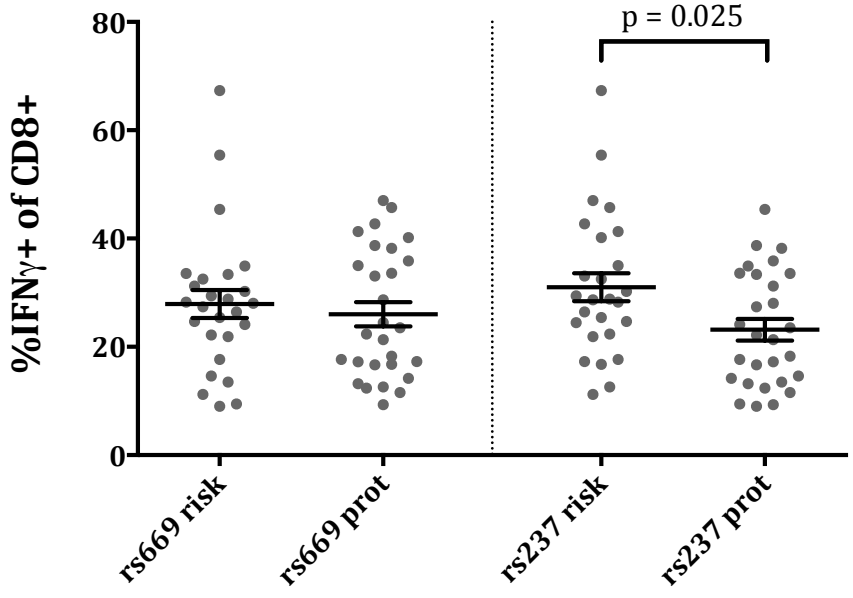
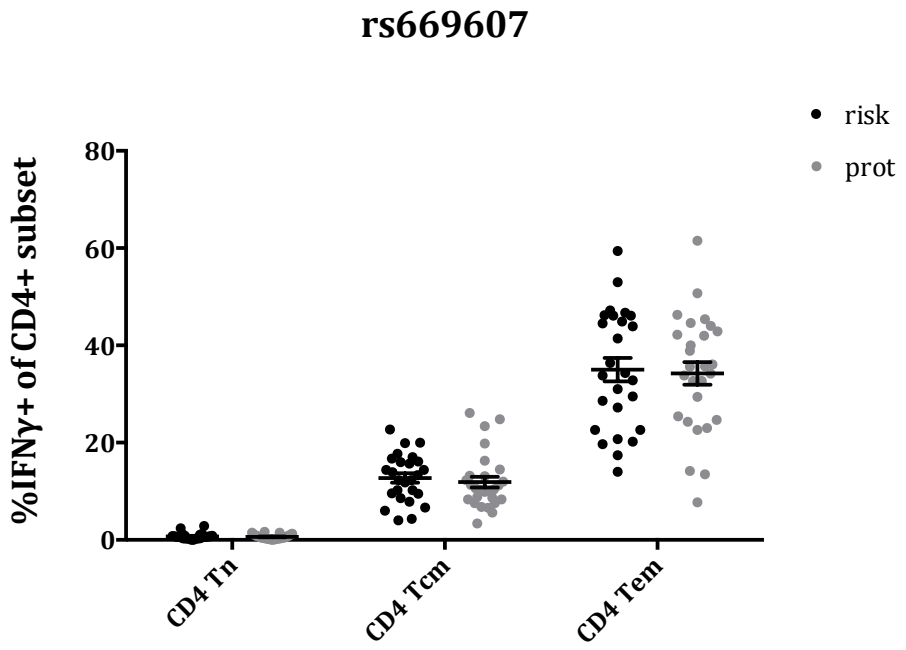


Fig 5.32. Analysis of IFN-γ expression in T cells by genotype. Following a 3h stimulation bulk CD4+ (A) and CD8+ (B) T cells were stained for surface markers and intracellular IFN-γ for analysis by genotype at rs669607 and rs2371108. P-values were calculated using the general linear model in SPSS and are uncorrected. Graphs show mean±SEM.

A.



B.

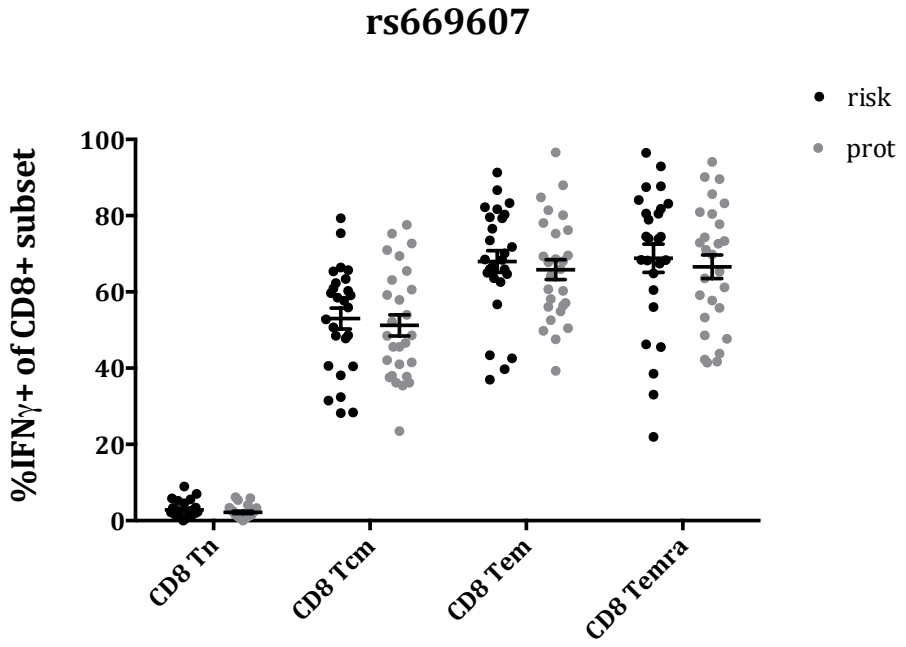
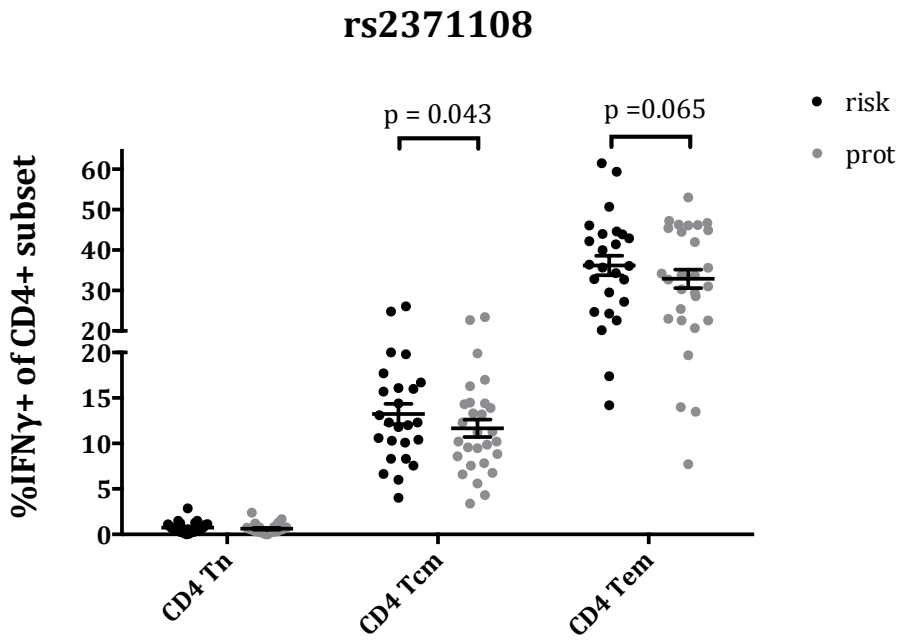


Fig 5.33. Analysis of IFN-γ expression in T cell subsets by genotype: rs669607. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular cytokines for analysis by genotype at rs669607. Graphs show mean±SEM.

A.



B.

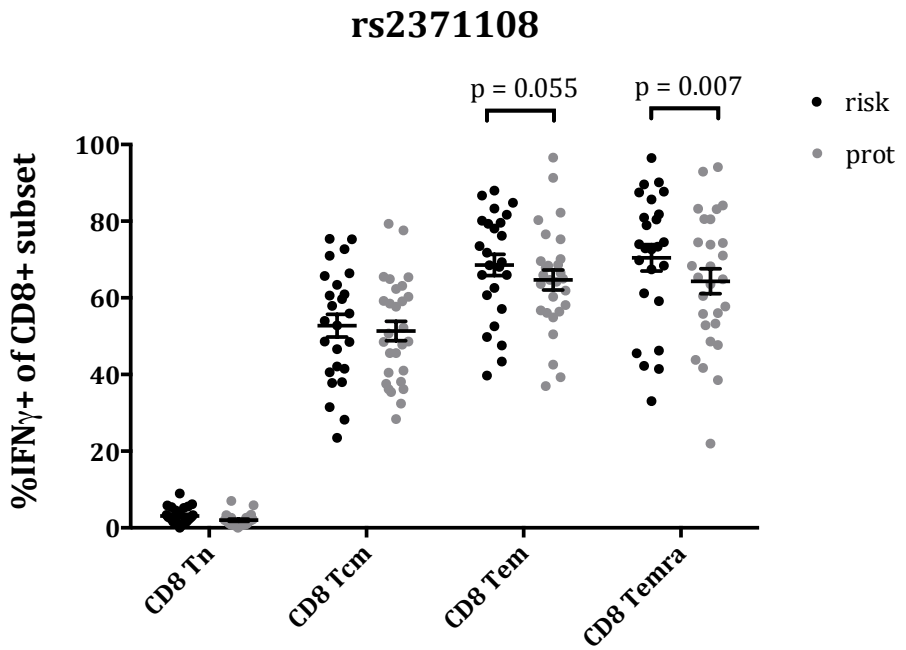


Fig 5.34. Analysis of IFN-γ expression in T cell subsets by genotype: rs2371108. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular cytokines for analysis by genotype at rs2371108. P-values were calculated using the general linear model in SPSS and are uncorrected. Graphs show mean±SEM.

5.2.10.2 IL-17 expression in CD4+ T cells by genotype at rs669607 and rs2371108

IL-17 expression was analysed in ex-vivo CD4+ and CD8+ T cells. A small number of IL-17+ CD8+ T cells were present, but were considered too few to accurately quantify. A greater proportion of CD4+ T cells expressed IL-17 (0.35±0.03%), arising mainly from the Tem population (1.85±0.13%) but also from the Tcm population (0.64±0.05%) (Fig 5.35 A). Intracellular staining revealed clear positive and negative populations.

The proportion of IL-17+ cells in CD4+ T cell subsets in donors homozygous for risk or protective alleles at each SNP locus was compared. No significant difference in the numbers of IL-17+ cells from the bulk CD4+ population (Fig 5.35 B) or the individual subsets (Fig 5.36) was observed by genotype at either rs669607 or rs2371108. In total CD4+ T cells, an apparent difference in the number of IL-17 expressing cells between rs2371108 risk and prot donors did not reach significance (risk 0.417±0.05% v prot 0.30±0.03%, p=0.047) and was not reflected on analysis of subpopulations.

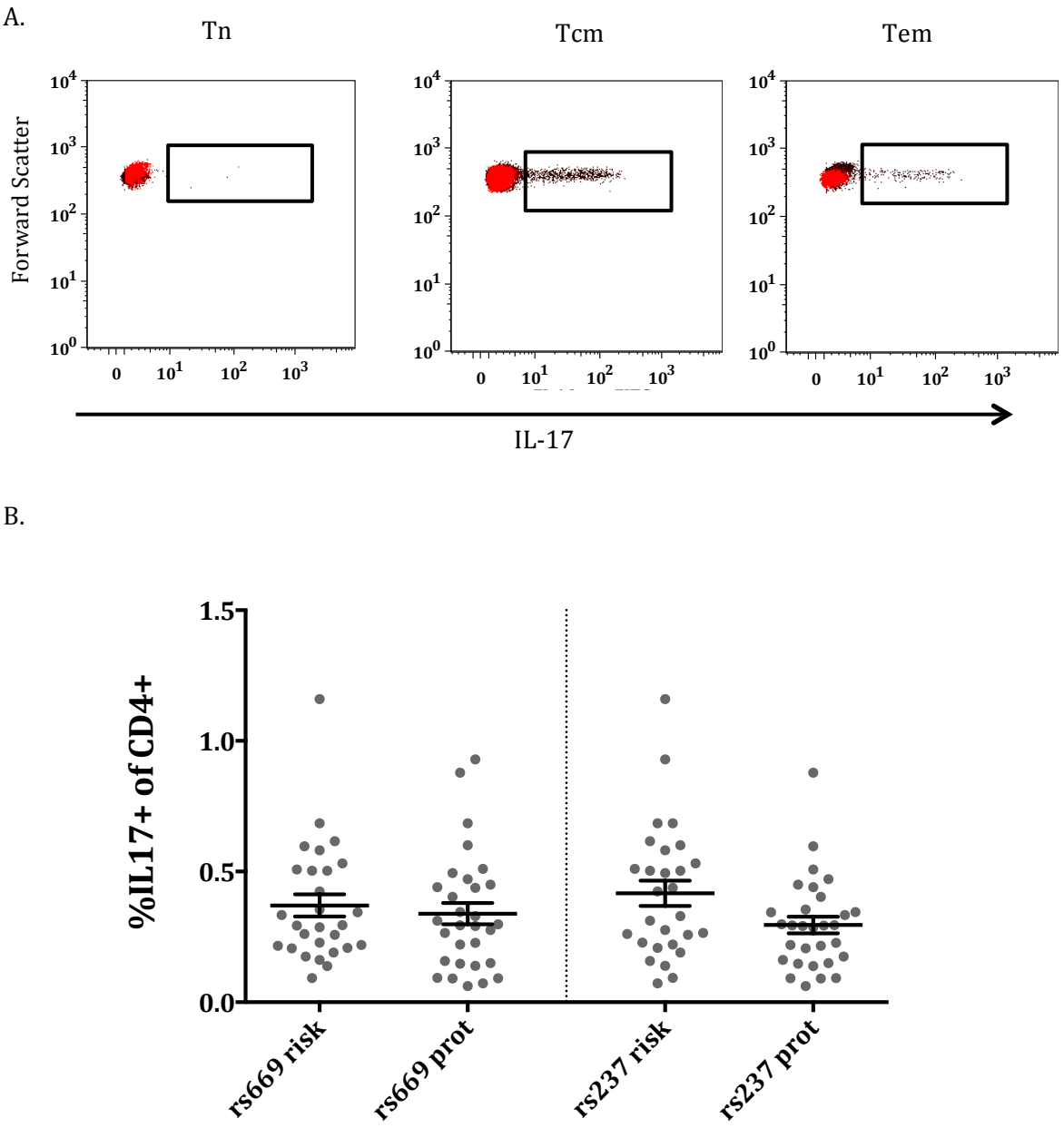
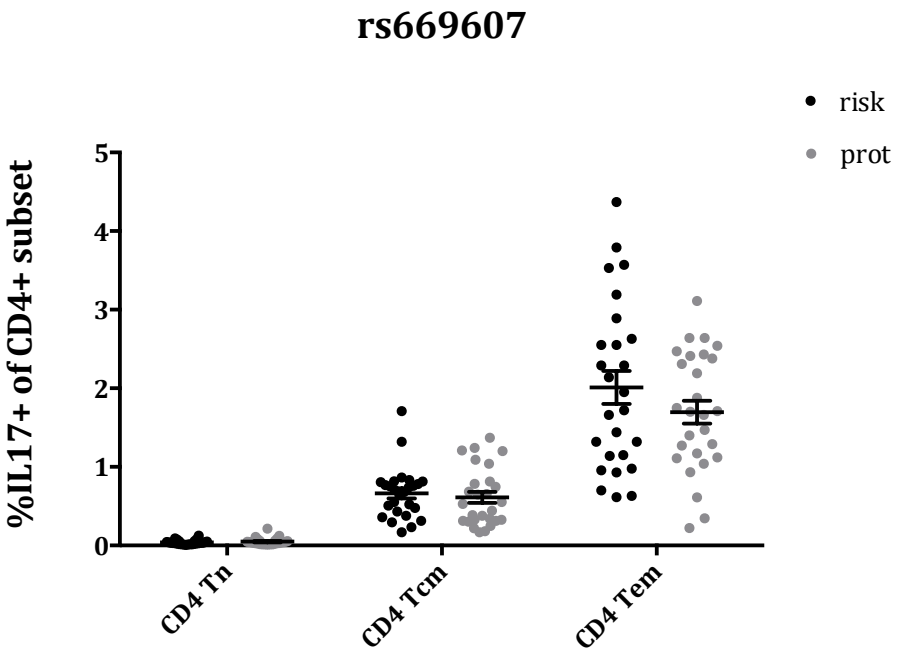


Fig 5.35. Analysis of IL-17 expression in CD4+ cells by genotype. **A**. Following a 3 h stimulation with PMA/ionomycin CD4+ T cells were stained for surface markers and intracellular IL-17. IL-17+ cells (black dot plots within gates) were defined by expression above the negative population and a fluorescence-minus-one (FMO) control (red dot plots). **B**. IL-17 expressing proportion of total CD4+ T cells by genotype at rs669607 and rs2371108. Graphs show mean±SEM.

A.



B.

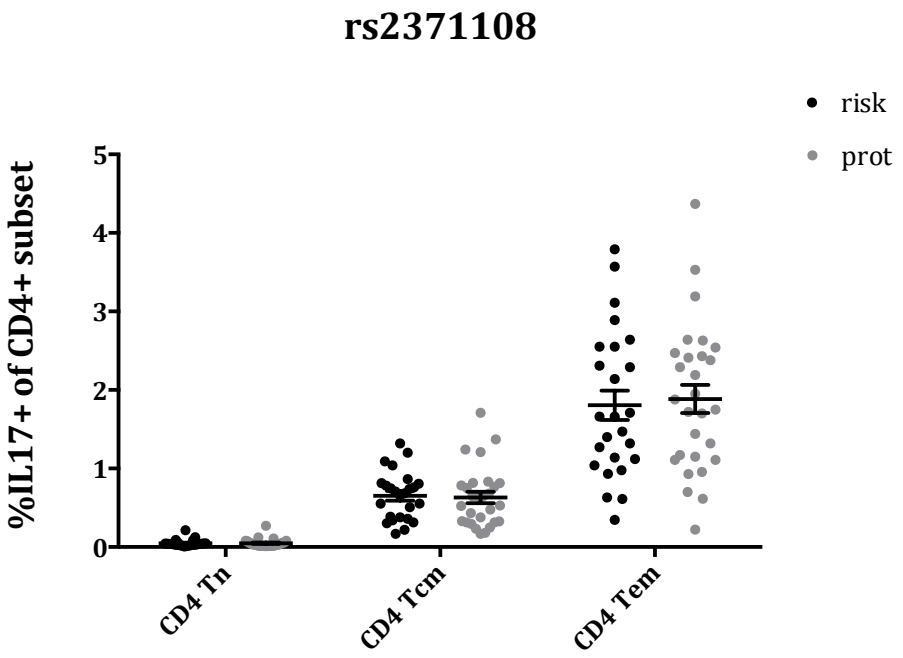


Fig 5.36. Analysis of IL-17 expression in CD4+ T cell subsets by genotype. Following stimulation PBMCs were stained for surface markers and intracellular cytokines for analysis by genotype at rs669607 (A) and rs2371108 (B). IL-17 expression was analysed by the proportion of subset expressing IL-17. Graphs show mean±SEM.

5.2.10.3 IL-2 expression in CD4+ and CD8+ T cells by genotype at rs669607 and rs2371108

IL-2 expressing cells were observed in all populations (Fig 5.37). In both CD4+ and CD8+ populations, the Tcm subset had the highest proportion of IL-2 expressing cells (CD4+ 43.7±1.0%, CD8+ 50.0±1.4%). The proportion of IL-2 expressing cells in each subset was compared by genotype at rs669607 and rs2371108, but did not reveal any significant difference in IL-2 expression between risk and protective homozygotes at either locus (Figs 5.38, 5.39).

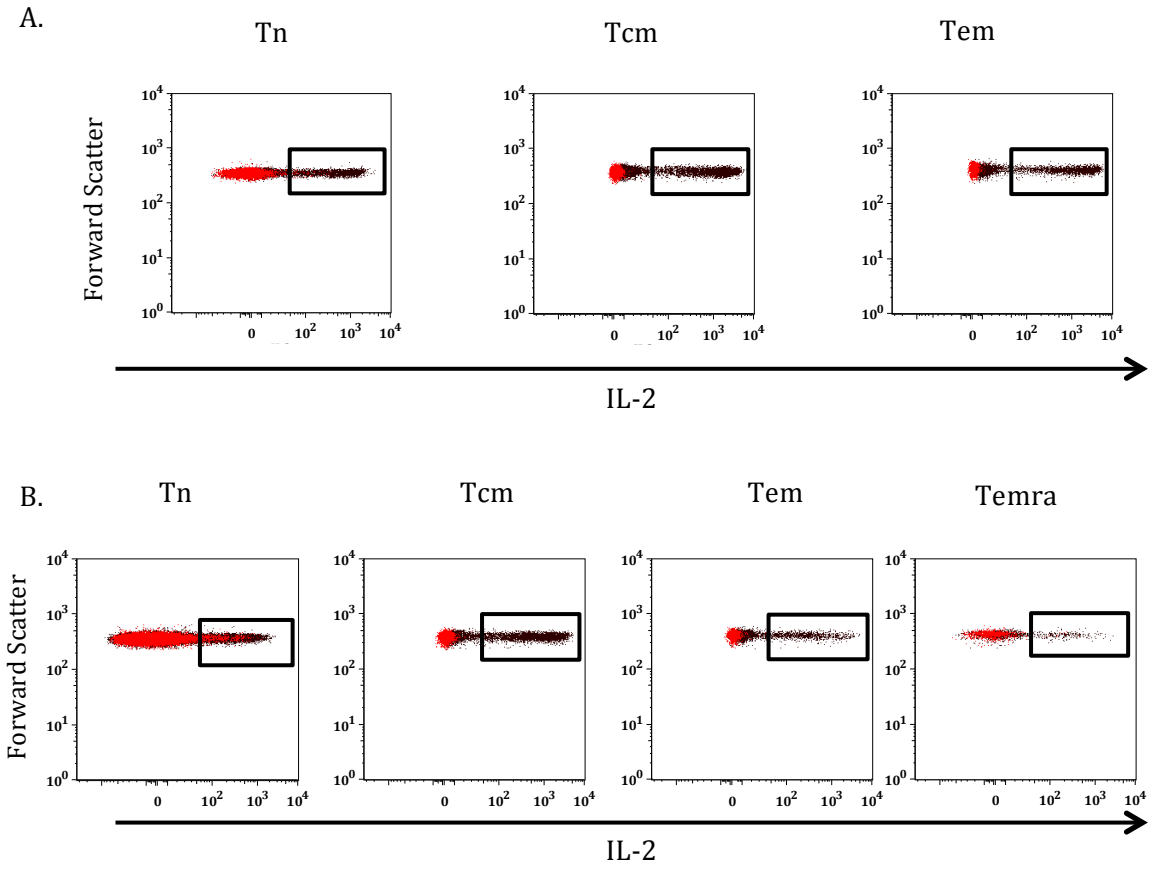
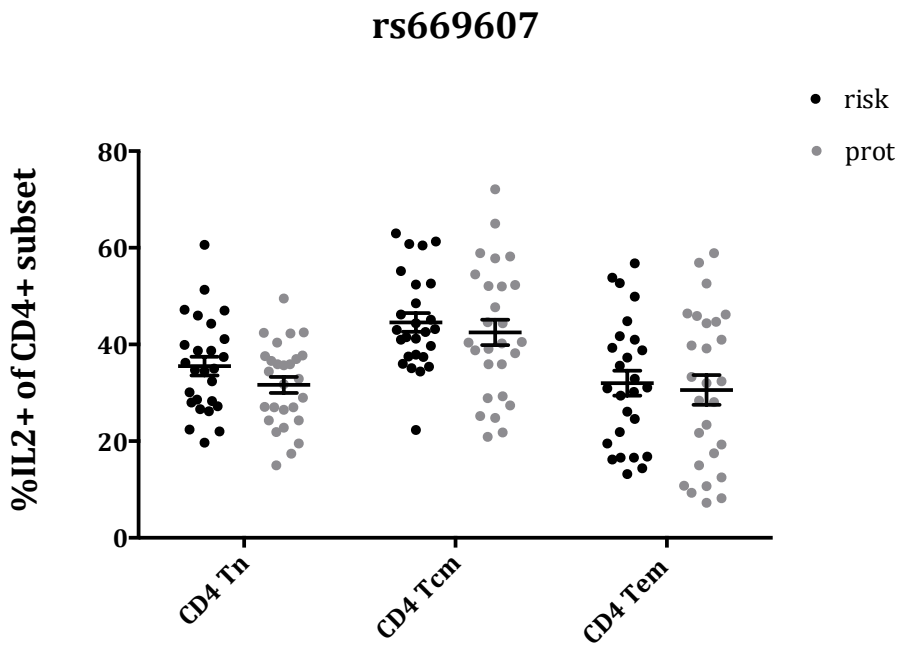


Fig 5.37. Flow cytometry analysis of IL-2 expression in T cell subsets. PBMCs were stimulated for 3h with PMA/ionomycin followed by staining for cell subset markers and intracellular IL-2. Effector/memory CD4+ (A) and CD8+ (B) subsets were gated by CD45RA and CCR7. IL-2-expressing cells were identified by expression above the negative population and a fluorescence-minus-one (FMO) control (red dot plots).

A.



B.

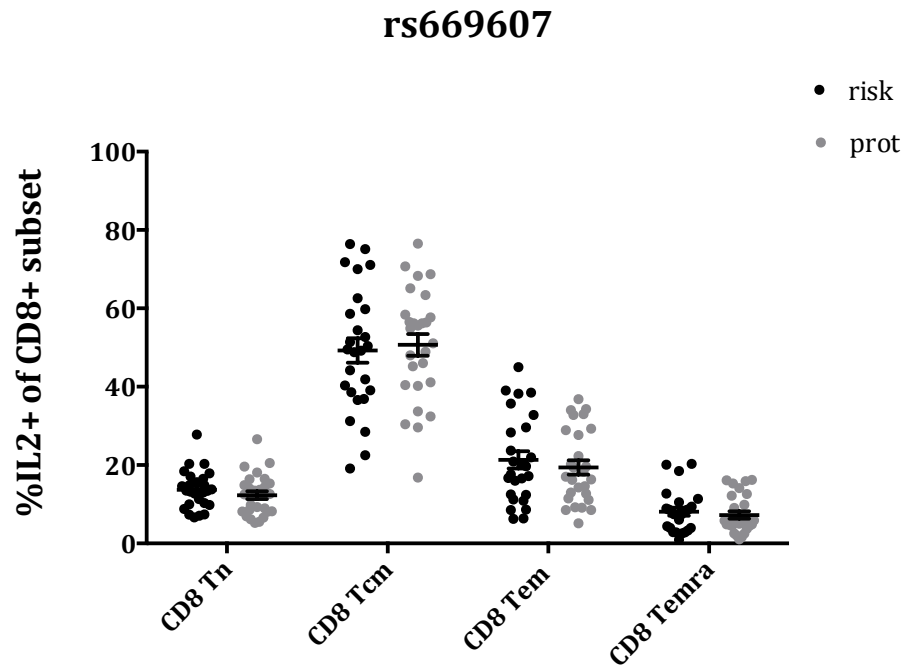
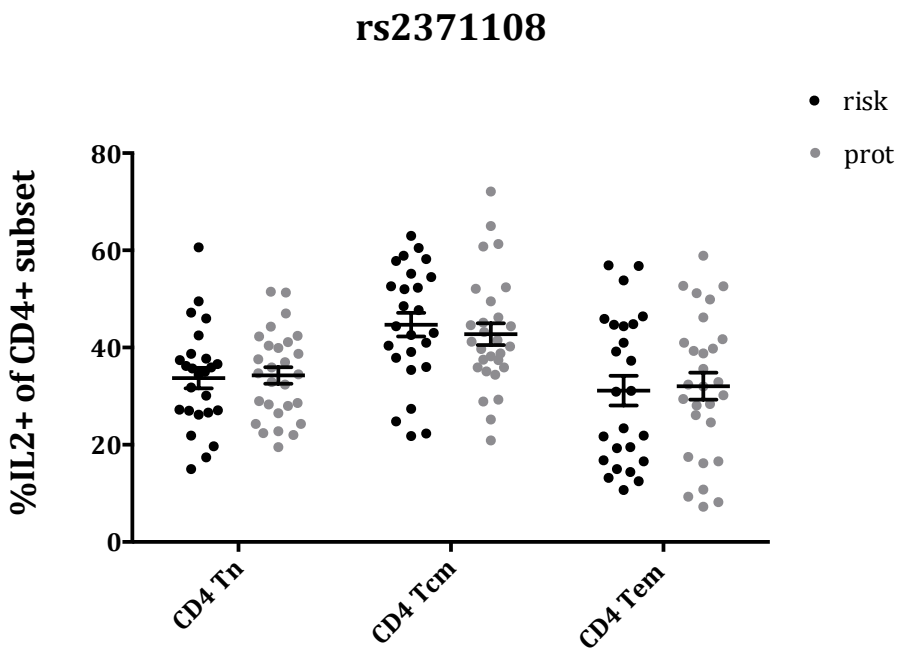


Fig 5.38. Analysis of IL-2 expression in T cell subsets by genotype at rs669607. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular IL-2 for analysis by genotype at rs669607. Graphs show mean±SEM.

A.



B.

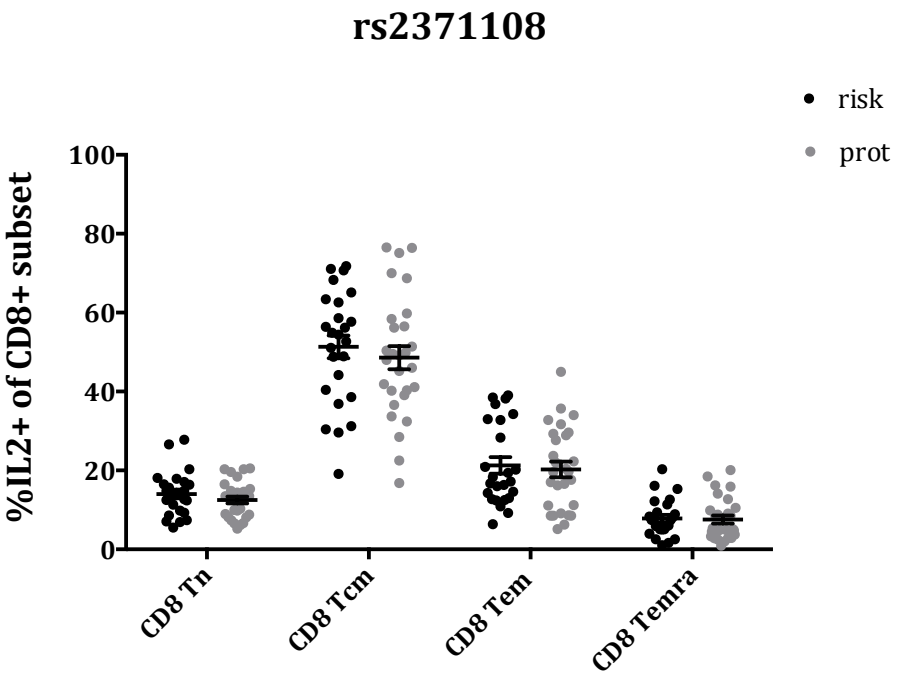


Fig 5.39. Analysis of IL-2 expression in T cell subsets by genotype at rs2371108. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular IL-2 for analysis by genotype at rs2371108. Graphs show mean±SEM.

5.2.10.4 TNF-α expression in CD4+ and CD8+ T cells by genotype at rs669607 and rs2371108

TNF-α expressing cells also arose from all populations (Fig 5.40). In CD4+ T cells TNF-α was expressed by a substantial proportion (25.4±0.2%) of naïve cells but a greater number of Tcm (58.8±2.5%) and Tem (59.9±0.6%) cells. In CD8+ T cells TNF-α was expressed by very few naïve cells (3.9±0.7%), but by higher proportions of Tcm, Tem and Temra cells (63.8±1.4, 69.0±0.4 and 72.4±2.8% respectively). The proportion of TNF-α-expressing cells in each subset was compared by genotype at rs669607 and rs2371108. No difference in expression between donors of different genotypes at either locus was observed in any of the subsets (Figs 5.41, 5.42).

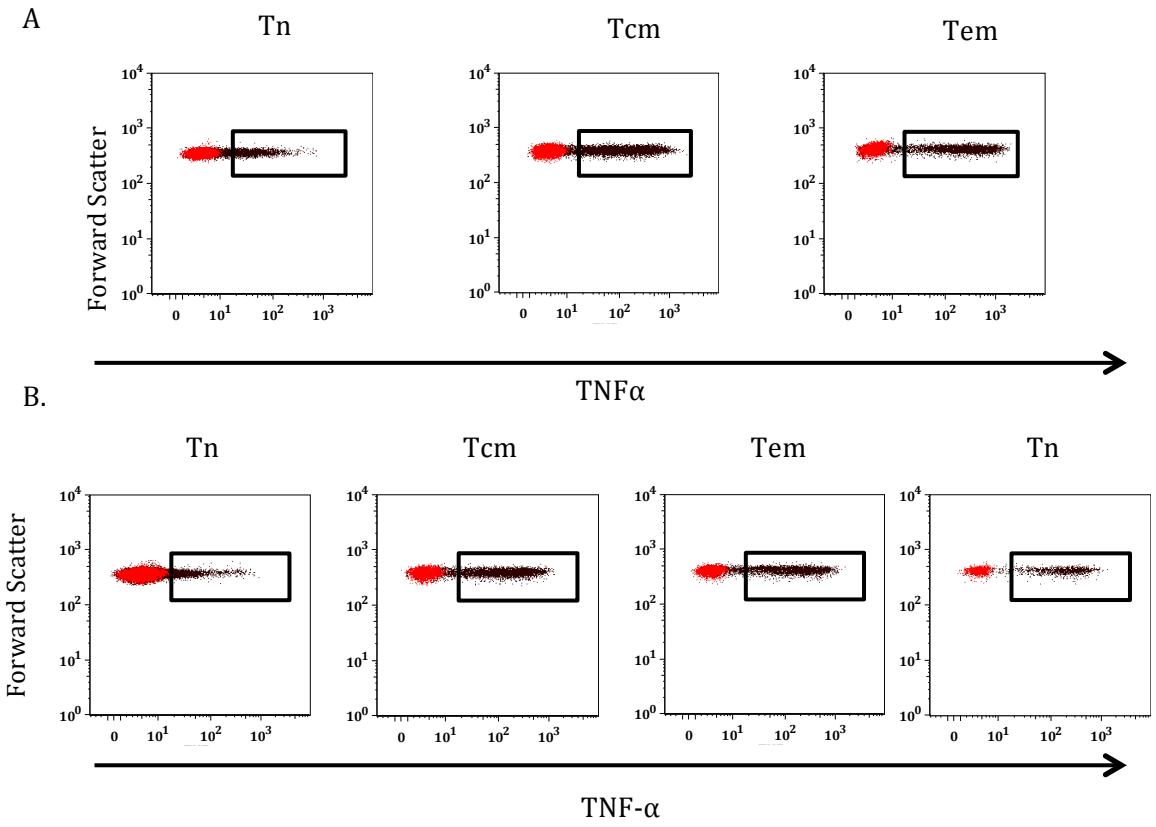
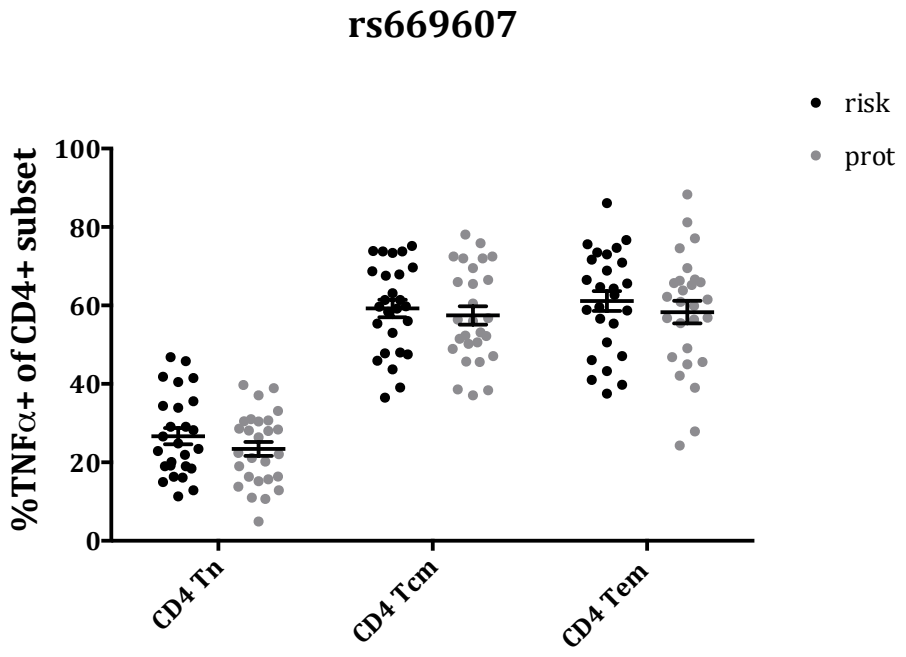


Fig 5.40. Flow cytometry analysis of TNF-α expression in T cell subsets. PBMCs were stimulated for 3h with PMA/ionomycin followed by staining for cell subset markers and intracellular TNF-α. Effector/memory CD4+ (A) and CD8+ (B) subsets were gated by CD45RA and CCR7. TNF-α-expressing cells were identified by expression above the negative population and a fluorescence-minus-one (FMO) control (red dot plots).

A.



B.

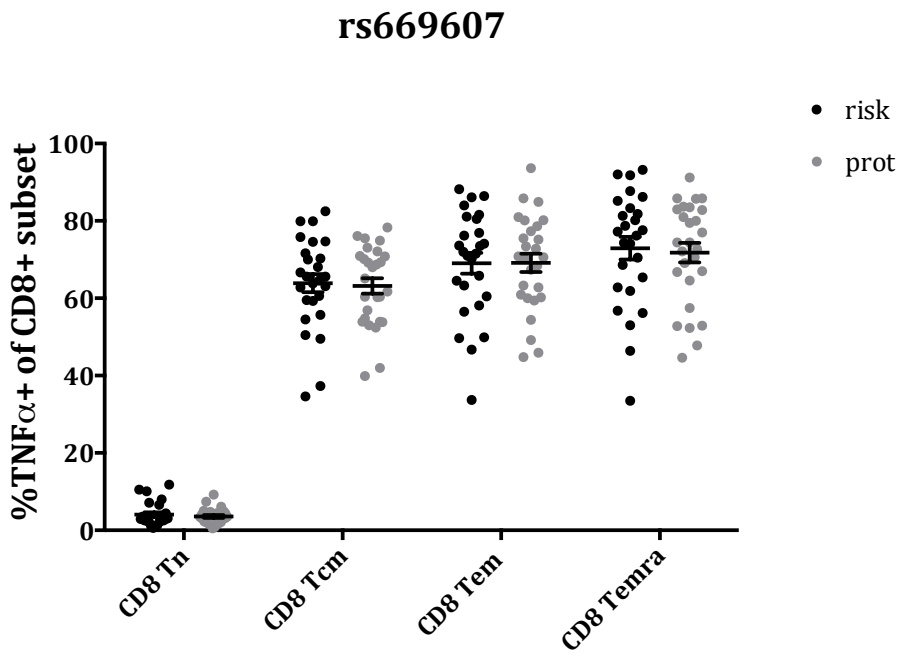
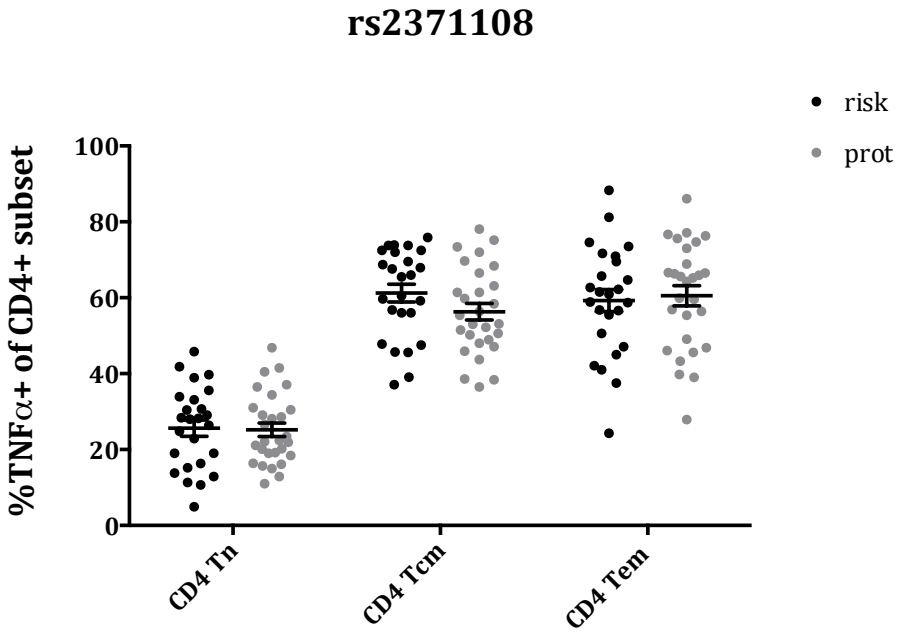


Fig 5.41. Analysis of TNF-α expression in T cell subsets by genotype: rs669607. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular TNF-α for analysis by genotype at rs669607. Graphs show mean±SEM.

A.



B.

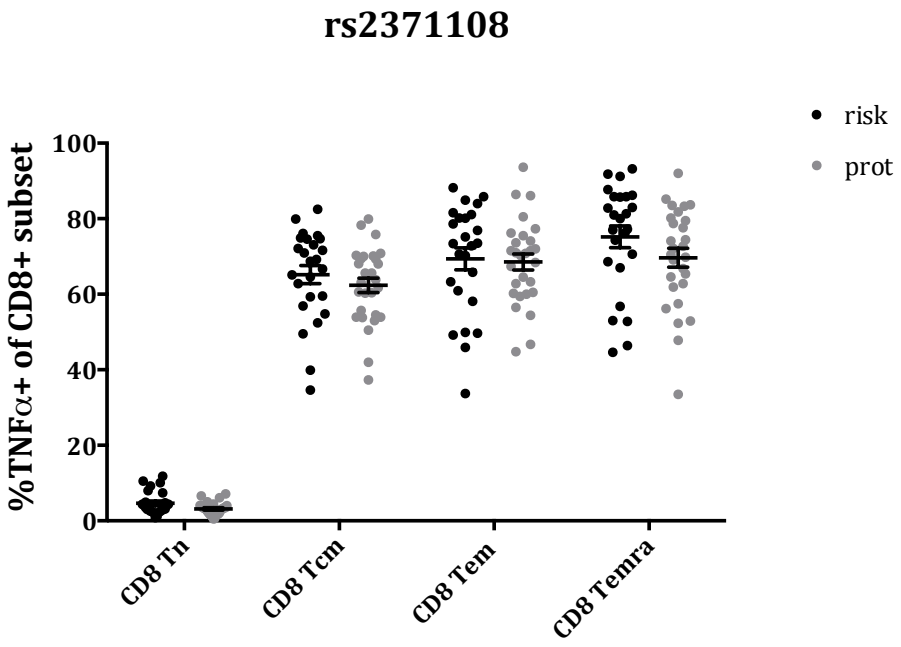


Fig 5.42. Analysis of TNF-α expression in T cell subsets by genotype: rs2371108. CD4+ (A) and CD8+ (B) T cells were stained for effector/memory subset surface markers and intracellular TNF-α for analysis by genotype at rs2371108. Graphs show mean±SEM.

5.3 Discussion

Independently published studies have found that SNPs in both MS associated regions are eQTL for Eomes at the mRNA level in LCLs and whole blood, as measured by microarray (Stranger et al. 2012, Westra et al. 2013). In these eQTL studies, risk genotype at the primary signal correlated with reduced Eomes expression (Stranger et al. 2012), whereas risk genotype at the secondary signal correlated with increased Eomes expression (Westra et al. 2013), suggesting that the association of these genetic regions with disease may be due to effects in different cell types or contexts.

Whether the mRNA expression differences by genotype are present at the protein level, and in which immune cell subsets, remained undetermined. For this study it was hypothesized, in accordance with the eQTL studies, that risk genotype at rs669607, tagging the primary signal, would correlate with reduced Eomes protein expression, and risk genotype at rs2371108, tagging the secondary signal, would correlate with increased Eomes protein expression. Given these opposing correlations, we proposed that genotype-dependent effects at the respective MS-associated SNPs would be observed in different cell types. We sought to elucidate these cell types, and in so doing to begin to explain the association of these variants with susceptibility to multiple sclerosis.

Analysis of Eomes expression in multiple immune subsets revealed apparently higher expression of Eomes in rs2371108 risk donors in effector memory CD8+ T cells, but this did not reach statistical significance. Analysis of downstream pathways detected trends towards higher IFN-γ expression in rs2371108 risk donors in both CD4+ and CD8+ T cells, but again this did not reach significance after adjustment of the significance threshold for multiple analyses. Correlation between genotype at rs669607 and Eomes expression was not observed in any of the cell types investigated.

The failure to replicate the published eQTL data in this study at the protein level has several possible causes. The nature of this type of study inevitably restricts the number of donors included for reasons of practicality; inter-individual variation therefore considerably affects a study's power, and as this was greater than anticipated from the pilot, the study may ultimately have been underpowered to detect an expression change due to variants with such a small odds ratio. A post hoc power calculation suggests that with the current observe inter-donor variation and difference between risk and protective genotype at rs2371108, an additional 30 donors per group would be required to detect a significant difference in Eomes expression in CD8+ Tem cells with a p-value threshold of 0.005.

A recent study of the immune systems of twins suggests that environmental effects on the immune system have a greater proportional effect than inherited factors (Brodin et al. 2015). This suggests that environmental effects increase as humans age, consistent with increasing exposure to numerous environmental agents; therefore, including donors over a wide range of ages (table 2.10) may have increased the inter-individual variability, reducing the power to detect an expression difference.

Studies of expression at the mRNA level do not take into account the degree of regulation at other levels. Steady-state protein levels are determined by a multitude of other factors, for instance regulation of translation initiation through microRNAs and RNA-binding proteins, and the rate of protein ubiquitination and degradation (Jackson et al. 2010, Vogel et al. 2012). In fact, very recent data suggests that mRNA level eQTL may be replicable at the protein level in fewer than half of cases (Battle et al. 2015); despite detection of an eQTL at the mRNA level, a similar effect on protein expression is not observed. In those cases where effects are found in both mRNA and protein, the effect on protein expression is often smaller. In primates, as well as more basic organisms, despite differing levels of a given mRNA transcript between species, little difference exists between protein levels, suggesting that evolutionary control of protein

levels is greater (Laurent et al. 2010, Khan et al. 2013). Additionally, a number of variants demonstrate eQTL properties at the protein level without detectable effects on mRNA expression, indicating that some variants exert their effect solely through post-transcriptional processing (Battle et al. 2015).

Transcriptional and post-transcriptional regulation may be tissue and context dependent, meaning that the effect of the variants on mRNA and protein expression levels may only be detectable in certain conditions. A study of peripheral immune cells looks at the immune system through a small window, restricted in both place and time. The situations of chronic infection or long-lasting sterile inflammation are difficult to recreate *in vitro*, and it may be only in these circumstances that an effect on Eomes protein expression is detectable. Therefore, it is feasible that stimulation of cells in the correct conditions may uncover protein level eQTLs that are not detectable in resting cells, as has been demonstrated at the mRNA level in monocytes (Fairfax et al. 2014), and may explain why these differences were not observed here. Furthermore, whole blood eQTL studies may be sufficiently powered to detect expression level differences in small populations of cells, such as iNKT or γδ-T cells, which were not investigated here.

A further possibility is that variants in the Eomes region contribute to susceptibility via an effect on neurons, which were not investigated in this study. Eomes is expressed as part of a transcription factor cascade during neural differentiation (Englund et al. 2005, Hodge et al. 2008). In the adult, neurogenesis plays a small role and is limited to few areas including the olfactory bulb and hippocampus. Given that the inflammation and neurodegeneration occurring in MS is widespread, it seems unlikely that an alteration of adult neurogenesis would significantly contribute to disease susceptibility. However, a role for neuronally expressed Eomes in CNS inflammation cannot be ruled out, as little is known about neuronal Eomes expression in this context. A further possibility is that in certain individuals, altered Eomes expression during brain development subtly alters the structure or composition of the brain, rendering some

individuals susceptible to future neurodegeneration. However, this would also be expected to contribute to disease severity; thus far, studies indicate a genetic basis for susceptibility but not severity (International Multiple Sclerosis Genetics Consortium 2011). The association of SNPs from the secondary signal in the *EOMES* region with other non-CNS autoimmune diseases suggests an immunological effect for these variants. However, the primary signal is MS-specific, raising the possibility that this locus is of importance in a cell type or context only relevant to MS.

The pilot study, performed to explore the difference between genotype groups and to calculate numbers that might be required, showed a significant difference in Eomes expression between risk and protective homozygotes at rs669607 in CD8+ Tcm cells, a finding that prompted further investigation and expansion of the study, but which was not replicated in a larger cohort. The p-value for this finding was 0.004, suggesting there may be reasons other than simple chance for the conflicting results of the pilot cohort and main cohort, in which the difference in CD8+ Tcm cells was not observed. These reasons are not immediately clear; risk and protective homozygotes were distributed evenly through the pilot study, ruling out an effect of day-to-day variability, and the analysis was blinded until the final collation of data to minimalise unconscious bias. An effect of time of year cannot be ruled out; the pilot study was conducted over the winter months, whereas the main cohort was assessed over spring and summer months. The incidence of viral illness in winter and allergic disease in spring may have influenced the results. It would be instructive to recall the same donors and repeat the assays in order to determine the robustness of the assay and whether the initial results were replicable.

However, it remains possible that the initial results occurred by chance, which reflects the importance of extending this type of functional genomics study to a larger cohort of donors to avoid a type 1 error due to low numbers. It also illustrates the limitations of small pilot studies with low power in this type of analysis.

Taking all donors from both cohorts into account, the final results suggested a trend toward higher Eomes expression in rs2371108 risk homozygotes in CD8+ Tem cells. This is consistent with published mRNA eQTL data (Westra et al. 2013) which suggested increased Eomes expression in whole blood mRNA in this genotype. No trends were seen in CD8+ T cells by genotype at rs669607 or in CD4+ T cells by genotype at either SNP.

In T cells, Eomes has multiple functions in both the acute phase response to infection, and in the development of immune memory (Pearce et al. 2003, Intlekofer et al. 2005, Cruz-Guilloty et al. 2009, Banerjee et al. 2010). It may also have a role in the determination of cell lineage commitment following exposure to antigen, particularly in the diversion of differentiating cells to an IFN-γ-producing phenotype rather than an IL-17 producing phenotype (Intlekofer et al. 2008); indeed in this cohort there was a trend toward higher IFN-γ expressing cells which was near to significance. Therefore there are multiple ways in which overexpression might lead to susceptibility to autoimmune disease. Increased levels of pro-inflammatory cytokines such as IFN-γ, as a result of Eomes over-expression, could lead to higher basal levels of immune activity, and the development of a constitutive inflammatory milieu in which only a small increased stimulus is required to activate an autoreactive T cell clone. In this way increased expression might contribute to overactivity of the immune system and the breakdown of peripheral self-tolerance. Higher Eomes expression may also lead to increased numbers of memory cells generated following exposure to a self-antigen such as a myelin component. This could contribute to an exaggerated response in the case of future exposure to the self-antigen, a "threshold" of tolerance being crossed and the causation of autoimmune disease.

As mentioned, HapMap data from LCLs suggested that risk genotype at rs669607 correlated with lower Eomes expression (Stranger, Montgomery et al. 2012). Here, no difference in expression was seen by genotype at this locus in T cells, which

may reflect the tissue-specific nature of genetic regulation, and that any regulatory elements involving or tagged by this locus do not exert an effect in CD8+ T cells.

Increased Eomes expression following exposure to antigen may lead to increased T cell survival and differentiation to memory precursor effector cells (MPECs), which localize to the secondary lymphoid organs (Rao et al. 2012). Access to these cells in healthy donors is obviously not possible, but might reveal genotype dependent differences in Eomes expression and MPEC numbers.

Eomes is uniformly and highly expressed by NK cells, as shown in Chapter 4. In these cells Eomes is likely to upregulate the cytotoxic molecules perforin and granzyme B, enabling NK cells' anti-viral and anti-tumour functions; loss of Eomes and T-bet results in reduced cytotoxicity, which can be regained by forced Eomes upregulation (Gill et al. 2012). Eomes is also thought to be required for the maintenance of NK cell maturity; this may be related to CD122 expression, which is highly expressed by NK cells and is a downstream target of Eomes (Intlekofer et al. 2005). In peripheral blood two main NK cell populations exist. Both the relative size of the populations and their Eomes expression was measured; a trend toward higher numbers of CD56++CD16- cells was seen in rs669607 risk donors, but this did not reach significance. Although no difference was seen in terms of Eomes expression, it is tempting to propose that the association of the primary signal with MS is due to an effect on NK cell differentiation, whereas the association of the secondary signal is due to an effect in CD8+ T cells. However, more evidence would be required to prove this definitively.

The role of NK cells in MS has not been thoroughly explored, and there is little literature in comparison with B cell or T cell research, which may be due to lack of evidence of direct NK cell involvement in the inflammatory lesions themselves (Durrenberger et al. 2012). However, there is some evidence that NK cells influence MS pathology, and relapses may coincide with lower NK cell activity(Kastrukoff et al. 1998). Intriguingly, the disease-ameliorating effects of daclizumab, an anti-CD25 monoclonal

antibody, have been suggested to occur via an increase in CNS CD56++CD16- NK cells, which are expanded in the CSF and peripheral blood of daclizumab-treated patients. *In vitro* these cells induce apoptosis of activated T cells (Bielekova et al. 2006). Eomes expression is highest in this NK population, and has been demonstrated to activate two apoptosis pathways albeit in other cell types (Eshima et al. 2012).

Therefore, it is possible that altered Eomes expression due to genotype at certain points during NK cell differentiation results in changes in the size of the CD56++CD16- NK cell population, or reduces their capacity to induce apoptosis in self-directed T cells, thereby permitting the loss of self-tolerance and the onset of autoimmune disease. The trend observed here was of increased numbers of CD56++CD16- cells in the peripheral blood of risk donors, which would be contrary to their regulatory role; however, the relative sizes of the two NK populations are known to be reversed in secondary lymphoid organs, and there remains the possibility that peripheral population sizes do not reflect the true numbers taking into account lymphoid tissue. Furthermore, changes in Eomes expression and NK cell function may only become apparent on activation of the relevant NK cells. Future work could further explore this possibility.

During the course of an immune response, particularly in the context of chronic infection, T cells may become "exhausted". This state is associated with loss of effector functions such as cytokine production and proliferation, and is marked by expression of inhibitory molecules such as PD-1 and certain transcription factors including Eomes, which is expressed highly by PD-1+ CD8+ T cells. T cell exhaustion is likely to be a progressive process, and may be reversible in the early stages(Larsen et al. 2011, Schietinger et al. 2014).

In MS this may be relevant to the immune response against chronic viral infection; increased T cell exhaustion may reduce the efficacy of viral eradication,

increasing the likelihood of autoimmunity. Evidence of the involvement of this pathway in MS comes from certain mouse models of MS, where activation of the PD-1/PD-1 ligand (PD-L1) pathway results in amelioration of EAE (Carter et al. 2007). In MS lesions, expression of PD-L1 is demonstrated by astrocytes and microglia, and is upregulated during inflammatory conditions (Pittet et al. 2011). However, only a minority of infiltrating CD8+ T cells express PD-1. Expression of PD-1 on T cells appears to correlate with disease activity, although whether this is cause or effect has not been explored (Trabattoni et al. 2009).

In this study no difference in Eomes expression was found in PD-1 expressing cells by genotype, nor any difference in the number of PD-1 positive cells. As PD-1 is also transiently expressed by activated effector cells, the population assessed may not consist entirely of exhausted cells. Furthermore, as these donors are healthy, the exhausted cell phenotype may not be fully developed, limiting the capacity of the study to detect genotype dependent differences in these cells.

CD122, the IL15R/IL2R β-chain, has been shown to be a downstream target of Eomes and is expressed highly in NK cells and to a lower level in CD8+ memory cells (Intlekofer et al. 2005). It may be particularly important for full effector function of NK cells, as cytotoxicity of these cells is reduced by blocking CD122 (Carson et al. 1994). Furthermore, IL-15 is required for the production of granzyme B in bystander-activated cells, responsiveness to which may be enabled by Eomes and CD122 upregulation (Marshall et al. 2010). In CD8+ T cells the strength of CD122 signaling appears to have a profound effect on subsequent differentiation and may determine the memory or effector function of the cell (Castro et al. 2011). Several murine studies have posited that certain CD122-expressing CD8+ T cells play a regulatory role, and are dependent on IL-15 for their development (Endharti et al. 2005, Lee et al. 2008, Endharti et al. 2011, Kim et al. 2011). It is tempting to postulate that altered CD122 expression, driven by

genotype dependent differences in Eomes levels, might influence the emergence of a regulatory CD8+ subset, altering the predisposition to immune disease. Alternatively it might have an effect in NK cells, reducing their effector function and capacity either to perform a regulatory role or eradicate viral infection.

Due to the prominent role of CD8+ T cells in MS, the CD122 analyses in this study focused on this population. No difference was seen by genotype. However, this may be due to the small proportion of CD122 expressing cells and the low levels of expression observed. CD122 is upregulated on activated cells (Alves et al. 2003), consistent with the finding that expression was highest in the terminally differentiated Temra subset. It may be that only under certain activating conditions, causing the upregulation of both Eomes and CD122, could a genotype dependent difference be observed. Another possibility is that NK cells are the relevant cell type for this assay and this would be a possible avenue of further investigation.

T-bet and Eomes, both master regulators of T cells, share many downstream targets such as CD122, IFN-γ, perforin and granzyme B (Intlekofer et al. 2008, Cruz-Guilloty et al. 2009). In addition they also have non-redundant functions, such as in NK cell lineage determination and differential expression in CD8+ effector and memory cells (Gordon et al. 2012, McLane et al. 2013, Daussy et al. 2014). It was postulated that a change in Eomes expression might lead to a corresponding change in T-bet levels, which might be detected in CD4+ and CD8+ T cells, populations that express both of these transcription factors as demonstrated in Chapter 4.

Analysis of the T-reg population, as defined by FoxP3 expression, was also performed. Both Eomes and FoxP3 divert differentiating CD4+ T cells away from a Th17 phenotype (Huber et al. 2008). Eomes predisposes to a Th1 phenotype (Intlekofer et al. 2008); accordingly an analysis was performed of the proportion of CD4+ T cells constituted by Tregs and Th17s, hypothesising that a change in Eomes expression might alter the size of CD4+ subsets. Altering the balance of CD4+ inflammatory and regulatory

populations might increase the susceptibility to autoimmune disease in a number of ways, for instance by reducing the number of regulatory cells, or increasing the production of proinflammatory Th1 or Th17 cytokines.

Comparison of risk and protective donors did not reveal any significant differences between the groups, suggesting that genotype at rs2371108 and rs669607 do not significantly influence the numbers of peripherally circulating Tregs or T-bet levels in peripheral T cells. This may be due to the multiple other influences on the expression of these transcription factors and the multiple other determinants of T cell differentiation.

The study went on to analyse the effect of genotype at rs2371108 and rs669607 on expression of effector molecules that were either downstream of Eomes, or were related cytokines. Eomes is thought to directly target the perforin and granzyme B genes (Pearce et al. 2003, Qui et al. 2011) to upregulate transcription, and also binds at the IFN-γ locus (Cruz-Guilloty et al. 2009). These downstream molecules are highly relevant to autoimmune disease; SNPs in the granzyme B region have been associated with susceptibility to vitiligo (Jin et al. 2010), and the perforin/granzyme pathway is one mechanism by which effector immune cells can induce apoptosis of target cells. It is feasible that reduced expression of perforin and granzyme B could lead to increased susceptibility to autoimmune disease, by reducing the capacity of regulatory cells to kill autoreactive T cells or virally infected cells. However, no difference in perforin or granzyme B positivity was seen by genotype in any of the CD8+ subsets. Due to the results of the pilot study we focused on CD8+ T cells; however, the expression of cytotoxic molecules is equally important to the function of NK cells and it might be more revealing to investigate the expression of these molecules in the NK populations.

SNPs in the IFN-γ region have been associated with susceptibility to inflammatory bowel disease (Franke et al. 2010, Jostins et al. 2012), and SNPs in the

regions of TNF-α and IL-2, and their receptors, have been associated with multiple autoimmune diseases (IGAS et al. 2013, International Multiple Sclerosis Genetics et al. 2013). Eomes is reported to have diverse roles in the differentiation of T cells into different cytokine-producing populations, and these populations were analysed by genotype. Significant differences in the number of IFN-γ-expressing cells by gender were observed; after adjusting for this, a trend toward higher numbers of IFN-γ-expressing CD8+ Temra cells was observed in rs2371108-risk donors. A trend was also seen in total CD4+ T cells as well as CD4+ T cell subsets. This is concordant with the trend in Eomes expression observed in CD8+ Tem cells, suggesting that higher Eomes expression is associated with higher number of IFN-γ positive cells. The observed effect of genotype is stronger on IFN-γ+ cells than on measured Eomes expression. This may suggest that the effect of the SNP is greatest during the polarisation of cells to a Th1 IFN-γ-secreting phenotype rather than on Eomes expression in resting peripheral cells.

IFN-γ is the archetypal type 1 pro-inflammatory cytokine and has wide ranging effects. It is produced during the initial innate immune response by NK cells and macrophages, and then later in large quantities by CD4+ and CD8+ T cells. It binds to the IFN-γ-receptor (IFNGR) which signals via the JAK-STAT pathway, primarily via STAT1, which interacts with IFN-response factors and translocates to the nucleus to activate transcription of multiple genes. Among its effects are an upregulation of MHC molecules, production of antiviral substances, activation of microbicidal effects of macrophages, and facilitation of leukocyte adhesion and trafficking via the upregulation of chemokines and adhesion molecules (Schroder et al. 2004, Reed et al. 2008). Increased numbers of IFN-γ producing effector cells could therefore contribute to a system-wide upregulation of inflammation, which might in turn lead to activation of bystander cells in the periphery and the loss of tolerance to self-peptides, causing autoimmunity. Early trials of IFN-γ in the treatment of MS resulted in exacerbation of disease (Panitch et al. 1987), although this reflects worsening the severity of already

existing disease rather than an effect on susceptibility. The role of IFN-γ in EAE is controversial; IFN-γ deficient mice have been shown to still be susceptible to EAE, and in certain cases the disease was more severe (Billiau et al. 1988, Ferber et al. 1996). This possibly reflects the differences between EAE and MS, but also that IFN-γ has multiple and complex roles that may be beneficial or detrimental depending on context.

The effect on leukocyte adhesion may be particularly pertinent in the case of MS, as leukocyte trafficking to the CNS across the blood brain barrier is a key event in the pathogenesis of the MS inflammatory lesion; drugs preventing leukocyte trafficking to the CNS, such as natalizumab and fingolimod, have proven highly effective in the prevention of new MS lesions.

IL-17 is a more recently characterised pro-inflammatory cytokine, production of which defines a subset of Type-17 T cells. Th17 cells are far more numerous than CD8+ Tc17 cells, although both are present in peripheral blood and in the lesions of MS (Tzartos et al. 2008, Walker et al. 2012). As Eomes diverts cells towards a Th1 phenotype and away from a Th17 phenotype it was thought that a change in IFN-γ producing Th1 cells might be reflected by a reciprocal change in Th17 cells. However, no difference by genotype was observed here in either CD8+ or CD4+ T cells. Peripheral IL-17+ CD8+ T cells were too few to accurately assess here; larger total numbers, or assessment in type-17 polarising conditions might be required to detect a genotype dependent change.

TNF-α and IL-2 also have wide ranging effects on the immune system. TNF-α signals primarily by TNFR1 and TNFR2 to activate the transcription factor NFkB and to induce cell death, among other effects (Chen et al. 2002, Wajant et al. 2003). NFkB has multiple pro-inflammatory target genes including IL-1, IL-2 and multiple chemokines. Although blockade of TNF-α has beneficial effects in many autoimmune diseases (Moreland et al. 1997, Targan et al. 1997), treatment with a soluble TNF-α receptor in MS resulted in the worsening of disease (Lenercept MS study group, 1999). IL-2 also has

wide ranging effects, particularly in promoting survival and proliferation of T cells. Blockade of IL-2Rα (CD25) with Daclizumab results in lower rates of relapse, reduced numbers of circulating and CSF CD4+ and CD8+ T cells and expansion of the CD56++CD16- NK cell population (Bielekova et al. 2006, Bielekova et al. 2011), thought to be due to increased availability of IL-2 for signaling via CD122 which is highly expressed by these cells (Martin, Perry et al. 2010).

In this study, no differences in the number of TNF-α or IL-2 expressing cells by genotype was observed, although consistent with the literature there was a significant difference in TNF-α and IL-2 expressing cells by gender.

Considering future work, ideally this study would be continued in a larger cohort of donors to ensure that the lack of statistical significance is not due to insufficient power and high inter-individual variation. Recruiting donors around the age at which MS susceptibility peaks, in the third decade, might also reduce variability and increase the likelihood of detecting a genotype effect on protein level. Further investigation of different contexts might elucidate conditions under which a genotype dependent change in expression becomes apparent, for instance stimulation with non-specific inflammatory stimuli, or with specific antigens. Ideally, samples from immune tissue besides peripheral blood, such as lymphoid tissue, would also be analysed though this is clearly not possible in the healthy population.

Given that eQTLs for SNPs in the primary association signal are detectable in EBV-transformed LCLs, it is possible that the change in expression becomes apparent in the context of EBV infection. In light of EBV infection being one of the strongest environmental factors in MS aetiology, this may be relevant; the effect of EBV transformation could be explored in future work on LCLs through analysis of DNase hypersensitivity and transcription factor binding.

In MS patients, Eomes and T-bet levels are thought to be reduced at the mRNA level (Parnell et al. 2014). This is in contrast to the trend observed here, as well as the published whole blood eQTL (Westra et al. 2013), that risk genotype is associated with increased expression, but in concordance with the published eQTL in LCLs (Stranger et al. 2012). However, in Parnell *et al* the patient samples were obtained 15-20 years following diagnosis, and therefore likely even longer since disease onset. It is possible that transcription factor levels, as well as a number of other immune parameters, may be considerably changed following this duration of autoimmune disease compared to levels at disease onset and, as mentioned above, altered mRNA levels may not reflect changes at the protein level. Further studies of Eomes levels in patients would be interesting given its wide-ranging effects on memory and effector function. Confirmation that Eomes is expressed by CD8+ or NK cells in active MS lesions would lend weight to the theory that this molecule has an important role in MS, and analysis of Eomes levels in immune cell subsets in CSF during relapse and remission, in conjunction with analysis of other inflammatory molecules, might indicate the pathways in which Eomes is important in the context of active autoimmune disease.

Chapter 6

The presence of MAIT cells in MS lesions and genotype-dependent MAIT cell Eomes expression

6.1 Introduction

A large proportion of both CD4+ and CD8+ T cells in MS lesions demonstrate IL-17 immunoreactivity (Tzartos et al. 2008). Subsequently, IL-17-producing CD8+ T cells have been identified as Mucosa-Associated Invariant T (MAIT) cells (Annibali et al. 2011). These cells arise from a pre-committed pool of progenitors already present in cord blood, and were given their name due to first being identified in the gut lamina propria (Treiner et al. 2003, Walker et al. 2012). They are identified by expression of the semi-invariant TCR Vα7.2-Jα33 and high levels of CD161 (CD161++), a surface marker of activation predominantly expressed by NK cells, and in peripheral blood comprise 1-10% of total T cells (Le Bourhis et al. 2010, Fergusson et al. 2011). MAIT cells are restricted to the MHC-related molecule MR1, thought to present vitamin B metabolites (Treiner et al. 2003); recognition of ligands in the context of MR1 necessitates restricted TCR diversity, and MAIT cells can be further identified by expression of a semi invariant Vα7.2 TCR (Treiner et al. 2005). Recent studies indicate that the MAIT TCR Jα-chain and β-chains are more diverse than previously thought, and suggest that rather than being truly innate, MAIT cells are able to differentiate between ligands and to a certain extent proliferate clonally while retaining a degree of innate-like pattern-recognition (Walker et al. 2012, Gold et al. 2014). Due to this and certain other characteristics, such as immediate effector function and development with a memory phenotype, MAIT cells

may be included under the umbrella of innate-like lymphocytes (Berg 2007). The majority of MAIT cells are CD8+ with a smaller proportion being CD4+ or double negative (DN)(Reantragoon et al. 2013).

MAIT cells display an unusual CD8+ effector phenotype; as well as containing all IL-17 producing CD8+ T cells, a significant proportion also have the capacity to produce IL-22, a cytokine important for the expression of antimicrobial genes and for tissue repair, as well as the inflammatory cytokines IFN-γ and TNF-α (Billerbeck et al. 2010, Dusseaux et al. 2011, McAleer et al. 2011). Although resting MAIT cells express low levels of the cytotoxic molecules perforin and granzyme B, they are able to rapidly induce high levels following stimulation with bacterially infected cells (Dusseaux et al. 2011, Le Bourhis et al. 2013, Kurioka et al. 2015). Initially described in the gut mucosa, they are capable of migrating to multiple tissues as evidenced by their expression of chemokine receptors CCR5, CCR6 and CXCR6 (Dusseaux et al. 2011), and are likely to play a particularly important role in the liver and lung (Gold et al. 2010, Tang et al. 2013, Harriff et al. 2014) Furthermore, they express high levels of IL18-receptor, a potent inducer of IFN-γ, in contrast to CD8+CD161+ or CD8+CD161- cells (Ussher et al. 2014). CD26, a T cell costimulatory molecule, has recently been described as a further specific marker of MAIT cells (Sharma et al. 2015).

It has been suggested that the peripheral abundance of this subset is altered in MS patients, with lower levels both during remission and to a greater extent during relapse when compared with healthy controls (Miyazaki et al. 2011). A possible explanation for this is that during periods of inflammation these cells "home" to regions of inflammation, such as the CNS. Evidence for involvement of this subset in the lesions of MS is from a study using clonotypic analysis, which identified the presence of the invariant TCR in MS lesions and CSF (Illes et al. 2004), and a study using immunohistochemistry to identify CD161+ CD8+ T cells in MS lesions (Annibali et al. 2011). However, at the time this work was done no study had identified these cells in

the inflammatory regions of MS lesions using both the accepted defining markers of Vα7.2-TCR and CD161, nor had the MAIT cell proportion of CD8+ cells in MS lesions been assessed. The CD161 expressing cells identified by Annibali *et al* might simply be CD161 expressing conventional CD8+ T cells. The primary aim of this work was to clarify whether MAIT cells, as defined by CD161 and Vα7.2 expression, were present in active MS lesions, and whether they were enriched in the lesions as a proportion of CD8+ T cells compared with peripheral blood, thereby possibly explaining the apparent reduction in number of circulating MAIT cells during MS relapse. The interest was in active lesions where a considerable T cell inflammatory infiltrate could be found; chronic lesions, which contain a sparse inflammatory infiltrate, were not assessed.

These cells were also of interest as their innate-like characteristics and effector-memory phenotype suggested they would express high levels of Eomes. This would be consistent with studies involving Tec-kinase knockout mice. The Tec family of tyrosine kinases are important in the development of T cells, and are thought to regulate the thresholds of TCR signaling determining positive and negative selection in the thymus. Mice lacking these enzymes develop an expanded population of thymic innate-like CD8+ cells with a memory phenotype, expressing high levels of Eomes and lacking T-bet (Atherly et al. 2006). A further aim of this part of the study therefore was to characterise Eomes expression in the MAIT population, and to investigate Eomes expression and MAIT cell abundance on a genotype dependent basis given the possible importance of this subset in multiple sclerosis pathogenesis.

6.2 Results

6.2.1 Characterisation of multiple sclerosis lesions.

Snap-frozen sections of post-mortem brain tissue from MS patients were obtained from the MS Society Tissue Bank, Imperial College London. All patient had secondary progressive MS at the time of death. Patient characteristics are shown in table 6.1. Initial characterisation of lesions was performed by the MS Society tissue bank; further identification of lesions and confirmation of lesion type was performed in-house by staining for MHC class II and counterstaining with Luxol fast blue (LFB) to delineate myelin. Acute lesions were identified by increased numbers of cells positive for MHC class II staining, distributed relatively evenly throughout the lesion, with largely uniform demyelination (Fig 6.1).

Chronic active lesions were identified by an area of clear demyelination variably populated by MHC class II positive cells, surrounded by a rim of increased numbers of MHCII+ cells, outside which preserved myelin was present with a gradient of inflammatory infiltrate away from the active region (Fig 6.2). Acute lesions were identifiable in 7 cases and chronic active lesions in 10 cases. Included lesions were cerebral white matter lesions; lesions with grey matter involvement were excluded.

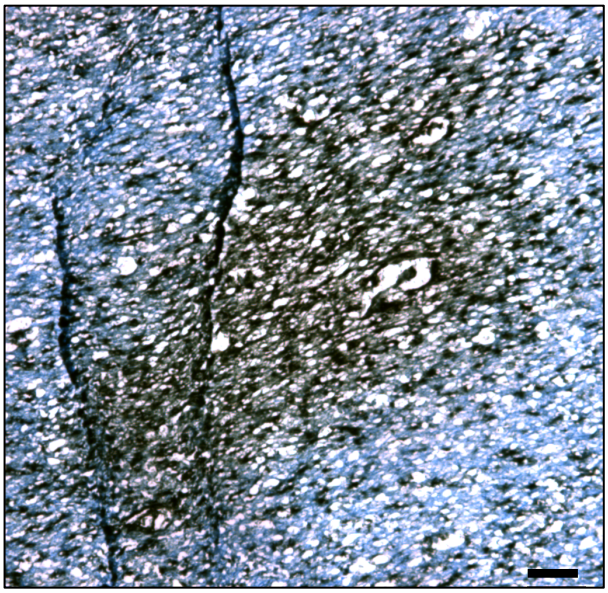


Fig 6.1. Characterisation of multiple sclerosis lesions. Acute/early active lesion demonstrated by relatively uniform demyelination and inflammatory MHCII-positive infiltrate, with a well-defined border and preserved myelin outside the lesion (magnification x4, scale bar 100um)

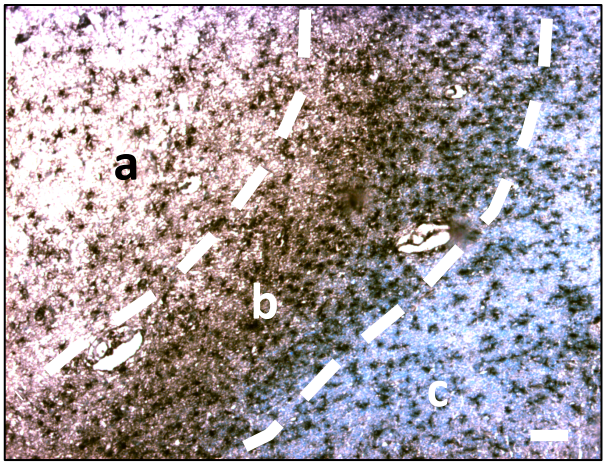


Fig 6.2. Characterisation of multiple sclerosis lesions. Chronic active lesions were identified by demyelinated central region (a), active rim with dense MHCII+ inflammatory infiltrate (b) and preserved myelin outside the lesion (c). (magnification x4, scale bar 100 μm).

Case No.	Age/ sex	MS subtype	Disease duration	PMI (h)	Cause of death	Lesion type (number)
MS100	46/M	SPMS	8y 9m	7	Bronchopneumonia	Chronic active (1)
MS 103	77/F	SPMS	21y	7	Bronchopneumonia	Acute (1) Chronic active (1)
MS 104	53/M	SPMS	11y	12	Urinary tract infection	Acute (2) Chronic active (2)
MS 106	39/F	probable SPMS	21y	18	Bronchopneumonia	Chronic active (1)
MS 107	38/M	SPMS	>16y	19	Aspiration pneumonia, pulmonary oedema	Acute (1) Chronic active (1)
MS 122	44/M	SPMS	> 10y	16	Bronchopneumonia	Acute (1) Chronic active (1)
MS 154	34/F	SPMS	11y	12	Bronchopneumonia	Acute (2) Chronic active (2)
MS 160	44/F	SPMS	15y 9m	18	Aspiration pneumonia, MS	Acute (1) Chronic Active (1)
MS 180	44/F	SPMS	> 10y	9	MS	Acute (3) Chronic active (1)
MS 203	53/F	SPMS	17y 10m	17	MS	Chronic active (1)

Table 6.1. Post-mortem tissue: patient data. Post-mortem brain tissue was obtained from the MS Society tissue bank, Imperial College London. Data relating to the donors are shown. Multiple lesion types were obtained from the same individual in most cases. SPMS: secondary progressive multiple sclerosis; PMI: post-mortem interval.

6.2.2 MAIT cells in MS lesions: Pilot study in thymus tissue

MAIT cells are present in the thymus where they undergo selection on the basis of MR1 expression by double positive thymocytes (Seach et al. 2013). They leave the thymus as naïve cells, proliferating and gaining memory phenotype once in the periphery(Gold et al. 2013). A pilot study in fixed, frozen healthy thymus tissue was

undertaken in order to optimize the use of CD8+, CD161 and Vα7.2 antibodies to identify MAIT cells. Vα7.2 and CD161 antibodies had been previously used by the laboratory of Prof Manuel Friese, who kindly provided starting working concentrations. Although in peripheral blood MAIT cells are defined as CD161++, the immunohistochemistry technique was not sensitive enough to distinguish between CD161+ and CD161++ populations, and therefore in this section all cells expressing CD161 are referred to as CD161+.

CD8+ MAIT cells, showing positivity for all three surface markers, were sparsely distributed through the tissue (Fig 6.3) in keeping with lower numbers of MAIT cells in the thymus compared with peripheral blood (Martin et al. 2009, Gold et al. 2013). A few single and double positive cells were also observed, but the majority of Vα7.2+ cells were also CD161+ and vice-versa, and there was little background staining. This indicated good specificity of the antibodies in this tissue.

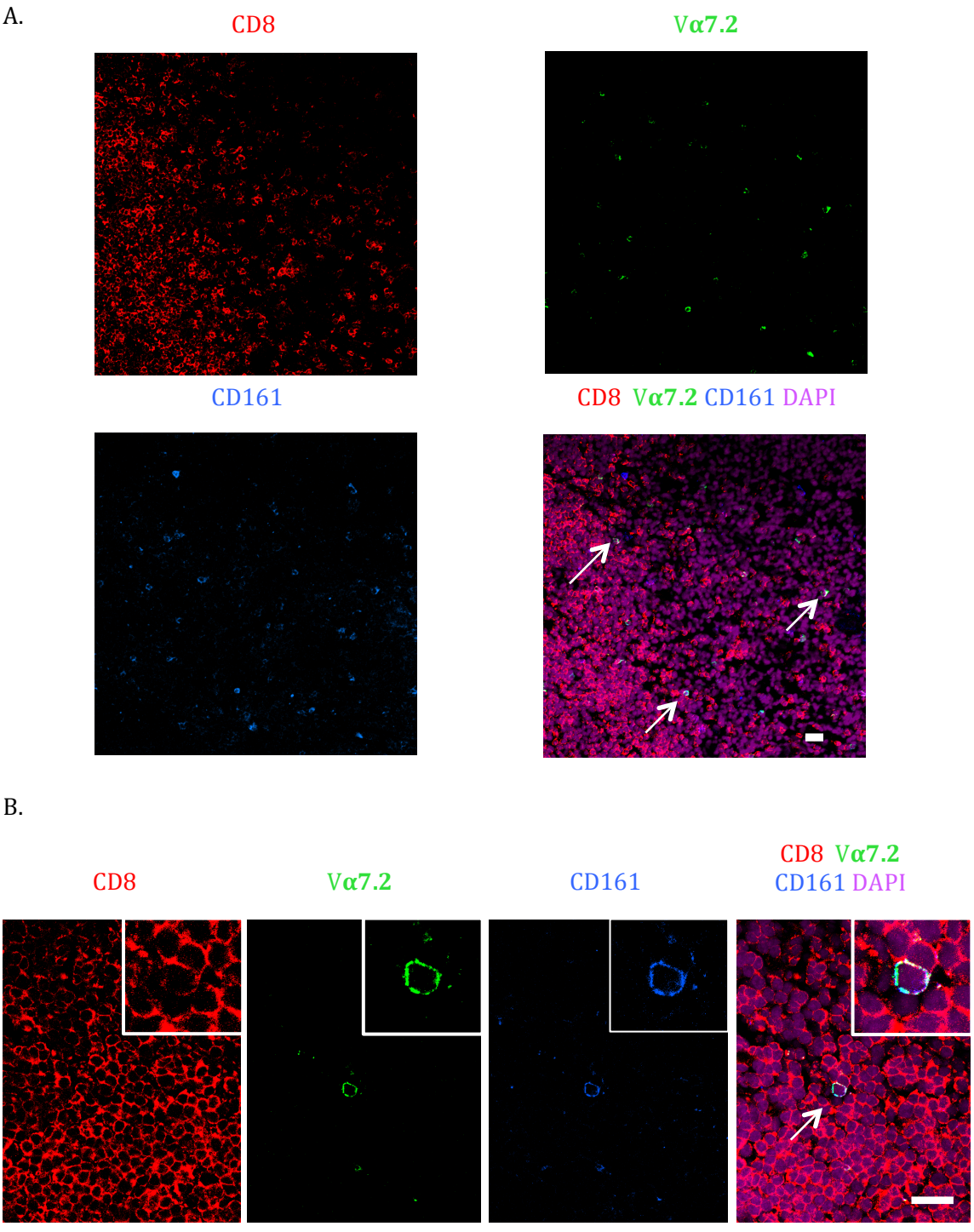


Fig 6.3. Pilot MAIT cell staining in thymus tissue. Thymus tissue was stained with antibodies against CD8 (red), Vα7.2 (green) and CD161 (blue) and with DAPI to show nuclei (purple). **A**. Arrows indicate sparsely distributed triple-positive cells. Magnification x25, scale bar 20 μm. **B**. Arrow indicates triple-positive cell. Magnification x63, scale bar 20 μm. Inset image is a digital magnification of the triple positive cell.

6.2.3 MAIT cells are present in acute and chronic active multiple sclerosis lesions

Fixed frozen sections from multiple sclerosis brain were stained for CD8, Vα7.2 TCR and CD161, surface markers that define the MAIT cell population. Potential non-specific binding of the secondary antibodies, streptavidin and tissue autofluorescence was assessed by performing a control staining omitting all primary antibodies. At x25 magnification (Fig 6.4 A) this showed some small areas of high intensity fluorescence in CD8, Vα7.2 and CD161 channels, suggestive of tissue autofluorescence most likely due to lipofuscin (Schnell et al. 1999). This was less obvious at x63 magnification (Fig 6.4 B). As the markers examined were all surface markers, genuine staining could be distinguished from autofluorescence by annular cellular morphology and by localization with cell nuclei.

To reduce bias, each lesion was scanned visually and images were taken of cells staining positive for CD8 without reference to CD161 or Vα7.2 staining, which was analysed at a later time post image acquisition. Both parenchymal and perivascular cells were included in the analysis, although it is likely that the majority of analysed cells were perivascular due to the higher density of cells in these regions. CD8+ cells were then individually outlined on image analysis software and analysed for the presence of Vα7.2 or CD161, and the proportion of CD8+ cells positive for all three markers was then calculated for each donor. MAIT cells were identified in acute lesions from all patients and in chronic active lesions in eight of ten patients (Fig 6.5 and 6.6).

There was substantial variability between individuals in the number of MAIT cells present in the lesions (acute lesions: mean 5.7%, range 2.9-12.7%; chronic active lesions: mean 6.4%, range 0-22%)(Willing et al. 2014). There was also significant variability in the post-mortem interval (PMI) between cases. To investigate the possibility that the PMI might affect surface antigen stability and MAIT cell detection, accounting for some of the variability in MAIT cell number, a linear regression analysis

of MAIT cell number and PMI was performed. No correlation was observed, suggesting that this was not a significant factor.

In addition to triple-positive cells, cells were observed that stained for CD8 and either Vα7.2 or CD161, but not both. However, the majority of Vα7.2+ cells were also CD161+ (Fig 6.7; AL mean 82% range 58.3-100%; CAL mean 68%, range 0-100%), whereas only a small proportion of Vα7.2- cells were CD161+ (AL mean 6.3%, range 2-13%; CAL mean 5.4%, range 0-18%) suggesting that the majority of Vα7.2+ cells in MS lesions are true MAIT cells, consistent with the pattern observed in peripheral blood where only a minority of Vα7.2+ cells are CD161- (Fig 6.8).

The overall percentage of CD8+ cells identified as MAIT cells in MS lesions was comparable to the frequencies observed in peripheral blood, which is also highly variable between individuals (Fig 6.9). There was no significant difference in MAIT cell number by type of lesion (Fig 6.7; acute lesions 5.3±3% v chronic active lesions 6.4±2.4%).

A.

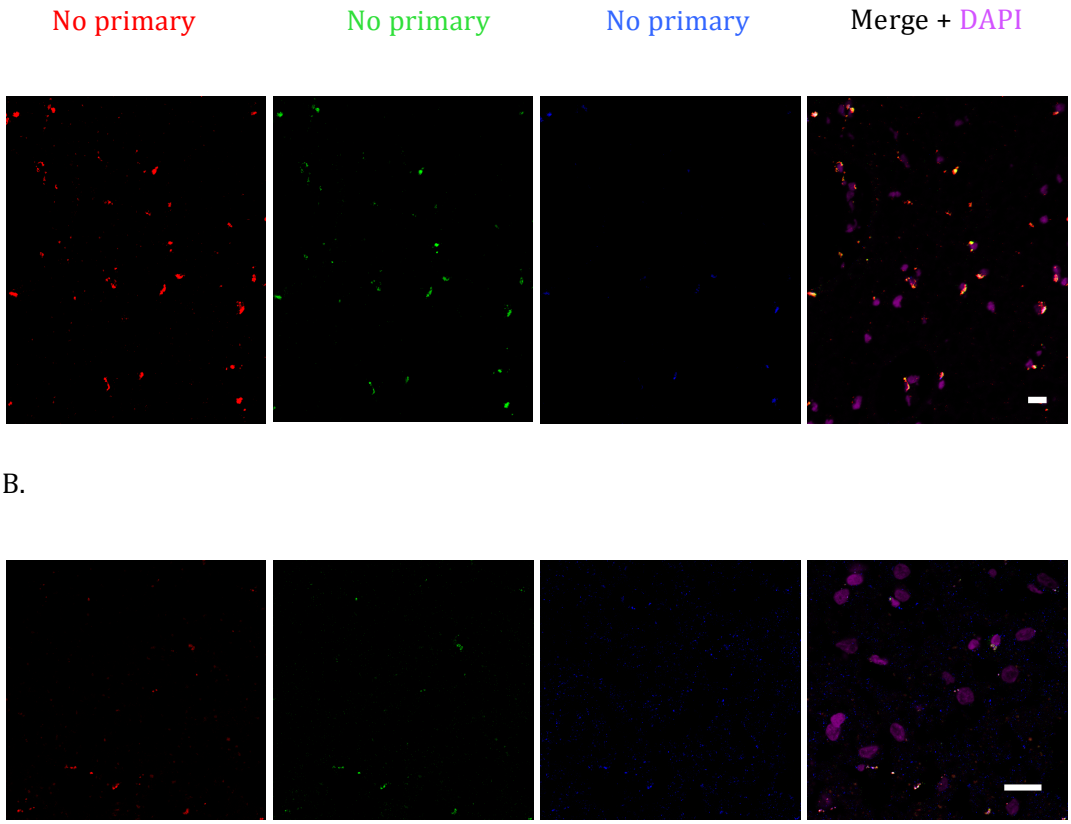


Fig 6.4. Control staining for fluorescent secondary antibodies. Primary antibodies were omitted to control for non-specific staining of the secondary antibodies in control brain tissue at x25 (A) and x63 (B) magnification. At x25 magnification regions of autofluorescence were observed (A); these were less obvious at x63 magnification (B) and were distinguished from true staining by morphology. Scale bars 20µm.

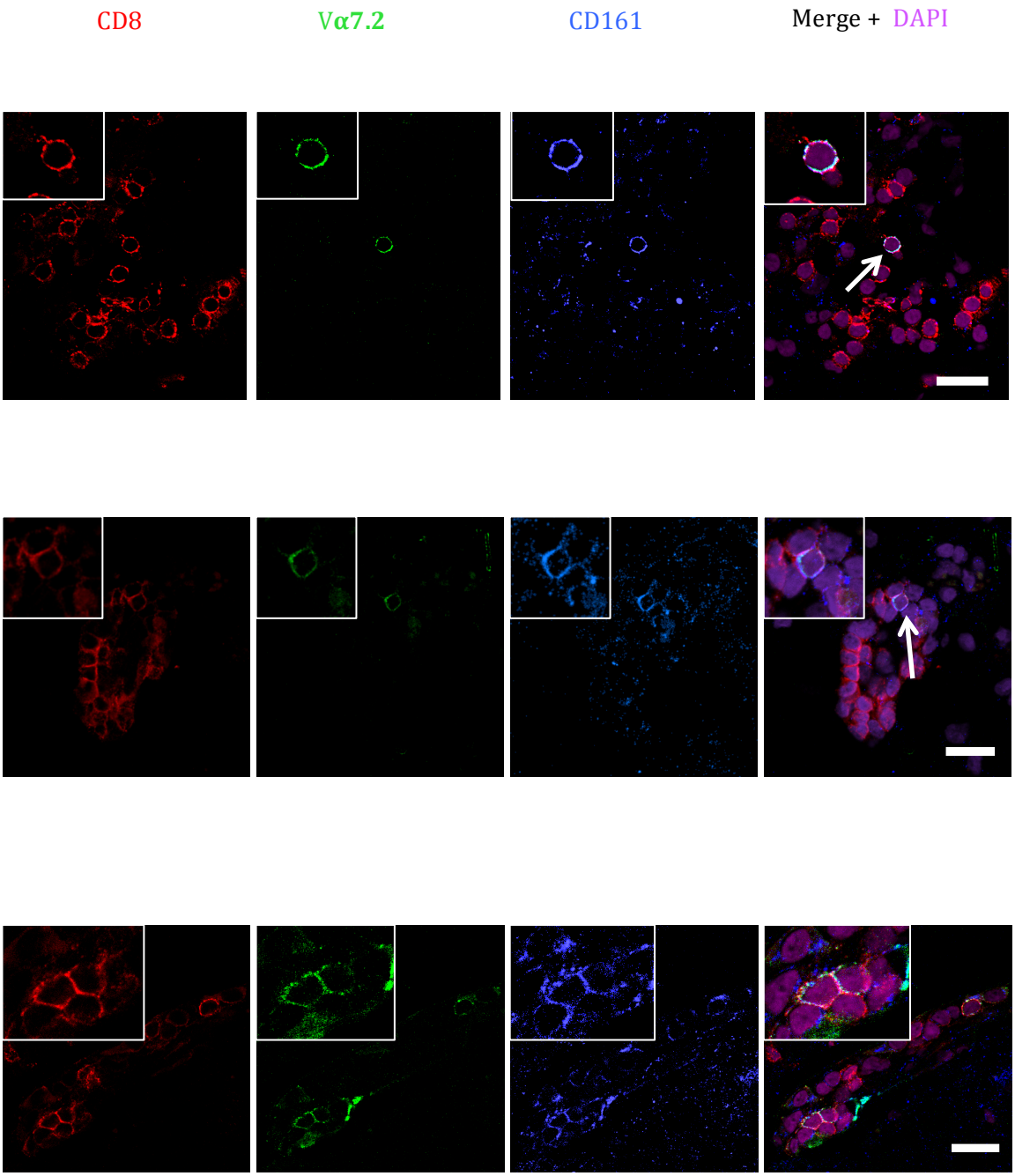


Fig 6.5. MAIT cell infiltrate acute MS lesions. CD8+ MAIT cells, defined by positive staining above background for CD8 (red), Vα7.2, and CD161 (blue) were observed in the vessel cuffs of acute MS lesions (white arrows). All images x63 magnification, scale bar 20μm. Inset images are digital magnifications of triple positive cells. (Willing et al. 2014).

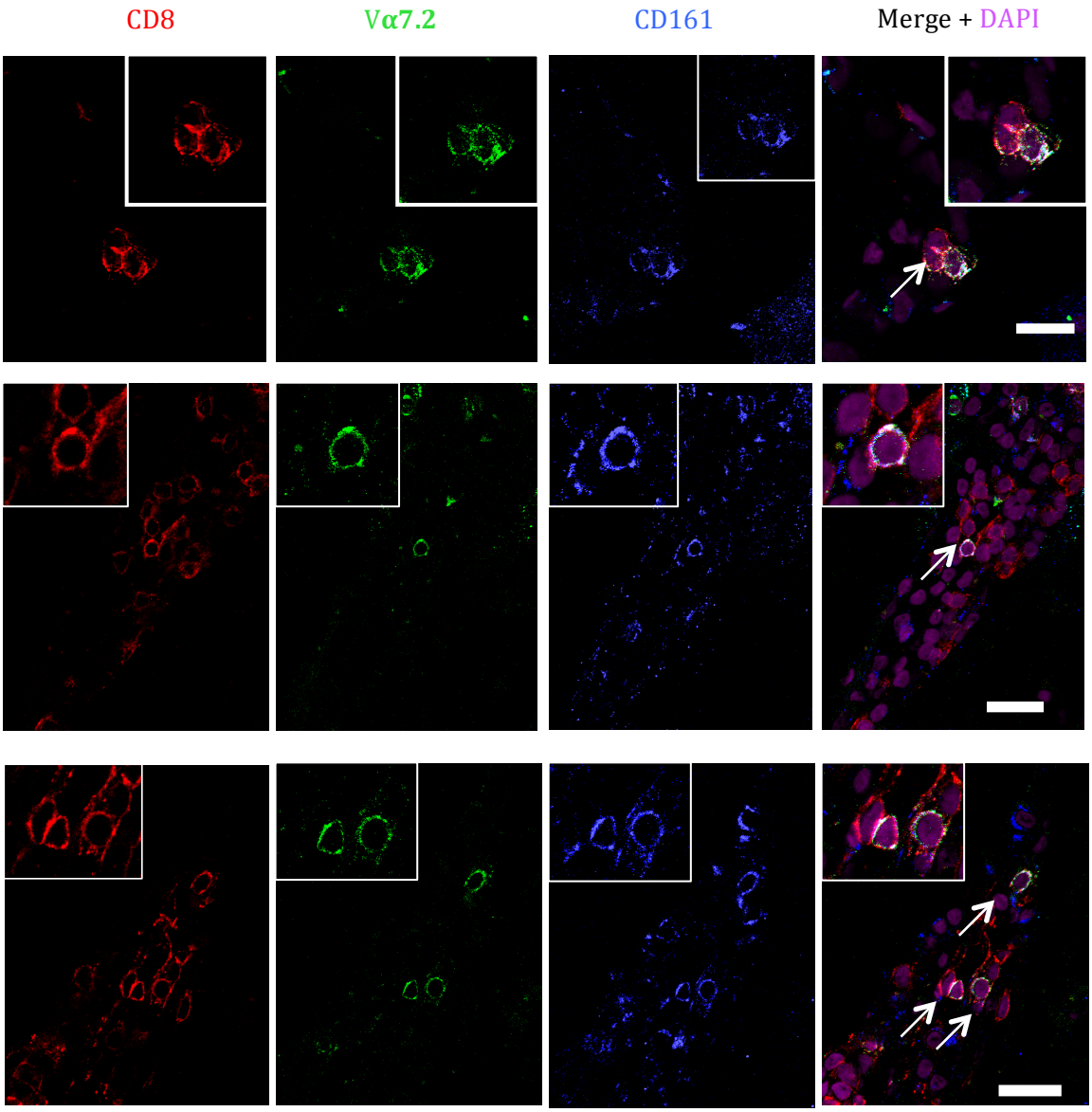
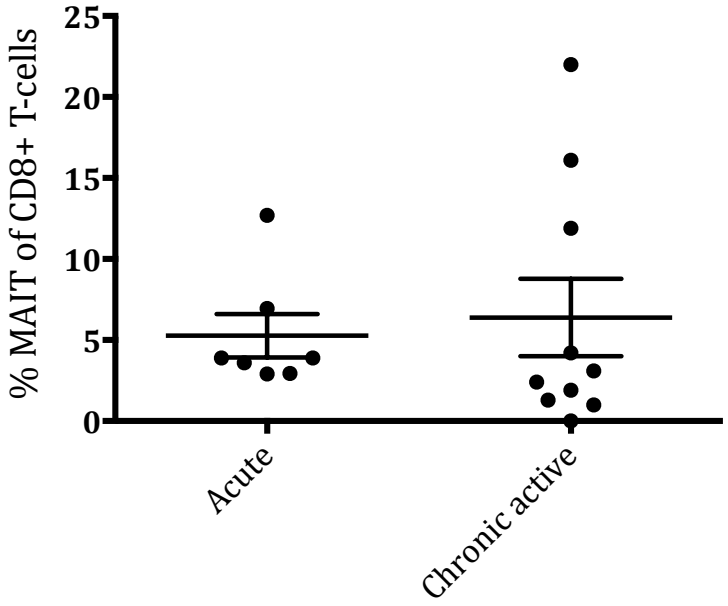


Fig 6.6 MAIT cells infiltrate chronic active MS lesions. CD8+ MAIT cells, defined by positive staining above background for CD8 (red), Vα7.2 (green), and CD161 (blue) were observed in the vessel cuffs of chronic active MS lesions (white arrows). All images x63 magnification, scale bar 20μm. Inset images are digital magnifications of triple positive cells.

A.



B.

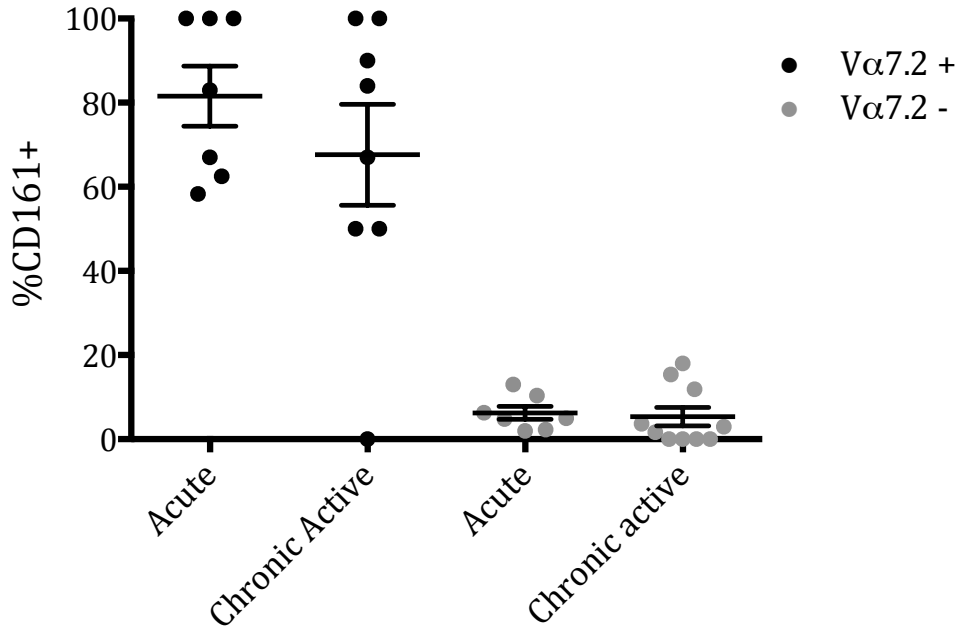


Fig 6.7. MAIT cell infiltrate in MS lesions. A. The proportion of CD8+ T cells that were CD8+MAIT cells, defined as CD8+CD161+Vα7.2+, was analysed in acute and chronic active lesions. B. The CD161+ proportion of CD8+Vα7.2+ and CD8+Vα7.2- cells was measured to assess whether cells expressing Vα7.2 were conventional CD8+ T cells or CD8+MAIT cells. Graphs show mean±SEM. (Willing et al. 2014).

6.2.4 Characterisation of Eomes expression in peripheral blood MAIT cells

The phenotype of MAIT cells led to the hypothesis that they would express high levels of Eomes, and due to their presence in multiple sclerosis lesions they were a candidate population through which a genotype effect on Eomes expression might alter susceptibility to MS.

CD8+ T cells were gated as in Fig 6.8, and MAIT cells in the CD8+ T cell population were identified as Vα7.2+ and CD161++. For comparison, two further populations were identified: the Vα7.2-CD161+ population is an effector memory population which shares many of the features of the MAIT cell transcriptional profile (Fergusson et al. 2014), and a Vα7.2+CD161- population representing conventional CD8+ T cells expressing the Vα7.2 TCR (Fig 6.8). Vα7.2- CD161++ cells were too few in number to provide a reliable comparison population.

MAIT cells were almost uniformly positive for Eomes expression (97.3±0.6%) compared with Vα7.2-CD161+ cells (71.3±2.3%) or Vα7.2+CD161- cells (35.9±2.6%)(Fig 6.9). There was also a difference in normalised median fluorescence (mean 50.48±3.5, 24.11±3.0 and 10.0±2.1 respectively)(Fig 6.9).

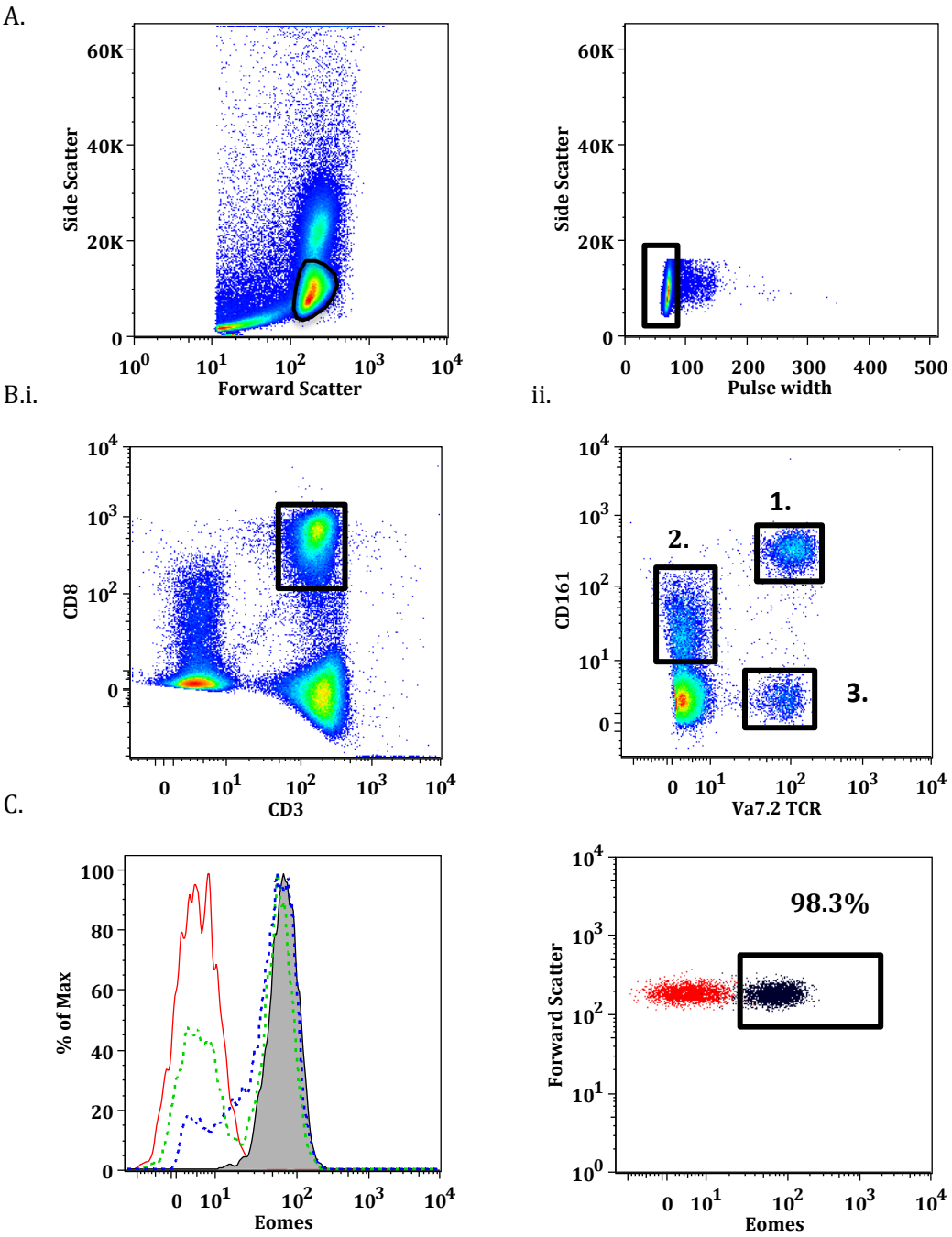
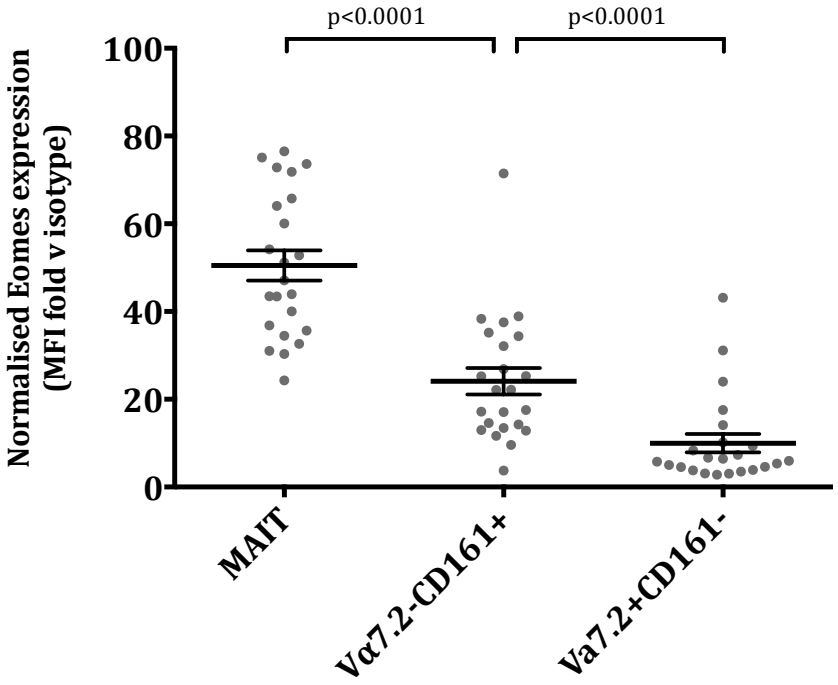


Fig 6.8. Gating of MAIT cells and related populations. A. Initial gating was performed on lymphocytes and singlets. **B.i**. CD8+ T cells were defined by CD3 and CD8 staining. **ii**. The Vα7.2+CD161++ population of MAIT cells (1) was clearly identified. Vα7.2-CD161+ (2) and Vα7.2+CD161- (3) populations were also defined. **C**. Eomes expression in MAIT (solid grey histogram), Vα7.2-CD161+ (blue dashed histogram) and Vα7.2+CD161- (green dashed histogram) cells compared with isotype control (red line). Eomes+ cells were defined as expression above isotype control – gating for MAIT cells is shown.

A.



B.

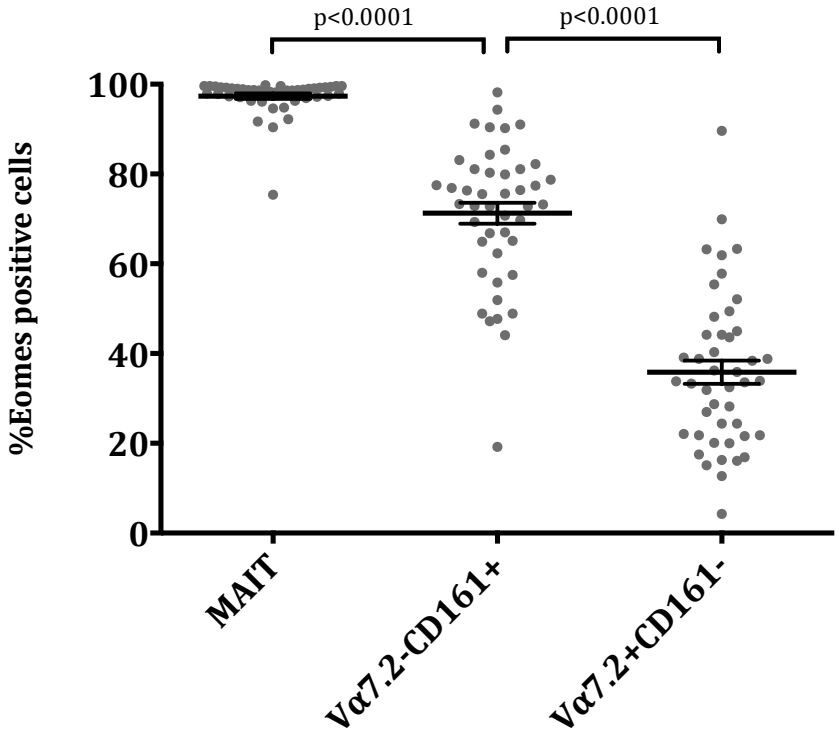


Fig 6.9. Eomes expression in peripheral blood MAIT cells from healthy donors. MAIT cells, CD8+ Vα7.2-CD161+ cells, and CD8+Vα7.2+CD161- cells were gated as shown in Fig. 6.8. Eomes expression was measured by both median fluorescence intensity normalised to isotype control (A) and % Eomes+ cells (B). P-values were calculated by Mann-Whitney test for non-parametric data

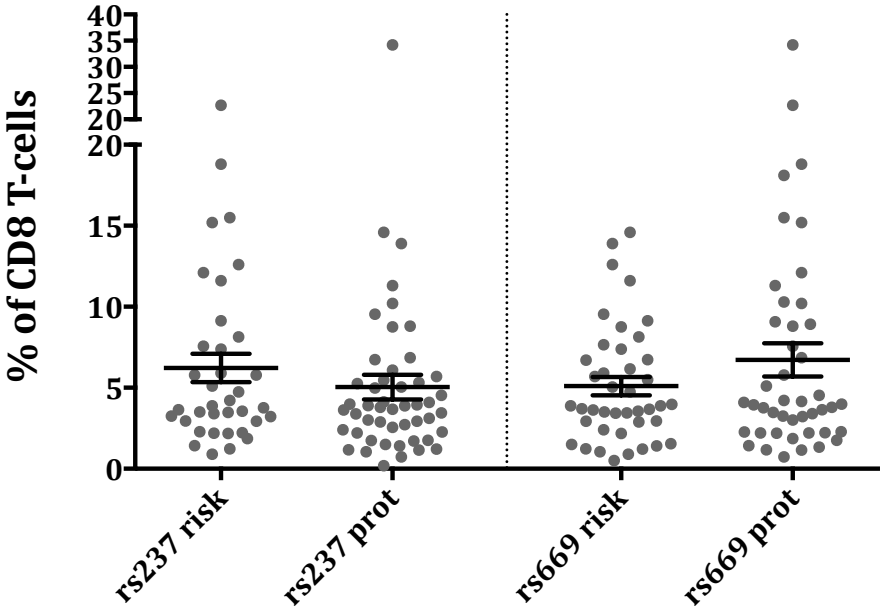
6.2.5 Genotype dependent analysis: MAIT cell abundance and Eomes expression

The uniformly high level of expression of Eomes in the MAIT cell population led to the hypothesis that Eomes was important for the development or maintenance of this subset, as has been described in NK cells (Gordon et al. 2012), and that changes in Eomes expression due to genotype at the MS-associated SNPs rs2371108 and rs669607 might alter the abundance of this subset as a proportion of total CD8+ T cells. We also hypothesised that the level of expression of Eomes in MAIT cells would be altered by genotype.

On analysis of MAIT cell abundance, no difference was observed between donors homozygous for risk or protective alleles at either rs2371108 (6.2±0.9% vs 5.0±0.8%) or rs669607 (5.1±0.6% vs 6.7±1.0%). There was considerable inter-individual variability (range 0.5-34.2% across all donors) which has been noted by other researchers (Walker et al. 2014).

Analysis of normalised Eomes expression in MAIT cells revealed a trend towards higher expression in rs2371108 risk homozygotes that did not reach significance after adjusting for multiple analyses (24.2±1.6 vs 18.9±1.3, unadjusted p-value 0.027) (Fig 6.9). This was consistent with the trend observed in the CD8+ effector memory subset (Fig.5 5).

A.



B.

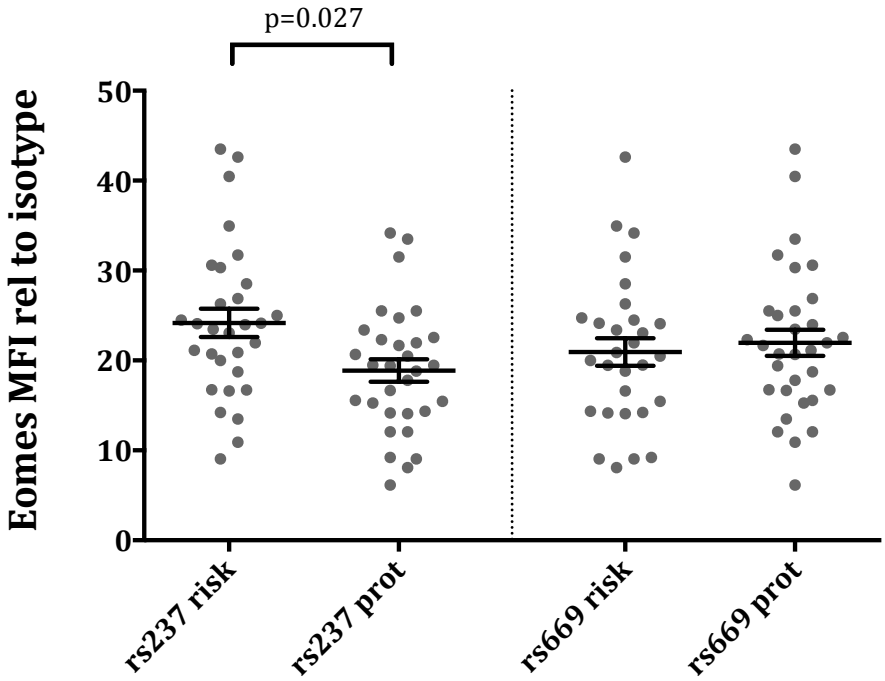


Fig 6.10 MAIT cell abundance and Eomes expression by genotype. **A**. MAIT cell levels as a proportion of total CD8+ T cells was analysed by risk or protective genotype at rs2371108 and rs669607. **B**. Eomes expression, measured as MFI normalised to isotype control, was analysed by genotype at rs2371108 and rs669607. P-values were calculated using the general linear model in SPSS and are uncorrected. Graphs show mean±SEM.

6.3 Discussion

This study demonstrated for the first time that MAIT cells, defined by both Vα7.2 TCR and CD161, are present in both acute and chronic active lesions of multiple sclerosis. This adds to the previous evidence from a PCR-based study that detected the invariant TCR transcript in the brain lesions and CSF from MS patients (Illes et al. 2004), and a small study that showed IFN-γ+CD161+ CD8+ cells in MS lesions (Annibali et al. 2011), further clarifying the phenotype and prevalence of these cells. A study of peripheral MAIT cells in MS patients suggest that MAIT cell abundance in blood is lower in MS patients than healthy controls, regardless of disease status, and that within MS patients MAIT cell numbers are lower in those patients with active disease when compared with those in remission (Miyazaki et al. 2011). As MAIT cells are tissue-homing cells, a possible explanation for this is that MAIT cells are recruited to sites of tissue inflammation.

Here, analysis of the numbers of cells demonstrated that the abundance of these cells is highly variable in the lesional CD8+ population, similar to the variability in MAIT cell number observed in peripheral blood of healthy donors. The overall abundance of MAIT cells as a proportion of CD8+ cells in the lesions was also comparable to that observed in peripheral blood in healthy donors, suggesting that there is no specific recruitment of MAIT cells to the CNS over other CD8+ T cells. However, the sample size was small, and to conclude this for definite would require paired blood and CNS samples from the same individual, which was not practically possible.

Furthermore, all of the patients included in the study had secondary progressive MS. While the lesions were well characterised, and showed features of ongoing active inflammation, there is a possibility that the nature of neuroinflammation is altered as the disease progresses, which may change the constituent cells of the

inflammatory infiltrate. There are no studies available looking at MAIT cells in the peripheral blood of patients with progressive MS and thus no direct comparison can be made in this regard. Post mortem tissue from patients in the early stages of relapsing-remitting disease is rare and difficult to obtain, as very few patients die during this early stage of the disease. Therefore, although MAIT cells were identified, specific recruitment of these cells cannot be confirmed or ruled out on the basis of this study. Ideally the study would have had an additional comparison group where a lymphocytic infiltrate is present, such as viral encephalitis, to investigate any differences in MAIT cell recruitment between CNS disease of different aetiology.

A more pertinent question is what role MAIT cells play in MS lesions, and whether they are activated in the inflammatory milieu even if not specifically recruited to lesions. In health, MAIT cells are likely to play an important role in mucosal immunity as rapid effectors against attack from various pathogens. They have been demonstrated to respond to a wide variety of bacteria including mycobacteria, gram-negative and gram-positive bacilli, and gram-positive cocci, with the ability to lyse bacterially infected cells (Gold et al. 2010, Chua et al. 2012, Le Bourhis et al. 2013, Kurioka et al. 2015). In addition they are able to react to yeast-infected cells, but appear to have no role in the response to viruses (Le Bourhis et al. 2010).

The ligand to which MAIT cells respond in the context of MR1 has been identified as a vitamin B metabolite, most likely derived from metabolic pathways unique to bacteria and yeast (Kjer-Nielsen et al. 2012, Lopez-Sagaseta et al. 2013). Only three such ligands have been identified, but given the previously unknown diversity of MAIT cell TCRs, and the ability of MR1 to present different ligands, it is likely that other MAIT cell activating molecules will be detected (Reantragoon et al. 2012, Gold et al. 2014). MR1 mRNA is ubiquitously expressed, but the protein is notoriously difficult to detect at the cell surface as it is mostly intracellular and only transiently expressed at the cell surface membrane. In the gut mucosa it is expressed by CD138 and CD38

expressing plasma cells, which most likely act as MAIT cell APCs in this tissue (Gozalbo-Lopez et al. 2009).

There is little evidence to suggest a role for yeast or bacterial infection in MS aetiology, but it remains possible that MR1 is expressed by APCs within the CNS that present an as-yet unknown ligand. However, immunostaining for MR1 is challenging and very few commercial antibodies are available. Staining of gut tissue was carried out prior to proposed staining of MS lesions. However, despite multiple attempts including the use of tyramide signal amplification, MR1 staining above background was not demonstrated and this was not taken further.

A further possibility is that MAIT cells undergo "bystander" activation. It has been demonstrated by separate groups that MAIT cells can be activated in a TCR and MR1-independent manner by IL-12 and IL-18, both of which are produced by macrophages and microglia, inducing the expression of both inflammatory cytokines and adhesion molecules in MAIT cells (Aloisi et al. 1997, Das et al. 2008, Miyoshi et al. 2008, Ussher et al. 2014, Willing et al. 2014). As MAIT cells have innate-like properties and are capable of rapidly upregulating effector molecules, it is possible that they play a significant role in the early development of T cell driven inflammation that follows initial infiltration of lesions by activated microglia and macrophages. Evidence of this could be sought by staining intralesional MAIT cells for high expression of IL-18 and IL-12 receptors, as well as looking for further immunohistochemical evidence that these cytokines are produced by macrophages or microglia within MS lesions.

Another intriguing question is whether a subset of MAIT cells plays a regulatory role in MS lesions. In mice, invariant T cells have been proposed to play an immunoregulatory role in EAE, which is exacerbated in MR1 knockout mice that lack MAIT cells. The effect is thought to be mediated by IL-10 (Croxford et al. 2006). CD8αα cells have also been shown to play a role in the regulation of experimental autoimmunity in mice (Tang et al. 2013). In humans, CD8αα cells constitute part of the

MAIT cell/CD161++ subset (Billerbeck et al. 2010), and MAIT cells have been shown to reduce IFN-γ production by T cells; this required cell-cell contact but the molecules involved remain unknown (Miyazaki et al. 2011). It is tempting therefore to posit a regulatory role for a subset of the MAIT cell population. In this project, the CD8 antibody used targeted the CD8α chain and therefore does not differentiate between CD8αα and CD8αβ cells. This could be addressed with further immunohistochemical studies using antibodies to CD8α and CD8β chains as well as Vα7.2. In addition, whether suppression of cytokine production is due to CD8αα or CD8αβ MAIT cells could be addressed by sorting these populations prior to performing suppression assays.

When considering a role for MAIT cells in MS it is pertinent to assess the evidence of their action in other autoimmune diseases. Indeed, in inflammatory bowel disease (IBD) MAIT cells have been demonstrated to increase in number in the inflamed gut, which correlates with reduced circulating numbers. In addition, the recruited cells demonstrate increased activation markers and the secretion of increased pro-inflammatory cytokines on stimulation (Serriari et al. 2014). In rheumatic diseases, reduced circulating numbers of MAIT cells have been described in both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), and in RA this appears to be due to increased recruitment to the inflamed synovial fluid (Cho et al. 2014).

SNPs in regions proximal to genes highly expressed by MAIT cells have been associated with susceptibility to multiple autoimmune diseases. Variants near the gene encoding CCR6, important for migration to tissues, have been associated with autoimmune thyroid disease, inflammatory bowel disease and rheumatoid arthritis (Cooper et al. 2012, Jostins et al. 2012, Okada et al. 2014). Variants in the region of CD161 have been identified with susceptibility to MS in two separate large GWAS (IMSGC et al. 2013, Sawcer et al. 2011). However, both of these markers are also expressed by multiple other cell types, including NK cells, γδ T cells and conventional IL-17 producing T cells.

Given their phenotype as effector cells, it was hypothesised that MAIT cells would express high levels of Eomes. It was demonstrated by flow cytometry that in healthy donors, MAIT cells are almost uniformly positive for Eomes expression consistent with previous studies of innate-like lymphocyte populations, such as γδ-T cells, in humans and mice (Chen et al. 2007, Knox et al. 2014, Prince et al. 2014). MAIT cells are capable of rapidly upregulating effector molecules such as IFN-γ, granzymes and perforin (Kurioka et al 2014) in response to stimulation, all of which are downstream targets of Eomes; therefore Eomes may be integral to the effector function of MAIT cells. Eomes was also expressed by the majority of $CD161^{mid}$ cells.

The importance and uniformly high expression of Eomes in these cells, as well as their potential role in MS, made them good candidates for analysis of genotype dependent changes in Eomes expression. This showed a trend towards higher expression of Eomes in risk homozygotes at rs2371108. Although this did not reach significance after correction for multiple analyses, it was consistent with the trend observed in effector memory CD8+ T cells (Chapter 5), suggesting that higher Eomes expression in effector memory cell populations, including MAIT cells, may correlate with MS susceptibility. As MAIT cells are not known to express other targets of Eomes, such as CD122, in the case of MAIT cells higher Eomes expression could increase disease susceptibility by increasing the transcription of downstream effector molecules mentioned above, particularly IFN-γ, which is present in MS lesions and is known to worsen the course of MS when given exogenously (Panitch et al. 1987, Lock et al. 2002). This would provide a mechanism by which the associated variants exert their effect on disease. The trend was not significant after correcting for multiple analyses; this in part was due to the unforeseen variability in Eomes expression over the course of the study which reduced the power to detect genotype-dependent differences.

A few limitations to this study have been considered. The CD161 antibody used for immunohistochemistry was initially designed for flow cytometry, and needed

to be used at high concentrations to obtain a robust signal when used for CNS tissue staining. Some sensitivity of the staining may therefore have been lost, and due to high background in some sections the threshold may have excluded some CD161+ cells. In addition, CNS tissue contains autofluorescent material as described above; as cells overlapping with regions of autofluorescence were excluded, this led to a reduction in the number of total cells analysed.

Unfortunately many of the studies that would be most interesting in MS are not feasible due to the difficulty of obtaining tissue early in disease. An interesting study would be to assess the presence of MAIT cells in MS lesions early in active disease, and to obtain contemporaneous samples of blood and CSF as well as samples during relapse and remission. This would give a true picture of the involvement of these cells in the disease and indicate any migration between different compartments. An alternative approach would be to assess involvement of these cells in EAE. Adoptive transfer of fluorescently-tagged MAIT cells prior to disease induction, followed by immunohistochemical analysis of CNS lesions, in conjunction with studies of peripheral blood and other tissues, would provide information about the CNS-specific recruitment of these cells. Investigation of the activation status of MAIT cells in MS lesions might further delineate their role. They are likely to produce IFN-γ (Annibali et al. 2011) but the production of other cytokines, particularly IL-17 and IL-10, and cytolytic molecules has not been assessed.

The effect of different MS therapies on MAIT cells is likely to vary greatly. MAIT cells have been shown to respond to IL-18 and TCR stimulation by upregulating α4-integrin, a molecule required for entry into the CNS and blocked by natalizumab, an anti-α4-integrin monoclonal antibody. This drug would therefore be expected to block MAIT cell entry into the CNS. Patients are often switched from natalizumab to fingolimod, a sphingosine-1 receptor modulator that sequesters T cells in secondary lymphoid organs, and is slightly less effective than natalizumab in preventing the

accrual of MS lesions. As MAIT cells do not enter lymph nodes, their levels remain unchanged in the peripheral blood of MS patients treated with fingolimod (Serpero et al. 2013) and it would be interesting to investigate differences in the abundance of these cells between fingolimod responders and non-responders. This may also have relevance for the second generation of sphingosine-phosphate receptor modulators and would allow for prediction of treatment effect; if for instance, patients with high MAIT cell counts were resistant to the immunosuppressive effects of these drugs, response could be predicted and alternative therapies tried in their place.

Both the immunohistochemical studies and the genotype dependent studies could be extended to other innate-like lymphocytes. γδ T cells also express high levels of Eomes (Knox et al. 2014), as well as CCR6 and CD161, and numbers of these cells may be higher in the CSF of MS relapse patients compared to other controls. This cell subset also presents a possible candidate in which to detect a genotype dependent change in expression and may be present in MS lesions.

General conclusion

Multiple sclerosis is a highly complex autoimmune and neurodegenerative disease with an as-yet unknown aetiology, involving complex interplay between genetic predisposition and environmental agents. Outside the HLA region there are a large number of single-nucleotide polymorphisms that have been demonstrated in GWAS to associate with multiple sclerosis, 90% of which lie in non-coding regions; only a small proportion of these variants have been extensively studied, and the mechanisms by which they might predispose to disease have only been uncovered in a few cases (Gregory et al. 2007, Dendrou et al. 2009, Gregory et al. 2012, Hartmann et al. 2014). There are many hurdles to jump when undertaking functional genomic studies. Evidence of the gene affected by the disease-associated variant, or variants, must first be sought. Determining as far as possible the causative variant in a disease association signal is also necessary to inform later functional studies. The cell type and the context in which the variant is relevant must also be determined in order to target functional studies appropriately. This project aimed to characterise the surrounding genetic landscape and the functional effects of two such variants in the Eomes region, both of which were reported in the two largest MS GWAS to date (Sawcer et al. 2011, International Multiple Sclerosis Genetics et al. 2013). This involved several separate strands of work, including computational studies, characterisation of peripheral blood expression, analysis of protein expression in a healthy cohort, and demonstrating the relevance of an Eomes-expressing subset.

Analysis of the genomic region revealed that the two associated variants arise from two blocks of high LD and that within each region there are up to 19 variants in high LD forming the "credible set", each of which might be the causal variant. The primary signal is approximately 300kb upstream of Eomes, and is unique to multiple

sclerosis; the lead SNP in this region is rs1813375. The secondary signal arises from an LD block further downstream and spanning the Eomes locus; the lead SNP in this region was rs5847475, which has not previously been reported. Conditional analysis of rheumatoid arthritis and ankylosing spondylitis-associated variants from the same region determined that although the reported lead SNP is different for each disease, they arise from the same region of LD. Furthermore this effect is concordant in each condition, suggesting that a common mechanism underlies the effect of this region on predisposition to these autoimmune diseases. A recent study suggested that around half of all common autoimmune disease associated loci are shared by more than one disease (Cotsapas et al. 2011). These shared susceptibility variants may provide valuable clues as to common mechanisms of pathogenesis in these common autoimmune diseases, which also often share an obscure underlying aetiology and lack of an identifiable autoantigen.

The complexity of the *EOMES* region is further underlined by correlations with eQTL studies. Both the primary and secondary MS-associated signals overlap with eQTLs, giving further credence to the theory that susceptibility is due to altered regulation of mRNA expression. However, the direction of the eQTL is different in each case; increased expression correlates with the protective allele for the primary signal, but with the risk allele for the secondary signal. While it has been previously described that a variant associated to more than one autoimmune disease may have opposing effects on susceptibility in those diseases (e.g. rs1800693/TNFRS1A in AS and MS), a conflicting effect on expression of the same gene by two variants associated with the same disease has not previously been reported. This finding might be explained by each regulatory region having a role specific to a cell type or context. That the primary association signal is only found in MS among autoimmune diseases suggests a process specific to MS where increased Eomes expression is beneficial rather than detrimental. A possible scenario is one of viral eradication; increased Eomes expression in NK cells

might lead to higher production of effector molecules, leading to more efficient eradication of viruses and a lower risk of chronic latent infection. However, in later life, the increased expression of effector molecules in CD8+ T cells might contribute to an inflammatory milieu causing detrimental activation of autoimmune cells and loss of tolerance.

Determining the precise causal variant in each region is a significant challenge due to haplotype structure. The technical possibility of using capture-C in primary cells was demonstrated here, and showed alignment of the secondary signal with peaks of DNA interaction (Fig 3.8), suggesting that this technique could be used in strategically targeted subsets to uncover interacting regions of DNA with even greater precision. Medications targeting epigenetic mechanisms have been licensed for a range of neoplastic conditions (Hideshima et al. 2011) and it is tempting to envisage the use of increasingly specific epigenetic modulators to disrupt, or repair, DNA interactions in autoimmune disease. However, significant inroads into the precise mechanisms underlying gene regulation must first be made.

During the next few decades, the reduction in cost of whole-genome sequencing (WGS) and the improvement in sequencing technology may allow it to be performed routinely, and we may see GWAS performed on WGS data, precluding the need for imputation and providing more accurate data on the contribution of rare variants to heritability. In conjunction with a growing bank of epigenetic studies in large numbers of people, this may contribute greatly to uncovering the mechanisms of gene regulation and how disruption of these processes is caused by small genetic variants. Functional studies will continue to illustrate the downstream effects of genetic variation and epigenetic disruption, highlighting pathways and possible targets for therapy.

Eomes expression was characterised in a number of peripheral immune subsets. However, despite observing several trends in Eomes expression, we were unable to definitively replicate eQTL findings at the protein level and thereby identify the relevant

cell types through which the disease association occurs. This may be due, in part, to difficulties estimating the sample size required to achieve sufficient power following a pilot study, but is also likely to reflect the highly contextual nature of genetic regulation. As the functional end product of gene expression, protein level is the most relevant parameter when seeking to determine the mechanism by which a SNP might alter disease susceptibility; however, studies suggest that protein levels are inherently more stable than mRNA levels, which may hinder the chances of detecting a protein-QTL outside the specific regulatory context (Battle et al. 2015). This issue also highlights the problem of small pilot studies that may give rise to coincidental results that are not borne out by a larger cohort. Given the ever-increasing number of disease-associated SNPs, and the difficulty inherent in uncovering their downstream effects, it may be some time before a comprehensive map of disease susceptibility pathways can be constructed.

Interestingly, Eomes was most highly and uniformly expressed by cells occupying the territory between the adaptive and innate immune systems, namely NK cells (Fig 4.13) and MAIT cells (Fig 6.9). MAIT cells were detected in both acute and chronic active multiple sclerosis lesions, although it was not possible to discern whether they were specifically recruited and enriched in the lesions. This population has previously been demonstrated to home to inflamed synovium (Cho et al. 2014), and it is tempting to postulate that this Eomes-expressing innate-like population provides the link between Eomes and susceptibility to autoimmune disease. Further analysis of the properties of these cells in synovial fluid, for instance their cytokine profile and surface marker expression, might provide clues as to their role in MS.

There are several possible lines of future work. Firstly, the mechanism of genetic regulation in this case remains to be elucidated; a strategy might be to first use ATAC-Seq (Buenrostro et al. 2013) as this requires small numbers of cells and could be performed in multiple cell types, including innate cell populations for which there is

little available data. Following this, Capture-C could be used to demonstrate a physical interaction between distant DNA regions.

In summary, the work presented here provides some evidence that genotype at the secondary MS association signal, tagged by rs2371108, correlates with Eomes expression, although the differences did not reach statistical significance after correction for multiple analyses. No further evidence was obtained that genotype at the primary association signal, tagged by rs669607, with multiple sclerosis occurs via an effect on expression of Eomes protein. As this association is unique to MS, it may occur due to an effect in neurons, or in a CNS-specific inflammatory context. The study illustrates the difficulty in building upon the findings of GWAS, and the importance of a measured, stepwise approach to determining the underlying mechanisms of the disease association of common variants.

The MS field continues to be hugely exciting for researchers and promising for patients; there are a number of treatments that have been recently licensed, such as fingolimod, dimethyl fumarate, teriflunomide and alemtuzumab, as well as a number of others that have recently shown efficacy in trials, such as ocrelizumab and daclizumab. If the side effects of these drugs can be minimized, and they can be used early in the disease course, the evolution of disability in cohorts of patients on long-term immunomodulatory therapy may help resolve the question of whether MS is an autoimmune or neurodegenerative disease. Their effect in primary progressive disease, which is currently suggestive of benefit (Romme Christensen et al. 2014), may also become clearer.

However, there will no doubt still be a need for neuroprotective treatments and remyelination strategies, and in this respect the continuing development of mesenchymal stem cell therapy and drug repositioning strategies may yield important results. The effects of polymorphisms in neurons are not well known, possibly due to the difficulty accessing primary neurons. The increasing expertise in generating neurons

from induced pluripotent stem cells (IPSCs) may allow neurons to be studied more closely for how genetic variants affect their function and survival, which may be particularly relevant for variants in the EOMES region, as well as other MS-associated genes expressed in neurons such as KIF21B and AHI1.

Perhaps the next big development is the advent of truly personalized medicine. MS is a highly heterogeneous disease both in terms of the pattern of disease and the response to therapy, variation in which remains largely unexplained. A greater understanding of varying response rates and an ability to predict these would enable different treatments to be targeted to those individuals in whom they are likely to be most effective, rather than applying them sequentially, as is the case in MS and other autoimmune diseases such as RA. As was the case for GWAS, study of treatment response needs to be performed on a larger scale in different populations and coordinated by consortia to enable the acquisition, prior to treatment, of samples that could be used for immune profiling, proteome analysis, genome sequencing and epigenetic studies.

Prior to then it will be intriguing to observe changes to MS incidence on a population-wide scale, which may occur as a result of public health measures such as vitamin D supplementation during pregnancy, or the prospective development of a vaccine for EBV.

Bibliography

1000 Genomes Project Consortium, Abecasis, G. R., Altshuler, D., et al. (2010). "A map of human genome variation from population-scale sequencing." Nature **467**(7319): 1061-1073.

1000 Genomes Project Consortium, Abecasis, G. R., Auton, A., et al. (2012). "An integrated map of genetic variation from 1,092 human genomes." Nature **491**(7422): 56-65.

't Hart, B. A., Gran, B. and Weissert, R. (2011). "EAE: imperfect but useful models of multiple sclerosis." Trends Mol Med **17**(3): 119-125.

Abrahamsson, S. V., Angelini, D. F., Dubinsky, A. N., et al. (2013). "Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis." Brain **136**(Pt 9): 2888-2903.

Abrous, D. N., Koehl, M. and Le Moal, M. (2005). "Adult neurogenesis: from precursors to network and physiology." Physiol Rev **85**(2): 523-569.

Agarwal, P., Raghavan, A., Nandiwada, S. L., et al. (2009). "Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory." J Immunol **183**(3): 1695-1704.

Aloisi, F., Penna, G., Cerase, J., et al. (1997). "IL-12 production by central nervous system microglia is inhibited by astrocytes." J Immunol **159**(4): 1604-1612.

Alter, M., Kahana, E., Zilber, N., et al. (2006). "Multiple sclerosis frequency in Israel's diverse populations." Neurology **66**(7): 1061-1066.

Alves, N. L., Hooibrink, B., Arosa, F. A., et al. (2003). "IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro." Blood **102**(7): 2541-2546.

American Association for Cancer Research Human Epigenome Task, F. and European Union, N. o. E. S. A. B. (2008). "Moving AHEAD with an international human epigenome project." Nature **454**(7205): 711-715.

Anderson, A. C., Nicholson, L. B., Legge, K. L., et al. (2000). "High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire." J Exp Med **191**(5): 761-770.

Anderson, C. A., Boucher, G., Lees, C. W., et al. (2011). "Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47." Nat Genet **43**(3): 246-252.

Annibali, V., Ristori, G., Angelini, D. F., et al. (2011). "CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis." Brain **134**(Pt 2): 542-554.

Appay, V., Zaunders, J. J., Papagno, L., et al. (2002). "Characterization of CD4(+) CTLs ex vivo." J Immunol **168**(11): 5954-5958.

Arnold, S. J., Huang, G. J., Cheung, A. F., et al. (2008). "The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone." Genes Dev **22**(18): 2479-2484.

Arnold, S. J., Sugnaseelan, J., Groszer, M., et al. (2009). "Generation and analysis of a mouse line harboring GFP in the Eomes/Tbr2 locus." Genesis **47**(11): 775-781.

Arsenio, J., Kakaradov, B., Metz, P. J., et al. (2014). "Early specification of CD8(+) T lymphocyte fates during adaptive immunity revealed by single-cell gene-expression analyses." Nat Immunol **15**(4): 365-372.

Arun, T., Tomassini, V., Sbardella, E., et al. (2013). "Targeting ASIC1 in primary progressive multiple sclerosis: evidence of neuroprotection with amiloride." Brain **136**(Pt 1): 106-115.

Ascherio, A. and Munger, K. L. (2007). "Environmental risk factors for multiple sclerosis. Part I: the role of infection." Ann Neurol **61**(4): 288-299.

Ascherio, A., Munger, K. L., White, R., et al. (2014). "Vitamin D as an Early Predictor of Multiple Sclerosis Activity and Progression." JAMA Neurol **71**(3): 306-314.

Astier, A. L., Meiffren, G., Freeman, S., et al. (2006). "Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis." J Clin Invest **116**(12): 3252-3257.

Atherly, L. O., Lucas, J. A., Felices, M., et al. (2006). "The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells." Immunity **25**(1): 79-91.

Attfield, K. E., Dendrou, C. A. and Fugger, L. (2012). "Bridging the gap from genetic association to functional understanding: the next generation of mouse models of multiple sclerosis." Immunol Rev **248**(1): 10-22.

Baala, L., Briault, S., Etchevers, H. C., et al. (2007). "Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis." Nat Genet **39**(4): 454-456.

Babbe, H., Roers, A., Waisman, A., et al. (2000). "Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction." J Exp Med **192**(3): 393-404.

Balashov, K. E., Rottman, J. B., Weiner, H. L., et al. (1999). "CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions." Proc Natl Acad Sci U S A **96**(12): 6873-6878.

Banchereau, J. and Steinman, R. M. (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.

Banerjee, A., Gordon, S. M., Intlekofer, A. M., et al. (2010). "Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche." J Immunol **185**(9): 4988-4992.

Bar-Or, A., Calabresi, P. A., Arnold, D., et al. (2008). "Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial." Ann Neurol **63**(3): 395-400.

Baranzini, S. E., Wang, J., Gibson, R. A., et al. (2009). "Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis." Hum Mol Genet **18**(4): 767-778.

Barnett, M. H. and Prineas, J. W. (2004). "Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion." Ann Neurol **55**(4): 458-468.

Barsh, G. S., Copenhaver, G. P., Gibson, G., et al. (2012). "Guidelines for genome-wide association studies." PLoS Genet **8**(7): e1002812.

Battle, A., Khan, Z., Wang, S. H., et al. (2015). "Genomic variation. Impact of regulatory variation from RNA to protein." Science **347**(6222): 664-667.

Battle, A., Mostafavi, S., Zhu, X., et al. (2014). "Characterizing the genetic basis of transcriptome diversity through RNA-sequencing of 922 individuals." Genome Res **24**(1): 14-24.

Benvenuto, R., Paroli, M., Buttinelli, C., et al. (1992). "Tumor necrosis factor-alpha and interferon-gamma synthesis by cerebrospinal fluid-derived T cell clones in multiple sclerosis." Ann N Y Acad Sci **650**: 341-346.

Berg, L. J. (2007). "Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development." Nat Rev Immunol **7**(6): 479-485.

Berg-Hansen, P., Moen, S. M., Sandvik, L., et al. (2015). "Prevalence of multiple sclerosis among immigrants in Norway." Mult Scler **21**(6): 695-702.

Berger, S. L. (2007). "The complex language of chromatin regulation during transcription." Nature **447**(7143): 407-412.

Berthelot, L., Laplaud, D. A., Pettre, S., et al. (2008). "Blood CD8+ T cell responses against myelin determinants in multiple sclerosis and healthy individuals." Eur J Immunol **38**(7): 1889-1899.

Bertrams, H. J. and Kuwert, E. K. (1976). "Association of histocompatibility haplotype HLA-A3-B7 with multiple sclerosis." J Immunol **117**(5 Pt.2): 1906-1912.

Berzins, S. P., Smyth, M. J. and Baxter, A. G. (2011). "Presumed guilty: natural killer T cell defects and human disease." Nat Rev Immunol **11**(2): 131-142.

Bhargava, P., Cassard, S., Steele, S. U., et al. (2014). "The vitamin D to ameliorate multiple sclerosis (VIDAMS) trial: study design for a multicenter, randomized, double-blind controlled trial of vitamin D in multiple sclerosis." Contemporary clinical trials **39**(2): 288-293.

Bielekova, B., Catalfamo, M., Reichert-Scrivner, S., et al. (2006). "Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis." Proc Natl Acad Sci U S A **103**(15): 5941-5946.

Bielekova, B., Richert, N., Herman, M. L., et al. (2011). "Intrathecal effects of daclizumab treatment of multiple sclerosis." Neurology **77**(21): 1877-1886.

Billerbeck, E., Kang, Y. H., Walker, L., et al. (2010). "Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties." Proc Natl Acad Sci U S A **107**(7): 3006-3011.

Billiau, A., Heremans, H., Vandekerckhove, F., et al. (1988). "Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma." J Immunol **140**(5): 1506-1510.

Bjorkstrom, N. K., Gonzalez, V. D., Malmberg, K. J., et al. (2008). "Elevated numbers of Fc gamma RIIIA+ (CD16+) effector CD8 T cells with NK cell-like function in chronic hepatitis C virus infection." J Immunol **181**(6): 4219-4228.

Bo, L., Mork, S., Kong, P. A., et al. (1994). "Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions." J Neuroimmunol **51**(2): 135-146.

Booss, J., Esiri, M. M., Tourtellotte, W. W., et al. (1983). "Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis." J Neurol Sci **62**(1-3): 219-232.

Bos, S. D., Page, C. M., Andreassen, B. K., et al. (2015). "Genome-wide DNA methylation profiles indicate CD8+ T cell hypermethylation in multiple sclerosis." PLoS One **10**(3): e0117403.

Bradfield, J. P., Qu, H. Q., Wang, K., et al. (2011). "A genome-wide meta-analysis of six type 1 diabetes cohorts identifies multiple associated loci." PLoS Genet **7**(9): e1002293.

Breij, E. C., Brink, B. P., Veerhuis, R., et al. (2008). "Homogeneity of active demyelinating lesions in established multiple sclerosis." Ann Neurol **63**(1): 16-25.

Brodin, P., Jojic, V., Gao, T., et al. (2015). "Variation in the human immune system is largely driven by non-heritable influences." Cell **160**(1-2): 37-47.

Bronnum-Hansen, H., Koch-Henriksen, N. and Hyllested, K. (1994). "Survival of patients with multiple sclerosis in Denmark: a nationwide, long-term epidemiologic survey." Neurology **44**(10): 1901-1907.

Bruck, W., Lucchinetti, C. and Lassmann, H. (2002). "The pathology of primary progressive multiple sclerosis." Mult Scler **8**(2): 93-97.

Brucklacher-Waldert, V., Stuerner, K., Kolster, M., et al. (2009). "Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis." Brain **132**(Pt 12): 3329-3341.

Bruno, R., Sabater, L., Sospedra, M., et al. (2002). "Multiple sclerosis candidate autoantigens except myelin oligodendrocyte glycoprotein are transcribed in human thymus." Eur J Immunol **32**(10): 2737-2747.

Brynedal, B., Duvefelt, K., Jonasdottir, G., et al. (2007). "HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis." PLoS One **2**(7): e664.

Buenrostro, J. D., Giresi, P. G., Zaba, L. C., et al. (2013). "Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position." Nature methods **10**(12): 1213-1218.

Buggert, M., Tauriainen, J., Yamamoto, T., et al. (2014). "T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection." PLoS Pathog **10**(7): e1004251.

Bulfone, A., Martinez, S., Marigo, V., et al. (1999). "Expression pattern of the Tbr2 (Eomesodermin) gene during mouse and chick brain development." Mech Dev **84**(1-2): 133-138.

Butzkueven, H., Kappos, L., Pellegrini, F., et al. (2014). "Efficacy and safety of natalizumab in multiple sclerosis: interim observational programme results." J Neurol Neurosurg Psychiatry **85**(11): 1190-1197.

Byun, H. M., Siegmund, K. D., Pan, F., et al. (2009). "Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns." Hum Mol Genet **18**(24): 4808-4817.

Calabrese, M., Filippi, M. and Gallo, P. (2010). "Cortical lesions in multiple sclerosis." Nat Rev Neurol **6**(8): 438-444.

Calabresi, P. A., Radue, E. W., Goodin, D., et al. (2014). "Safety and efficacy of fingolimod in patients with relapsing-remitting multiple sclerosis (FREEDOMS II): a double-blind, randomised, placebo-controlled, phase 3 trial." Lancet Neurol **13**(6): 545-556.

Caliskan, M., Cusanovich, D. A., Ober, C., et al. (2011). "The effects of EBV transformation on gene expression levels and methylation profiles." Hum Mol Genet **20**(8): 1643-1652.

Campbell, J. J., Qin, S., Unutmaz, D., et al. (2001). "Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire." J Immunol **166**(11): 6477-6482.

Carson, W. E., Giri, J. G., Lindemann, M. J., et al. (1994). "Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor." J Exp Med **180**(4): 1395-1403.

Carter, K. L., Cahir-McFarland, E. and Kieff, E. (2002). "Epstein-barr virus-induced changes in B-lymphocyte gene expression." J Virol **76**(20): 10427-10436.

Carter, L. L., Leach, M. W., Azoitei, M. L., et al. (2007). "PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis." J Neuroimmunol **182**(1-2): 124-134.

Carty, S. A., Koretzky, G. A. and Jordan, M. S. (2014). "Interleukin-4 regulates eomesodermin in CD8+ T cell development and differentiation." PLoS One **9**(9): e106659.

Castro, I., Yu, A., Dee, M. J., et al. (2011). "The basis of distinctive IL-2- and IL-15-dependent signaling: weak CD122-dependent signaling favors CD8+ T central-memory cell survival but not T effector-memory cell development." J Immunol **187**(10): 5170-5182.

Chae, D. W., Nosaka, Y., Strom, T. B., et al. (1996). "Distribution of IL-15 receptor alpha-chains on human peripheral blood mononuclear cells and effect of immunosuppressive drugs on receptor expression." J Immunol **157**(7): 2813-2819.

Chan, A., Hong, D. L., Atzberger, A., et al. (2007). "CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts." J Immunol **179**(1): 89-94.

Chang, J. T., Palanivel, V. R., Kinjyo, I., et al. (2007). "Asymmetric T lymphocyte division in the initiation of adaptive immune responses." Science **315**(5819): 1687-1691.

Chen, G. and Goeddel, D. V. (2002). "TNF-R1 signaling: a beautiful pathway." Science **296**(5573): 1634-1635.

Chen, L., He, W., Kim, S. T., et al. (2007). "Epigenetic and transcriptional programs lead to default IFN-gamma production by gammadelta T cells." J Immunol **178**(5): 2730-2736.

Cho, J. H., Kim, H. O., Kim, K. S., et al. (2013). "Unique features of naive CD8+ T cell activation by IL-2." J Immunol **191**(11): 5559-5573.

Cho, Y. N., Kee, S. J., Kim, T. J., et al. (2014). "Mucosal-associated invariant T cell deficiency in systemic lupus erythematosus." J Immunol **193**(8): 3891-3901.

Choi, J. W., Gardell, S. E., Herr, D. R., et al. (2011). "FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation." Proc Natl Acad Sci U S A **108**(2): 751-756.

Choi, Y., Sims, G. E., Murphy, S., et al. (2012). "Predicting the functional effect of amino acid substitutions and indels." PLoS One **7**(10): e46688.

Chua, W. J., Truscott, S. M., Eickhoff, C. S., et al. (2012). "Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection." Infect Immun **80**(9): 3256-3267.

Clemenceau, B., Vivien, R., Berthome, M., et al. (2008). "Effector memory alphabeta T lymphocytes can express FcgammaRIIIa and mediate antibody-dependent cellular cytotoxicity." J Immunol **180**(8): 5327-5334.

Cohen, J. A., Coles, A. J., Arnold, D. L., et al. (2012). "Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial." Lancet **380**(9856): 1819-1828.

Coles, A. (2009). "Multiple sclerosis." Pract Neurol **9**(2): 118-126.

Coles, A. J., Cox, A., Le Page, E., et al. (2006). "The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy." J Neurol **253**(1): 98-108.

Coles, A. J., Twyman, C. L., Arnold, D. L., et al. (2012). "Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial." Lancet **380**(9856): 1829-1839.

Colombo, M., Dono, M., Gazzola, P., et al. (2000). "Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients." J Immunol **164**(5): 2782-2789.

Compston, A. (2005). McAlpine's multiple sclerosis. Philadelphia, Churchill Livingstone Elsevier.

Confavreux, C., O'Connor, P., Comi, G., et al. (2014). "Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial." Lancet Neurol **13**(3): 247-256.

Confavreux, C. and Vukusic, S. (2006). "Age at disability milestones in multiple sclerosis." Brain **129**(Pt 3): 595-605.

Confavreux, C., Vukusic, S. and Adeleine, P. (2003). "Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process." Brain **126**(Pt 4): 770-782.

Confavreux, C., Vukusic, S., Moreau, T., et al. (2000). "Relapses and progression of disability in multiple sclerosis." N Engl J Med **343**(20): 1430-1438.

Cooper, J. D., Simmonds, M. J., Walker, N. M., et al. (2012). "Seven newly identified loci for autoimmune thyroid disease." Hum Mol Genet **21**(23): 5202-5208.

Cooper, M. A., Colonna, M. and Yokoyama, W. M. (2009). "Hidden talents of natural killers: NK cells in innate and adaptive immunity." EMBO reports **10**(10): 1103-1110.

Cooper, M. A., Elliott, J. M., Keyel, P. A., et al. (2009). "Cytokine-induced memory-like natural killer cells." Proc Natl Acad Sci U S A **106**(6): 1915-1919.

Cooper, M. A., Fehniger, T. A. and Caligiuri, M. A. (2001). "The biology of human natural killer-cell subsets." Trends Immunol **22**(11): 633-640.

Cooper, M. A., Fehniger, T. A., Turner, S. C., et al. (2001). "Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset." Blood **97**(10): 3146-3151.

Cornish, G. H., Sinclair, L. V. and Cantrell, D. A. (2006). "Differential regulation of T-cell growth by IL-2 and IL-15." Blood **108**(2): 600-608.

Correale, J. and Villa, A. (2010). "Role of CD8+ CD25+ Foxp3+ regulatory T cells in multiple sclerosis." Ann Neurol **67**(5): 625-638.

Correale, J., Ysrraelit, M. C. and Gaitan, M. I. (2009). "Immunomodulatory effects of Vitamin D in multiple sclerosis." Brain **132**(Pt 5): 1146-1160.

Cortes, A. and Brown, M. A. (2011). "Promise and pitfalls of the Immunochip." Arthritis Res Ther **13**(1): 101.

Cotsapas, C., Voight, B. F., Rossin, E., et al. (2011). "Pervasive sharing of genetic effects in autoimmune disease." PLoS Genet **7**(8): e1002254.

Couturier, N., Bucciarelli, F., Nurtdinov, R. N., et al. (2011). "Tyrosine kinase 2 variant influences T lymphocyte polarization and multiple sclerosis susceptibility." Brain **134**(Pt 3): 693-703.

Croxford, J. L., Miyake, S., Huang, Y. Y., et al. (2006). "Invariant V(alpha)19i T cells regulate autoimmune inflammation." Nat Immunol **7**(9): 987-994.

Cruz-Guilloty, F., Pipkin, M. E., Djuretic, I. M., et al. (2009). "Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs." J Exp Med **206**(1): 51-59.

Cui, W., Liu, Y., Weinstein, J. S., et al. (2011). "An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells." Immunity **35**(5): 792-805.

Curtsinger, J. M., Agarwal, P., Lins, D. C., et al. (2012). "Autocrine IFN-gamma promotes naive CD8 T cell differentiation and synergizes with IFN-alpha to stimulate strong function." J Immunol **189**(2): 659-668.

Dai, H., Wan, N., Zhang, S., et al. (2010). "Cutting edge: programmed death-1 defines CD8+CD122+ T cells as regulatory versus memory T cells." J Immunol **185**(2): 803-807.

Dalbeth, N. and Callan, M. F. (2002). "A subset of natural killer cells is greatly expanded within inflamed joints." Arthritis Rheum **46**(7): 1763-1772.

Das, S., Mishra, M. K., Ghosh, J., et al. (2008). "Japanese Encephalitis Virus infection induces IL-18 and IL-1beta in microglia and astrocytes: correlation with in vitro cytokine responsiveness of glial cells and subsequent neuronal death." J Neuroimmunol **195**(1-2): 60-72.

Daussy, C., Faure, F., Mayol, K., et al. (2014). "T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow." J Exp Med **211**(3): 563-577.

De Jager, P. L., Baecher-Allan, C., Maier, L. M., et al. (2009). "The role of the CD58 locus in multiple sclerosis." Proc Natl Acad Sci U S A **106**(13): 5264-5269.

De Jager, P. L., Jia, X., Wang, J., et al. (2009). "Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci." Nat Genet **41**(7): 776-782.

Delaneau, O., Marchini, J., Genomes Project, C., et al. (2014). "Integrating sequence and array data to create an improved 1000 Genomes Project haplotype reference panel." Nat Commun **5**: 3934.

DeLuca, G. C., Kimball, S. M., Kolasinski, J., et al. (2013). "Review: the role of vitamin D in nervous system health and disease." Neuropathol Appl Neurobiol **39**(5): 458-484.

DeLuca, G. C., Williams, K., Evangelou, N., et al. (2006). "The contribution of demyelination to axonal loss in multiple sclerosis." Brain **129**(Pt 6): 1507-1516.

Dendrou, C. A., Plagnol, V., Fung, E., et al. (2009). "Cell-specific protein phenotypes for the autoimmune locus IL2RA using a genotype-selectable human bioresource." Nat Genet **41**(9): 1011-1015.

Deng, W., Aimone, J. B. and Gage, F. H. (2010). "New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?" Nat Rev Neurosci **11**(5): 339-350.

Deng, W., Rupon, J. W., Krivega, I., et al. (2014). "Reactivation of developmentally silenced globin genes by forced chromatin looping." Cell **158**(4): 849-860.

Dhib-Jalbut, S. (2003). "Glatiramer acetate (Copaxone) therapy for multiple sclerosis." Pharmacology & therapeutics **98**(2): 245-255.

Dhib-Jalbut, S. and Marks, S. (2010). "Interferon-beta mechanisms of action in multiple sclerosis." Neurology **74 Suppl 1**: S17-24.

Diogo, D., Bastarache, L., Liao, K. P., et al. (2015). "TYK2 Protein-Coding Variants Protect against Rheumatoid Arthritis and Autoimmunity, with No Evidence of Major Pleiotropic Effects on Non-Autoimmune Complex Traits." PLoS One **10**(4): e0122271.

Disanto, G., Kjetil Sandve, G., Ricigliano, V. A., et al. (2014). "DNase hypersensitive sites and association with multiple sclerosis." Hum Mol Genet **23**(4): 942-948.

Disanto, G., Morahan, J. M., Barnett, M. H., et al. (2012). "The evidence for a role of B cells in multiple sclerosis." Neurology **78**(11): 823-832.

Disanto, G., Pakpoor, J., Morahan, J. M., et al. (2013). "Epstein-Barr virus, latitude and multiple sclerosis." Mult Scler **19**(3): 362-365.

Disanto, G., Sandve, G. K., Berlanga-Taylor, A. J., et al. (2012). "Genomic regions associated with multiple sclerosis are active in B cells." PLoS One **7**(3): e32281.

Dobson, R., Giovannoni, G. and Ramagopalan, S. (2013). "The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude." J Neurol Neurosurg Psychiatry **84**(4): 427-432.

Doering, T. A., Crawford, A., Angelosanto, J. M., et al. (2012). "Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory." Immunity **37**(6): 1130-1144.

Dogan, R. I., Getoor, L., Wilbur, W. J., et al. (2007). "SplicePort--an interactive splice-site analysis tool." Nucleic Acids Res **35**(Web Server issue): W285-291.

Dominguez-Villar, M., Baecher-Allan, C. M. and Hafler, D. A. (2011). "Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease." Nat Med **17**(6): 673-675.

Duhen, T., Duhen, R., Lanzavecchia, A., et al. (2012). "Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells." Blood **119**(19): 4430-4440.

Durrenberger, P. F., Ettorre, A., Kamel, F., et al. (2012). "Innate immunity in multiple sclerosis white matter lesions: expression of natural cytotoxicity triggering receptor 1 (NCR1)." J Neuroinflammation **9**(1): 1.

Dusseaux, M., Martin, E., Serriari, N., et al. (2011). "Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells." Blood **117**(4): 1250-1259.

Ebers, G. C., Bulman, D. E., Sadovnick, A. D., et al. (1986). "A population-based study of multiple sclerosis in twins." N Engl J Med **315**(26): 1638-1642.

Edwards, E. S., Smith, C. and Khanna, R. (2014). "Phenotypic and transcriptional profile correlates with functional plasticity of antigen-specific CD4+ T cells." Immunol Cell Biol **92**(2): 181-190.

Eikelenboom, M. J., Killestein, J., Izeboud, T., et al. (2002). "Chemokine receptor expression on T cells is related to new lesion development in multiple sclerosis." J Neuroimmunol **133**(1-2): 225-232.

Endharti, A. T., Okuno, Y., Shi, Z., et al. (2011). "CD8+CD122+ regulatory T cells (Tregs) and CD4+ Tregs cooperatively prevent and cure CD4+ cell-induced colitis." J Immunol **186**(1): 41-52.

Endharti, A. T., Rifa, I. M., Shi, Z., et al. (2005). "Cutting edge: CD8+CD122+ regulatory T cells produce IL-10 to suppress IFN-gamma production and proliferation of CD8+ T cells." J Immunol **175**(11): 7093-7097.

Endo, Y., Iwamura, C., Kuwahara, M., et al. (2011). "Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3." Immunity **35**(5): 733-745.

Englund, C., Fink, A., Lau, C., et al. (2005). "Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex." J Neurosci **25**(1): 247-251.

Ernst, A., Alkass, K., Bernard, S., et al. (2014). "Neurogenesis in the striatum of the adult human brain." Cell **156**(5): 1072-1083.

Ernst, J., Kheradpour, P., Mikkelsen, T. S., et al. (2011). "Mapping and analysis of chromatin state dynamics in nine human cell types." Nature **473**(7345): 43-49.

Eshima, K., Chiba, S., Suzuki, H., et al. (2012). "Ectopic expression of a T-box transcription factor, eomesodermin, renders CD4(+) Th cells cytotoxic by activating both perforin- and FasL-pathways." Immunol Lett **144**(1-2): 7-15.

Esteller, M. (2008). "Epigenetics in cancer." N Engl J Med **358**(11): 1148-1159.

Fairfax, B. P., Humburg, P., Makino, S., et al. (2014). "Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression." Science **343**(6175): 1246949.

Farez, M. F., Fiol, M. P., Gaitan, M. I., et al. (2015). "Sodium intake is associated with increased disease activity in multiple sclerosis." J Neurol Neurosurg Psychiatry **86**(1): 26-31.

Farh, K. K., Marson, A., Zhu, J., et al. (2015). "Genetic and epigenetic fine mapping of causal autoimmune disease variants." Nature **518**(7539): 337-343.

Fehniger, T. A., Cooper, M. A., Nuovo, G. J., et al. (2003). "CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity." Blood **101**(8): 3052-3057.

Ferber, I. A., Brocke, S., Taylor-Edwards, C., et al. (1996). "Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE)." J Immunol **156**(1): 5-7.

Ferguson, B., Matyszak, M. K., Esiri, M. M., et al. (1997). "Axonal damage in acute multiple sclerosis lesions." Brain **120 (Pt 3)**: 393-399.

Fergusson, J. R., Fleming, V. M. and Klenerman, P. (2011). "CD161-expressing human T cells." Front Immunol **2**: 36.

Fergusson, J. R., Smith, K. E., Fleming, V. M., et al. (2014). "CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages." Cell Rep **9**(3): 1075-1088.

Fiddes, B., Wason, J., Kemppinen, A., et al. (2013). "Confounding underlies the apparent month of birth effect in multiple sclerosis." Ann Neurol **73**(6): 714-720.

Filippi, M., Rocca, M. A., Barkhof, F., et al. (2012). "Association between pathological and MRI findings in multiple sclerosis." Lancet Neurol **11**(4): 349-360.

Fox, R. J., Miller, D. H., Phillips, J. T., et al. (2012). "Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis." N Engl J Med **367**(12): 1087-1097.

Franke, A., McGovern, D. P., Barrett, J. C., et al. (2010). "Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci." Nat Genet **42**(12): 1118-1125.

Friese, M. A., Craner, M. J., Etzensperger, R., et al. (2007). "Acid-sensing ion channel-1 contributes to axonal degeneration in autoimmune inflammation of the central nervous system." Nat Med **13**(12): 1483-1489.

Friese, M. A., Jakobsen, K. B., Friis, L., et al. (2008). "Opposing effects of HLA class I molecules in tuning autoreactive CD8+ T cells in multiple sclerosis." Nat Med **14**(11): 1227-1235.

Frischer, J. M., Bramow, S., Dal-Bianco, A., et al. (2009). "The relation between inflammation and neurodegeneration in multiple sclerosis brains." Brain **132**(Pt 5): 1175-1189.

Gallegos, A. M. and Bevan, M. J. (2004). "Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation." J Exp Med **200**(8): 1039-1049.

Ganguly, D., Haak, S., Sisirak, V., et al. (2013). "The role of dendritic cells in autoimmunity." Nat Rev Immunol **13**(8): 566-577.

Gattinoni, L., Lugli, E., Ji, Y., et al. (2011). "A human memory T cell subset with stem cell-like properties." Nat Med **17**(10): 1290-1297.

Gill, S., Vasey, A. E., De Souza, A., et al. (2012). "Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells." Blood **119**(24): 5758-5768.

Giovannoni, G., Gold, R., Selmaj, K., et al. (2014). "Daclizumab high-yield process in relapsing-remitting multiple sclerosis (SELECTION): a multicentre, randomised, double-blind extension trial." Lancet Neurol **13**(5): 472-481.

Gold, M. C., Cerri, S., Smyk-Pearson, S., et al. (2010). "Human mucosal associated invariant T cells detect bacterially infected cells." PLoS Biol **8**(6): e1000407.

Gold, M. C., Eid, T., Smyk-Pearson, S., et al. (2013). "Human thymic MR1-restricted MAIT cells are innate pathogen-reactive effectors that adapt following thymic egress." Mucosal Immunol **6**(1): 35-44.

Gold, M. C., McLaren, J. E., Reistetter, J. A., et al. (2014). "MR1-restricted MAIT cells display ligand discrimination and pathogen selectivity through distinct T cell receptor usage." J Exp Med **211**(8): 1601-1610.

Gold, R., Kappos, L., Arnold, D. L., et al. (2012). "Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis." N Engl J Med **367**(12): 1098-1107.

Gordon, S. M., Carty, S. A., Kim, J. S., et al. (2011). "Requirements for eomesodermin and promyelocytic leukemia zinc finger in the development of innate-like CD8+ T cells." J Immunol **186**(8): 4573-4578.

Gordon, S. M., Chaix, J., Rupp, L. J., et al. (2012). "The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation." Immunity **36**(1): 55-67.

Gothard, L. Q., Hibbard, J. C. and Seyfred, M. A. (1996). "Estrogen-mediated induction of rat prolactin gene transcription requires the formation of a chromatin loop between the distal enhancer and proximal promoter regions." Mol Endocrinol **10**(2): 185-195.

Gozalbo-Lopez, B., Gomez del Moral, M., Campos-Martin, Y., et al. (2009). "The MHC-related protein 1 (MR1) is expressed by a subpopulation of CD38+, IgA+ cells in the human intestinal mucosa." Histol Histopathol **24**(11): 1439-1449.

Grange, M., Verdeil, G., Arnoux, F., et al. (2013). "Active STAT5 regulates T-bet and eomesodermin expression in CD8 T cells and imprints a T-bet-dependent Tc1 program with repressed IL-6/TGF-beta1 signaling." J Immunol **191**(7): 3712-3724.

Greer, J. M., Csurhes, P. A., Cameron, K. D., et al. (1997). "Increased immunoreactivity to two overlapping peptides of myelin proteolipid protein in multiple sclerosis." Brain **120 (Pt 8)**: 1447-1460.

Gregory, A. P., Dendrou, C. A., Attfield, K. E., et al. (2012). "TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis." Nature **488**(7412): 508-511.

Gregory, S. G., Schmidt, S., Seth, P., et al. (2007). "Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis." Nat Genet **39**(9): 1083-1091.

Greter, M., Heppner, F. L., Lemos, M. P., et al. (2005). "Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis." Nat Med **11**(3): 328-334.

Groom, J. R. and Luster, A. D. (2011). "CXCR3 in T cell function." Exp Cell Res **317**(5): 620-631.

Grundberg, E., Small, K. S., Hedman, A. K., et al. (2012). "Mapping cis- and trans-regulatory effects across multiple tissues in twins." Nat Genet **44**(10): 1084-1089.

Guimond, C., Lee, J. D., Ramagopalan, S. V., et al. (2014). "Multiple sclerosis in the Iranian immigrant population of BC, Canada: prevalence and risk factors." Mult Scler **20**(9): 1182-1188.

Gyodi, E., Benczur, M., Palffy, G., et al. (1982). "Association between HLA B7, DR2 and dysfunction of natural- and antibody-mediated cytotoxicity without connection with the deficient interferon production in multiple sclerosis." Hum Immunol **4**(3): 209-217.

Hafler, D. A., Compston, A., Sawcer, S., et al. (2007). "Risk alleles for multiple sclerosis identified by a genomewide study." N Engl J Med **357**(9): 851-862.

Haile, Y., Simmen, K. C., Pasichnyk, D., et al. (2011). "Granule-derived granzyme B mediates the vulnerability of human neurons to T cell-induced neurotoxicity." J Immunol **187**(9): 4861-4872.

Haines, J. L., Terwedow, H. A., Burgess, K., et al. (1998). "Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group." Hum Mol Genet **7**(8): 1229-1234.

Hampton, D. W., Serio, A., Pryce, G., et al. (2013). "Neurodegeneration progresses despite complete elimination of clinical relapses in a mouse model of multiple sclerosis." Acta neuropathologica communications **1**(1): 84.

Hao, J., Liu, R., Piao, W., et al. (2010). "Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology." J Exp Med **207**(9): 1907-1921.

Harriff, M. J., Cansler, M. E., Toren, K. G., et al. (2014). "Human lung epithelial cells contain Mycobacterium tuberculosis in a late endosomal vacuole and are efficiently recognized by CD8(+) T cells." PLoS One **9**(5): e97515.

Hartmann, F. J., Khademi, M., Aram, J., et al. (2014). "Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells." Nat Commun **5**: 5056.

Hauser, S. L., Bhan, A. K., Gilles, F., et al. (1986). "Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions." Ann Neurol **19**(6): 578-587.

He, W., Hao, J., Dong, S., et al. (2010). "Naturally activated V gamma 4 gamma delta T cells play a protective role in tumor immunity through expression of eomesodermin." J Immunol **185**(1): 126-133.

Hedstrom, A. K., Baarnhielm, M., Olsson, T., et al. (2009). "Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis." Neurology **73**(9): 696-701.

Hedstrom, A. K., Hillert, J., Olsson, T., et al. (2013). "Smoking and multiple sclerosis susceptibility." European journal of epidemiology **28**(11): 867-874.

Hedstrom, A. K., Sundqvist, E., Baarnhielm, M., et al. (2011). "Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis." Brain **134**(Pt 3): 653-664.

Heng, T. S., Painter, M. W. and Immunological Genome Project, C. (2008). "The Immunological Genome Project: networks of gene expression in immune cells." Nat Immunol **9**(10): 1091-1094.

Hess Michelini, R., Doedens, A. L., Goldrath, A. W., et al. (2013). "Differentiation of CD8 memory T cells depends on Foxo1." J Exp Med **210**(6): 1189-1200.

Hideshima, T., Richardson, P. G. and Anderson, K. C. (2011). "Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma." Molecular cancer therapeutics **10**(11): 2034-2042.

Hintzen, R. (2013). "Multiple sclerosis: Month of birth effect in MS--fact or artefact?" Nat Rev Neurol **9**(9): 489-490.

Hirsch, R. L., Panitch, H. S. and Johnson, K. P. (1985). "Lymphocytes from multiple sclerosis patients produce elevated levels of gamma interferon in vitro." J Clin Immunol **5**(6): 386-389.

Hochmeister, S., Grundtner, R., Bauer, J., et al. (2006). "Dysferlin is a new marker for leaky brain blood vessels in multiple sclerosis." Journal of neuropathology and experimental neurology **65**(9): 855-865.

Hodge, R. D., Kowalczyk, T. D., Wolf, S. A., et al. (2008). "Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output." J Neurosci **28**(14): 3707-3717.

Hogquist, K. A., Baldwin, T. A. and Jameson, S. C. (2005). "Central tolerance: learning self-control in the thymus." Nat Rev Immunol **5**(10): 772-782.

Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., et al. (2006). "The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis." Neurol Sci **27 Suppl 1**: S1-7.

Hu, Y., Turner, M. J., Shields, J., et al. (2009). "Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model." Immunology **128**(2): 260-270.

Huber, A. K., Wang, L., Han, P., et al. (2014). "Dysregulation of the IL-23/IL-17 axis and myeloid factors in secondary progressive MS." Neurology **83**(17): 1500-1507.

Huber, M., Brustle, A., Reinhard, K., et al. (2008). "IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype." Proc Natl Acad Sci U S A **105**(52): 20846-20851.

Huber, M., Heink, S., Grothe, H., et al. (2009). "A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity." Eur J Immunol **39**(7): 1716-1725.

Huber, M., Heink, S., Pagenstecher, A., et al. (2013). "IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis." J Clin Invest **123**(1): 247-260.

Hughes, J. R., Lower, K. M., Dunham, I., et al. (2013). "High-resolution analysis of cis-acting regulatory networks at the alpha-globin locus." Philos Trans R Soc Lond B Biol Sci **368**(1620): 20120361.

Hughes, J. R., Roberts, N., McGowan, S., et al. (2014). "Analysis of hundreds of cis-regulatory landscapes at high resolution in a single, high-throughput experiment." Nat Genet **46**(2): 205-214.

Huseby, E. S., Liggitt, D., Brabb, T., et al. (2001). "A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis." J Exp Med **194**(5): 669-676.

Ichiyama, K., Sekiya, T., Inoue, N., et al. (2011). "Transcription factor Smad-independent T helper 17 cell induction by transforming-growth factor-beta is mediated by suppression of eomesodermin." Immunity **34**(5): 741-754.

IFNB Multiple Sclerosis Study Group (1993). "Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group." Neurology **43**(4): 655-661.

IGAS, Cortes, A., Hadler, J., et al. (2013). "Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci." Nat Genet **45**(7): 730-738.

Illes, Z., Kondo, T., Newcombe, J., et al. (2000). "Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy." J Immunol **164**(8): 4375-4381.

Illes, Z., Shimamura, M., Newcombe, J., et al. (2004). "Accumulation of Valpha7.2-Jalpha33 invariant T cells in human autoimmune inflammatory lesions in the nervous system." Int Immunol **16**(2): 223-230.

International HapMap, C. (2005). "A haplotype map of the human genome." Nature **437**(7063): 1299-1320.

International Multiple Sclerosis Genetics, C., Beecham, A. H., Patsopoulos, N. A., et al. (2013). "Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis." Nat Genet **45**(11): 1353-1360.

International Multiple Sclerosis Genetics Consortium (2011). "Genome-wide association study of severity in multiple sclerosis." Genes Immun **12**(8): 615-625.

Intlekofer, A. M., Banerjee, A., Takemoto, N., et al. (2008). "Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin." Science **321**(5887): 408-411.

Intlekofer, A. M., Takemoto, N., Wherry, E. J., et al. (2005). "Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin." Nat Immunol **6**(12): 1236-1244.

Isobe, N., Damotte, V., Lo Re, V., et al. (2013). "Genetic burden in multiple sclerosis families." Genes Immun **14**(7): 434-440.

Iwata, Y., Matsushita, T., Horikawa, M., et al. (2011). "Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells." Blood **117**(2): 530-541.

Jackson, R. J., Hellen, C. U. and Pestova, T. V. (2010). "The mechanism of eukaryotic translation initiation and principles of its regulation." Nature reviews. Molecular cell biology **11**(2): 113-127.

Jackson, S. J., Giovannoni, G. and Baker, D. (2011). "Fingolimod modulates microglial activation to augment markers of remyelination." J Neuroinflammation **8**: 76.

Jacobs, L., O'Malley, J., Freeman, A., et al. (1982). "Intrathecal interferon in multiple sclerosis." Arch Neurol **39**(10): 609-615.

James, E., Dobson, R., Kuhle, J., et al. (2013). "The effect of vitamin D-related interventions on multiple sclerosis relapses: a meta-analysis." Mult Scler **19**(12): 1571-1579.

Janeway, C. A., Jr. and Medzhitov, R. (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.

Janossy, G., Bofill, M., Rowe, D., et al. (1989). "The tissue distribution of T lymphocytes expressing different CD45 polypeptides." Immunology **66**(4): 517-525.

Jiang, W., Chai, N. R., Maric, D., et al. (2011). "Unexpected role for granzyme K in CD56bright NK cell-mediated immunoregulation of multiple sclerosis." J Immunol **187**(2): 781-790.

Jick, S. S., Li, L., Falcone, G. J., et al. (2014). "Mortality of patients with multiple sclerosis: a cohort study in UK primary care." J Neurol **261**(8): 1508-1517.

Jilek, S., Schluep, M., Harari, A., et al. (2012). "HLA-B7-restricted EBV-specific CD8+ T cells are dysregulated in multiple sclerosis." J Immunol **188**(9): 4671-4680.

Jin, Y., Birlea, S. A., Fain, P. R., et al. (2010). "Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo." N Engl J Med **362**(18): 1686-1697.

Johnson, H. L., Willenbring, R. C., Jin, F., et al. (2014). "Perforin competent CD8 T cells are sufficient to cause immune-mediated blood-brain barrier disruption." PLoS One **9**(10): e111401.

Johnson, K. P., Arrigo, S. C., Nelson, B. J., et al. (1977). "Agarose electrophoresis of cerebrospinal fluid in multiple sclerosis. A simplified method for demonstrating cerebrospinal fluid oligoclonal immunoglobulin bands." Neurology **27**(3): 273-277.

Johnson, K. P., Brooks, B. R., Cohen, J. A., et al. (1995). "Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group." Neurology **45**(7): 1268-1276.

Johnson, T. A., Lapierre, Y., Bar-Or, A., et al. (2010). "Distinct properties of circulating CD8+ T cells in FTY720-treated patients with multiple sclerosis." Arch Neurol **67**(12): 1449-1455.

Jones, J. L. and Coles, A. J. (2014). "Mode of action and clinical studies with alemtuzumab." Exp Neurol **262 Pt A**: 37-43.

Joseph, F. G., Hirst, C. L., Pickersgill, T. P., et al. (2009). "CSF oligoclonal band status informs prognosis in multiple sclerosis: a case control study of 100 patients." J Neurol Neurosurg Psychiatry **80**(3): 292-296.

Jostins, L., Ripke, S., Weersma, R. K., et al. (2012). "Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease." Nature **491**(7422): 119-124.

Junker, A., Ivanidze, J., Malotka, J., et al. (2007). "Multiple sclerosis: T-cell receptor expression in distinct brain regions." Brain **130**(Pt 11): 2789-2799.

Jurewicz, A., Biddison, W. E. and Antel, J. P. (1998). "MHC class I-restricted lysis of human oligodendrocytes by myelin basic protein peptide-specific CD8 T lymphocytes." J Immunol **160**(6): 3056-3059.

Kamada, N., Seo, S. U., Chen, G. Y., et al. (2013). "Role of the gut microbiota in immunity and inflammatory disease." Nat Rev Immunol **13**(5): 321-335.

Kappos, L., Hartung, H. P., Freedman, M. S., et al. (2014). "Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial." Lancet Neurol **13**(4): 353-363.

Kappos, L., Li, D., Calabresi, P. A., et al. (2011). "Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial." Lancet **378**(9805): 1779-1787.

Kappos, L., Polman, C. H., Freedman, M. S., et al. (2006). "Treatment with interferon beta-1b delays conversion to clinically definite and McDonald MS in patients with clinically isolated syndromes." Neurology **67**(7): 1242-1249.

Kappos, L., Radue, E. W., O'Connor, P., et al. (2010). "A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis." N Engl J Med **362**(5): 387-401.

Kastrukoff, L. F., Morgan, N. G., Zecchini, D., et al. (1998). "A role for natural killer cells in the immunopathogenesis of multiple sclerosis." J Neuroimmunol **86**(2): 123-133.

Khan, Z., Ford, M. J., Cusanovich, D. A., et al. (2013). "Primate transcript and protein expression levels evolve under compensatory selection pressures." Science **342**(6162): 1100-1104.

Kim, H. J. and Cantor, H. (2011). "Regulation of self-tolerance by Qa-1-restricted CD8(+) regulatory T cells." Semin Immunol **23**(6): 446-452.

Kim, H. J., Wang, X., Radfar, S., et al. (2011). "CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice." Proc Natl Acad Sci U S A **108**(5): 2010-2015.

Kim, H. S. and Chung, D. H. (2012). "TLR4-mediated IL-12 production enhances IFN-gamma and IL-1beta production, which inhibits TGF-beta production and promotes antibody-induced joint inflammation." Arthritis Res Ther **14**(5): R210.

Kim, K., Bang, S. Y., Lee, H. S., et al. (2015). "High-density genotyping of immune loci in Koreans and Europeans identifies eight new rheumatoid arthritis risk loci." Ann Rheum Dis **74**(3): e13.

King, C. (2009). "New insights into the differentiation and function of T follicular helper cells." Nat Rev Immunol **9**(11): 757-766.

Kinjyo, I., Gordon, S. M., Intlekofer, A. M., et al. (2010). "Cutting edge: Lymphoproliferation caused by Fas deficiency is dependent on the transcription factor eomesodermin." J Immunol **185**(12): 7151-7155.

Kjer-Nielsen, L., Patel, O., Corbett, A. J., et al. (2012). "MR1 presents microbial vitamin B metabolites to MAIT cells." Nature **491**(7426): 717-723.

Klein, J. and Sato, A. (2000). "The HLA system. First of two parts." N Engl J Med **343**(10): 702-709.

Klein, R. J., Zeiss, C., Chew, E. Y., et al. (2005). "Complement factor H polymorphism in age-related macular degeneration." Science **308**(5720): 385-389.

Kleinewietfeld, M., Manzel, A., Titze, J., et al. (2013). "Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells." Nature **496**(7446): 518-522.

Knox, J. J., Cosma, G. L., Betts, M. R., et al. (2014). "Characterization of T-bet and eomes in peripheral human immune cells." Front Immunol **5**: 217.

Knudson, K. M., Goplen, N. P., Cunningham, C. A., et al. (2013). "Low-affinity T cells are programmed to maintain normal primary responses but are impaired in their recall to low-affinity ligands." Cell Rep **4**(3): 554-565.

Kobelt, G., Berg, J., Lindgren, P., et al. (2006). "Costs and quality of life of patients with multiple sclerosis in Europe." J Neurol Neurosurg Psychiatry **77**(8): 918-926.

Koch, M. W., Metz, L. M., Agrawal, S. M., et al. (2013). "Environmental factors and their regulation of immunity in multiple sclerosis." J Neurol Sci **324**(1-2): 10-16.

Komai-Koma, M., Gilchrist, D. S. and Xu, D. (2009). "Direct recognition of LPS by human but not murine CD8+ T cells via TLR4 complex." Eur J Immunol **39**(6): 1564-1572.

Komiyama, Y., Nakae, S., Matsuki, T., et al. (2006). "IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis." J Immunol **177**(1): 566-573.

Konjevic, G., Schlesinger, B., Cheng, L., et al. (1995). "Analysis of perforin expression in human peripheral blood lymphocytes, CD56+ natural killer cell subsets and its induction by interleukin-2." Immunological investigations **24**(3): 499-507.

Kooi, E. J., Geurts, J. J., van Horssen, J., et al. (2009). "Meningeal inflammation is not associated with cortical demyelination in chronic multiple sclerosis." Journal of neuropathology and experimental neurology **68**(9): 1021-1028.

Kurioka, A., Ussher, J. E., Cosgrove, C., et al. (2015). "MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets." Mucosal Immunol **8**(2): 429-440.

Kurtzke, J. (2000). "Epidemiology of multiple sclerosis. Does this really point toward an etiology? Lectio Doctoralis." Neurol Sci **21**: 383-403.

Kurtzke, J. F. (1977). "Geography in multiple sclerosis." J Neurol **215**(1): 1-26.

Kutzelnigg, A., Faber-Rod, J. C., Bauer, J., et al. (2007). "Widespread demyelination in the cerebellar cortex in multiple sclerosis." Brain Pathol **17**(1): 38-44.

Kutzelnigg, A., Lucchinetti, C. F., Stadelmann, C., et al. (2005). "Cortical demyelination and diffuse white matter injury in multiple sclerosis." Brain **128**(Pt 11): 2705-2712.

Lande, R., Gafa, V., Serafini, B., et al. (2008). "Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta." Journal of neuropathology and experimental neurology **67**(5): 388-401.

Lang, H. L., Jacobsen, H., Ikemizu, S., et al. (2002). "A functional and structural basis for TCR cross-reactivity in multiple sclerosis." Nat Immunol **3**(10): 940-943.

Langrish, C. L., Chen, Y., Blumenschein, W. M., et al. (2005). "IL-23 drives a pathogenic T cell population that induces autoimmune inflammation." J Exp Med **201**(2): 233-240.

Larsen, M., Sauce, D., Deback, C., et al. (2011). "Exhausted cytotoxic control of Epstein-Barr virus in human lupus." PLoS Pathog **7**(10): e1002328.

Lassmann, H. (2011). "Review: the architecture of inflammatory demyelinating lesions: implications for studies on pathogenesis." Neuropathol Appl Neurobiol **37**(7): 698-710.

Lassmann, H., Bruck, W. and Lucchinetti, C. F. (2007). "The immunopathology of multiple sclerosis: an overview." Brain Pathol **17**(2): 210-218.

Lassmann, H., van Horssen, J. and Mahad, D. (2012). "Progressive multiple sclerosis: pathology and pathogenesis." Nat Rev Neurol **8**(11): 647-656.

Laurent, J. M., Vogel, C., Kwon, T., et al. (2010). "Protein abundances are more conserved than mRNA abundances across diverse taxa." Proteomics **10**(23): 4209-4212.

Le Bourhis, L., Dusseaux, M., Bohineust, A., et al. (2013). "MAIT cells detect and efficiently lyse bacterially-infected epithelial cells." PLoS Pathog **9**(10): e1003681.

Le Bourhis, L., Martin, E., Peguillet, I., et al. (2010). "Antimicrobial activity of mucosal-associated invariant T cells." Nat Immunol **11**(8): 701-708.

Lee, C. W., Choi, J. W. and Chun, J. (2010). "Neurological S1P signaling as an emerging mechanism of action of oral FTY720 (fingolimod) in multiple sclerosis." Archives of pharmacal research **33**(10): 1567-1574.

Lee, S. H., Wray, N. R., Goddard, M. E., et al. (2011). "Estimating missing heritability for disease from genome-wide association studies." Am J Hum Genet **88**(3): 294-305.

Lee, Y. H., Ishida, Y., Rifa'i, M., et al. (2008). "Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis." J Immunol **180**(2): 825-832.

Leikfoss, I. S., Mero, I. L., Dahle, M. K., et al. (2013). "Multiple sclerosis-associated single-nucleotide polymorphisms in CLEC16A correlate with reduced SOCS1 and DEXI expression in the thymus." Genes Immun **14**(1): 62-66.

Lenercept MS study group (1999). "TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group." Neurology **53**(3): 457-465.

Leray, E., Yaouanq, J., Le Page, E., et al. (2010). "Evidence for a two-stage disability progression in multiple sclerosis." Brain **133**(Pt 7): 1900-1913.

Lettau, M., Schmidt, H., Kabelitz, D., et al. (2007). "Secretory lysosomes and their cargo in T and NK cells." Immunol Lett **108**(1): 10-19.

Link, J., Kockum, I., Lorentzen, A. R., et al. (2012). "Importance of human leukocyte antigen (HLA) class I and II alleles on the risk of multiple sclerosis." PLoS One **7**(5): e36779.

Link, J., Lorentzen, A. R., Kockum, I., et al. (2010). "Two HLA class I genes independently associated with multiple sclerosis." J Neuroimmunol **226**(1-2): 172-176.

Litwin, V., Gumperz, J., Parham, P., et al. (1993). "Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type." J Exp Med **178**(4): 1321-1336.

Lock, C., Hermans, G., Pedotti, R., et al. (2002). "Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis." Nat Med **8**(5): 500-508.

Lopez-Sagaseta, J., Dulberger, C. L., Crooks, J. E., et al. (2013). "The molecular basis for Mucosal-Associated Invariant T cell recognition of MR1 proteins." Proc Natl Acad Sci U S A **110**(19): E1771-1778.

Lovato, L., Willis, S. N., Rodig, S. J., et al. (2011). "Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis." Brain **134**(Pt 2): 534-541.

Lucchinetti, C., Bruck, W., Parisi, J., et al. (2000). "Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination." Ann Neurol **47**(6): 707-717.

Lucchinetti, C. F., Bruck, W. and Lassmann, H. (2004). "Evidence for pathogenic heterogeneity in multiple sclerosis." Ann Neurol **56**(2): 308.

Lugli, E., Gattinoni, L., Roberto, A., et al. (2013). "Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells." Nat Protoc **8**(1): 33-42.

Lundstrom, W., Highfill, S., Walsh, S. T., et al. (2013). "Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity." Proc Natl Acad Sci U S A **110**(19): E1761-1770.

Lunemann, A., Tackenberg, B., DeAngelis, T., et al. (2011). "Impaired IFN-gamma production and proliferation of NK cells in multiple sclerosis." Int Immunol **23**(2): 139-148.

Mack, C. L., Vanderlugt-Castaneda, C. L., Neville, K. L., et al. (2003). "Microglia are activated to become competent antigen presenting and effector cells in the inflammatory environment of the Theiler's virus model of multiple sclerosis." J Neuroimmunol **144**(1-2): 68-79.

Mackenzie, I. S., Morant, S. V., Bloomfield, G. A., et al. (2014). "Incidence and prevalence of multiple sclerosis in the UK 1990-2010: a descriptive study in the General Practice Research Database." J Neurol Neurosurg Psychiatry **85**(1): 76-84.

Maeder, M. L., Angstman, J. F., Richardson, M. E., et al. (2013). "Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins." Nat Biotechnol **31**(12): 1137-1142.

Magliozzi, R., Howell, O., Vora, A., et al. (2007). "Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology." Brain **130**(Pt 4): 1089-1104.

Magliozzi, R., Howell, O. W., Reeves, C., et al. (2010). "A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis." Ann Neurol **68**(4): 477-493.

Manolio, T. A., Collins, F. S., Cox, N. J., et al. (2009). "Finding the missing heritability of complex diseases." Nature **461**(7265): 747-753.

Marchini, J., Howie, B., Myers, S., et al. (2007). "A new multipoint method for genome-wide association studies by imputation of genotypes." Nat Genet **39**(7): 906-913.

Markowitz, C. E. (2007). "Interferon-beta: mechanism of action and dosing issues." Neurology **68**(24 Suppl 4): S8-11.

Marshall, H. D., Prince, A. L., Berg, L. J., et al. (2010). "IFN-alpha beta and self-MHC divert CD8 T cells into a distinct differentiation pathway characterized by rapid acquisition of effector functions." J Immunol **185**(3): 1419-1428.

Martin, E., Treiner, E., Duban, L., et al. (2009). "Stepwise development of MAIT cells in mouse and human." PLoS Biol **7**(3): e54.

Martin, J. F., Perry, J. S., Jakhete, N. R., et al. (2010). "An IL-2 paradox: blocking CD25 on T cells induces IL-2-driven activation of CD56(bright) NK cells." J Immunol **185**(2): 1311-1320.

Martirosyan, A., Von Bargen, K., Arce Gorvel, V., et al. (2013). "In vivo identification and characterization of CD4(+) cytotoxic T cells induced by virulent Brucella abortus infection." PLoS One **8**(12): e82508.

Maurano, M. T., Humbert, R., Rynes, E., et al. (2012). "Systematic localization of common disease-associated variation in regulatory DNA." Science **337**(6099): 1190-1195.

McAleer, J. P. and Kolls, J. K. (2011). "Mechanisms controlling Th17 cytokine expression and host defense." J Leukoc Biol **90**(2): 263-270.

McClain, M. T., Heinlen, L. D., Dennis, G. J., et al. (2005). "Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry." Nat Med **11**(1): 85-89.

McLane, L. M., Banerjee, P. P., Cosma, G. L., et al. (2013). "Differential localization of T-bet and Eomes in CD8 T cell memory populations." J Immunol **190**(7): 3207-3215.

Medana, I., Martinic, M. A., Wekerle, H., et al. (2001). "Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes." Am J Pathol **159**(3): 809-815.

Medzhitov, R. (2001). "Toll-like receptors and innate immunity." Nat Rev Immunol **1**(2): 135-145.

Meinl, E., Weber, F., Drexler, K., et al. (1993). "Myelin basic protein-specific T lymphocyte repertoire in multiple sclerosis. Complexity of the response and dominance of nested epitopes due to recruitment of multiple T cell clones." J Clin Invest **92**(6): 2633-2643.

Metzger, T. C. and Anderson, M. S. (2011). "Control of central and peripheral tolerance by Aire." Immunol Rev **241**(1): 89-103.

Miller, D. H., Chard, D. T. and Ciccarelli, O. (2012). "Clinically isolated syndromes." Lancet Neurol **11**(2): 157-169.

Mills, R. E., Pittard, W. S., Mullaney, J. M., et al. (2011). "Natural genetic variation caused by small insertions and deletions in the human genome." Genome Res **21**(6): 830-839.

Minguillon, C. and Logan, M. (2003). "The comparative genomics of T-box genes." Briefings in functional genomics & proteomics **2**(3): 224-233.

Miyazaki, Y., Miyake, S., Chiba, A., et al. (2011). "Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis." Int Immunol **23**(9): 529-535.

Miyoshi, K., Obata, K., Kondo, T., et al. (2008). "Interleukin-18-mediated microglia/astrocyte interaction in the spinal cord enhances neuropathic pain processing after nerve injury." J Neurosci **28**(48): 12775-12787.

Montaldo, E., Del Zotto, G., Della Chiesa, M., et al. (2013). "Human NK cell receptors/markers: a tool to analyze NK cell development, subsets and function." Cytometry A **83**(8): 702-713.

Moreland, L. W., Baumgartner, S. W., Schiff, M. H., et al. (1997). "Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein." N Engl J Med **337**(3): 141-147.

Mumford, C. J., Wood, N. W., Kellar-Wood, H., et al. (1994). "The British Isles survey of multiple sclerosis in twins." Neurology **44**(1): 11-15.

Munger, K. L., Levin, L. I., Hollis, B. W., et al. (2006). "Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis." Jama **296**(23): 2832-2838.

Munger, K. L., Zhang, S. M., O'Reilly, E., et al. (2004). "Vitamin D intake and incidence of multiple sclerosis." Neurology **62**(1): 60-65.

Murphy, K., Travers, P., Walport, M., et al. (2012). Janeway's immunobiology. New York, Garland Science.

Naismith, R. T., Piccio, L., Lyons, J. A., et al. (2010). "Rituximab add-on therapy for breakthrough relapsing multiple sclerosis: a 52-week phase II trial." Neurology **74**(23): 1860-1867.

National Institute for Health and Care Excellence (2007). "NICE TA127: Natalizumab for the treatment of adults with highly active relapsing–remitting multiple sclerosis." from http://www.nice.org.uk/guidance/ta127.

National Institute for Health and Care Excellence (2014). "NICE TA303: teriflunomide for treating relapsing remitting multiple sclerosis." from http://www.nice.org.uk/guidance/ta303.

Ng, P. C. and Henikoff, S. (2003). "SIFT: Predicting amino acid changes that affect protein function." Nucleic Acids Res **31**(13): 3812-3814.

NHS England Clinical Reference Group for Neurosciences (2014, 16 June 2014). "Clinical Commissioning Policy: Disease Modifying Therapies for Patients with Multiple Sclerosis (MS)." from https://http://www.england.nhs.uk/commissioning/spec-services/npc-crg/group-d/d04/.

Nielsen, T. R., Rostgaard, K., Nielsen, N. M., et al. (2007). "Multiple sclerosis after infectious mononucleosis." Arch Neurol **64**(1): 72-75.

Nikic, I., Merkler, D., Sorbara, C., et al. (2011). "A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis." Nat Med **17**(4): 495-499.

Okada, Y., Wu, D., Trynka, G., et al. (2014). "Genetics of rheumatoid arthritis contributes to biology and drug discovery." Nature **506**(7488): 376-381.

Oksenberg, J. R., Baranzini, S. E., Sawcer, S., et al. (2008). "The genetics of multiple sclerosis: SNPs to pathways to pathogenesis." Nat Rev Genet **9**(7): 516-526.

Okuda, Y., Okuda, M., Apatoff, B. R., et al. (2005). "The activation of memory CD4(+) T cells and CD8(+) T cells in patients with multiple sclerosis." J Neurol Sci **235**(1-2): 11-17.

Olerup, O. and Hillert, J. (1991). "HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation." Tissue Antigens **38**(1): 1-15.

Ono, C., Yu, Z., Kasahara, Y., et al. (2014). "Fluorescently activated cell sorting followed by microarray profiling of helper T cell subtypes from human peripheral blood." PLoS One **9**(11): e111405.

Ouyang, Q., Baerlocher, G., Vulto, I., et al. (2007). "Telomere length in human natural killer cell subsets." Ann N Y Acad Sci **1106**: 240-252.

Owens, G. P., Ritchie, A. M., Burgoon, M. P., et al. (2003). "Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid." J Immunol **171**(5): 2725-2733.

Pabst, O., Herbrand, H., Friedrichsen, M., et al. (2006). "Adaptation of solitary intestinal lymphoid tissue in response to microbiota and chemokine receptor CCR7 signaling." J Immunol **177**(10): 6824-6832.

Palace, J. (2001). "Making the diagnosis of multiple sclerosis." J Neurol Neurosurg Psychiatry **71 Suppl 2**: ii3-8.

Paley, M. A., Kroy, D. C., Odorizzi, P. M., et al. (2012). "Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection." Science **338**(6111): 1220-1225.

Panitch, H. S., Hirsch, R. L., Haley, A. S., et al. (1987). "Exacerbations of multiple sclerosis in patients treated with gamma interferon." Lancet **1**(8538): 893-895.

Panitch, H. S., Hirsch, R. L., Schindler, J., et al. (1987). "Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system." Neurology **37**(7): 1097-1102.

Papeix, C. and Lubetzki, C. (2013). "If I had a clinically isolated syndrome with MRI diagnostic of MS, I would take vitamin D 10,000 IU daily: No." Mult Scler **19**(2): 140-142.

Parnell, G. P., Gatt, P. N., Krupa, M., et al. (2014). "The autoimmune disease-associated transcription factors EOMES and TBX21 are dysregulated in multiple sclerosis and define a molecular subtype of disease." Clin Immunol **151**(1): 16-24.

Patsopoulos, N. A., Esposito, F., Reischl, J., et al. (2011). "Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci." Ann Neurol **70**(6): 897-912.

Pearce, E. L., Mullen, A. C., Martins, G. A., et al. (2003). "Control of effector CD8+ T cell function by the transcription factor Eomesodermin." Science **302**(5647): 1041-1043.

Perchellet, A., Brabb, T. and Goverman, J. M. (2008). "Crosspresentation by nonhematopoietic and direct presentation by hematopoietic cells induce central tolerance to myelin basic protein." Proc Natl Acad Sci U S A **105**(37): 14040-14045.

Peters, A., Pitcher, L. A., Sullivan, J. M., et al. (2011). "Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation." Immunity **35**(6): 986-996.

Phillips, J. T. and Fox, R. J. (2013). "BG-12 in multiple sclerosis." Seminars in neurology **33**(1): 56-65.

Pipkin, M. E., Sacks, J. A., Cruz-Guilloty, F., et al. (2010). "Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells." Immunity **32**(1): 79-90.

Pitt, D., Werner, P. and Raine, C. S. (2000). "Glutamate excitotoxicity in a model of multiple sclerosis." Nat Med **6**(1): 67-70.

Pittet, C. L., Newcombe, J., Antel, J. P., et al. (2011). "The majority of infiltrating CD8 T lymphocytes in multiple sclerosis lesions is insensitive to enhanced PD-L1 levels on CNS cells." Glia **59**(5): 841-856.

Poli, A., Michel, T., Theresine, M., et al. (2009). "CD56bright natural killer (NK) cells: an important NK cell subset." Immunology **126**(4): 458-465.

Polman, C. H., O'Connor, P. W., Havrdova, E., et al. (2006). "A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." N Engl J Med **354**(9): 899-910.

Polman, C. H., Reingold, S. C., Banwell, B., et al. (2011). "Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria." Ann Neurol **69**(2): 292-302.

Popescu, I., Pipeling, M. R., Shah, P. D., et al. (2014). "T-bet:Eomes balance, effector function, and proliferation of cytomegalovirus-specific CD8+ T cells during primary infection differentiates the capacity for durable immune control." J Immunol **193**(11): 5709-5722.

Pribyl, T. M., Campagnoni, C., Kampf, K., et al. (1996). "The major myelin protein genes are expressed in the human thymus." Journal of neuroscience research **45**(6): 812-819.

Prince, A. L., Kraus, Z., Carty, S. A., et al. (2014). "Development of innate CD4+ and CD8+ T cells in Itk-deficient mice is regulated by distinct pathways." J Immunol **193**(2): 688-699.

Pruim, R. J., Welch, R. P., Sanna, S., et al. (2010). "LocusZoom: regional visualization of genome-wide association scan results." Bioinformatics **26**(18): 2336-2337.

Qui, H. Z., Hagymasi, A. T., Bandyopadhyay, S., et al. (2011). "CD134 plus CD137 dual costimulation induces Eomesodermin in CD4 T cells to program cytotoxic Th1 differentiation." J Immunol **187**(7): 3555-3564.

Rado-Trilla, N. and Alba, M. (2012). "Dissecting the role of low-complexity regions in the evolution of vertebrate proteins." BMC Evol Biol **12**: 155.

Raivich, G. and Banati, R. (2004). "Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease." Brain research. Brain research reviews **46**(3): 261-281.

Ransohoff, R. M. and Engelhardt, B. (2012). "The anatomical and cellular basis of immune surveillance in the central nervous system." Nat Rev Immunol **12**(9): 623-635.

Rao, R. R., Li, Q., Odunsi, K., et al. (2010). "The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin." Immunity **32**(1): 67-78.

Rao, R. R., Li, Q. S., Bupp, M. R. G., et al. (2012). "Transcription Factor Foxo1 Represses T-bet-Mediated Effector Functions and Promotes Memory CD8(+) T Cell Differentiation." Immunity **36**(3): 374-387.

Reantragoon, R., Corbett, A. J., Sakala, I. G., et al. (2013). "Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells." J Exp Med **210**(11): 2305-2320.

Reantragoon, R., Kjer-Nielsen, L., Patel, O., et al. (2012). "Structural insight into MR1-mediated recognition of the mucosal associated invariant T cell receptor." J Exp Med **209**(4): 761-774.

Reed, J. M., Branigan, P. J. and Bamezai, A. (2008). "Interferon gamma enhances clonal expansion and survival of CD4+ T cells." Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research **28**(10): 611-622.

Reich, D. E., Cargill, M., Bolk, S., et al. (2001). "Linkage disequilibrium in the human genome." Nature **411**(6834): 199-204.

Reich, D. E. and Lander, E. S. (2001). "On the allelic spectrum of human disease." Trends Genet **17**(9): 502-510.

Renowden, S. (2014). "Imaging in multiple sclerosis and related disorders." Pract Neurol **14**(5): e3.

Richards, S. J., Jones, R. A., Roberts, B. E., et al. (1990). "Relationships between 2H4 (CD45RA) and UCHL1 (CD45RO) expression by normal blood CD4+CD8-, CD4-CD8+, CD4-CD8dim+, CD3+CD4-CD8- and CD3-CD4-CD8- lymphocytes." Clin Exp Immunol **81**(1): 149-155.

Roche, P. A. and Furuta, K. (2015). "The ins and outs of MHC class II-mediated antigen processing and presentation." Nat Rev Immunol **15**(4): 203-216.

Romagnani, C., Juelke, K., Falco, M., et al. (2007). "CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation." J Immunol **178**(8): 4947-4955.

Romme Christensen, J., Bornsen, L., Hesse, D., et al. (2012). "Cellular sources of dysregulated cytokines in relapsing-remitting multiple sclerosis." J Neuroinflammation **9**: 215.

Romme Christensen, J., Bornsen, L., Ratzer, R., et al. (2013). "Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression." PLoS One **8**(3): e57820.

Romme Christensen, J., Ratzer, R., Bornsen, L., et al. (2014). "Natalizumab in progressive MS: results of an open-label, phase 2A, proof-of-concept trial." Neurology **82**(17): 1499-1507.

Ryan, K., Butler, K., Bellefroid, E., et al. (1998). "Xenopus eomesodermin is expressed in neural differentiation." Mech Dev **75**(1-2): 155-158.

Ryan, K., Garrett, N., Mitchell, A., et al. (1996). "Eomesodermin, a key early gene in Xenopus mesoderm differentiation." Cell **87**(6): 989-1000.

Sabet-Baktach, M., Eggenhofer, E., Rovira, J., et al. (2013). "Double deficiency for RORgammat and T-bet drives Th2-mediated allograft rejection in mice." J Immunol **191**(8): 4440-4446.

Sachidanandam, R., Weissman, D., Schmidt, S. C., et al. (2001). "A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms." Nature **409**(6822): 928-933.

Sadaba, M. C., Tzartos, J., Paino, C., et al. (2012). "Axonal and oligodendrocyte-localized IgM and IgG deposits in MS lesions." J Neuroimmunol **247**(1-2): 86-94.

Sallusto, F., Geginat, J. and Lanzavecchia, A. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol **22**: 745-763.

Saruhan-Direskeneli, G., Weber, F., Meinl, E., et al. (1993). "Human T cell autoimmunity against myelin basic protein: CD4+ cells recognizing epitopes of the T cell receptor beta chain from a myelin basic protein-specific T cell clone." Eur J Immunol **23**(2): 530-536.

Sathaliyawala, T., Kubota, M., Yudanin, N., et al. (2013). "Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets." Immunity **38**(1): 187-197.

Sawcer, S., Franklin, R. J. and Ban, M. (2014). "Multiple sclerosis genetics." Lancet Neurol **13**(7): 700-709.

Sawcer, S., Hellenthal, G., Pirinen, M., et al. (2011). "Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis." Nature **476**(7359): 214-219.

Scalfari, A., Knappertz, V., Cutter, G., et al. (2013). "Mortality in patients with multiple sclerosis." Neurology **81**(2): 184-192.

Scalfari, A., Neuhaus, A., Daumer, M., et al. (2013). "Early relapses, onset of progression, and late outcome in multiple sclerosis." JAMA Neurol **70**(2): 214-222.

Schietinger, A. and Greenberg, P. D. (2014). "Tolerance and exhaustion: defining mechanisms of T cell dysfunction." Trends Immunol **35**(2): 51-60.

Schneider-Hohendorf, T., Rossaint, J., Mohan, H., et al. (2014). "VLA-4 blockade promotes differential routes into human CNS involving PSGL-1 rolling of T cells and MCAM-adhesion of TH17 cells." J Exp Med **211**(9): 1833-1846.

Schnell, S. A., Staines, W. A. and Wessendorf, M. W. (1999). "Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue." The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society **47**(6): 719-730.

Schroder, K., Hertzog, P. J., Ravasi, T., et al. (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Seach, N., Guerri, L., Le Bourhis, L., et al. (2013). "Double-positive thymocytes select mucosal-associated invariant T cells." J Immunol **191**(12): 6002-6009.

Serafini, B., Rosicarelli, B., Franciotta, D., et al. (2007). "Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain." J Exp Med **204**(12): 2899-2912.

Serafini, B., Rosicarelli, B., Magliozzi, R., et al. (2004). "Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis." Brain Pathol **14**(2): 164-174.

Serafini, B., Rosicarelli, B., Magliozzi, R., et al. (2006). "Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells." Journal of neuropathology and experimental neurology **65**(2): 124-141.

Serafini, B., Severa, M., Columba-Cabezas, S., et al. (2010). "Epstein-Barr virus latent infection and BAFF expression in B cells in the multiple sclerosis brain: implications for viral persistence and intrathecal B-cell activation." Journal of neuropathology and experimental neurology **69**(7): 677-693.

Serpero, L. D., Filaci, G., Parodi, A., et al. (2013). "Fingolimod modulates peripheral effector and regulatory T cells in MS patients." Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology **8**(5): 1106-1113.

Serriari, N. E., Eoche, M., Lamotte, L., et al. (2014). "Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases." Clin Exp Immunol **176**(2): 266-274.

Sharma, P. K., Wong, E. B., Napier, R. J., et al. (2015). "High expression of CD26 accurately identifies human bacteria-reactive MR1-restricted MAIT cells." Immunology **145**(3): 443-453.

Shen, P., Roch, T., Lampropoulou, V., et al. (2014). "IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases." Nature **507**(7492): 366-370.

Sheridan, J. P., Zhang, Y., Riester, K., et al. (2011). "Intermediate-affinity interleukin-2 receptor expression predicts CD56(bright) natural killer cell expansion after daclizumab treatment in the CHOICE study of patients with multiple sclerosis." Mult Scler **17**(12): 1441-1448.

Shi, G., Cox, C. A., Vistica, B. P., et al. (2008). "Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells." J Immunol **181**(10): 7205-7213.

Shields, R. (2011). "Common disease: are causative alleles common or rare?" PLoS Biol **9**(1): e1001009.

Shirani, A., Zhao, Y., Karim, M. E., et al. (2012). "Association between use of interferon beta and progression of disability in patients with relapsing-remitting multiple sclerosis." Jama **308**(3): 247-256.

Simpson, J. E., Newcombe, J., Cuzner, M. L., et al. (2000). "Expression of the interferon-gamma-inducible chemokines IP-10 and Mig and their receptor, CXCR3, in multiple sclerosis lesions." Neuropathol Appl Neurobiol **26**(2): 133-142.

Simpson, S., Jr., Taylor, B., Blizzard, L., et al. (2010). "Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis." Ann Neurol **68**(2): 193-203.

Slieker, R. C., Bos, S. D., Goeman, J. J., et al. (2013). "Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array." Epigenetics & chromatin **6**(1): 26.

Sorensen, P. S., Lisby, S., Grove, R., et al. (2014). "Safety and efficacy of ofatumumab in relapsing-remitting multiple sclerosis: a phase 2 study." Neurology **82**(7): 573-581.

Stadelmann, C. (2011). "Multiple sclerosis as a neurodegenerative disease: pathology, mechanisms and therapeutic implications." Curr Opin Neurol **24**(3): 224-229.

Steiner, D. F., Thomas, M. F., Hu, J. K., et al. (2011). "MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells." Immunity **35**(2): 169-181.

Stranger, B. E., Montgomery, S. B., Dimas, A. S., et al. (2012). "Patterns of cis regulatory variation in diverse human populations." PLoS Genet **8**(4): e1002639.

Stys, P. K., Zamponi, G. W., van Minnen, J., et al. (2012). "Will the real multiple sclerosis please stand up?" Nat Rev Neurosci **13**(7): 507-514.

Su, A. I., Wiltshire, T., Batalov, S., et al. (2004). "A gene atlas of the mouse and human protein-encoding transcriptomes." Proc Natl Acad Sci U S A **101**(16): 6062-6067.

Sun, D., Qin, Y., Chluba, J., et al. (1988). "Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T-T cell interactions." Nature **332**(6167): 843-845.

Sun, D., Whitaker, J. N., Huang, Z., et al. (2001). "Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice." J Immunol **166**(12): 7579-7587.

Suzuki, M., Jagger, A. L., Konya, C., et al. (2012). "CD8+CD45RA+CCR7+FOXP3+ T Cells with Immunosuppressive Properties: A Novel Subset of Inducible Human Regulatory T Cells." J Immunol **189**(5): 2118-2130.

Suzuki, M. M. and Bird, A. (2008). "DNA methylation landscapes: provocative insights from epigenomics." Nat Rev Genet **9**(6): 465-476.

Svenningsson, A., Hansson, G. K., Andersen, O., et al. (1993). "Adhesion molecule expression on cerebrospinal fluid T lymphocytes: evidence for common recruitment mechanisms in multiple sclerosis, aseptic meningitis, and normal controls." Ann Neurol **34**(2): 155-161.

Takahashi, K., Aranami, T., Endoh, M., et al. (2004). "The regulatory role of natural killer cells in multiple sclerosis." Brain **127**(Pt 9): 1917-1927.

Takemoto, N., Intlekofer, A. M., Northrup, J. T., et al. (2006). "Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation." J Immunol **177**(11): 7515-7519.

Tang, X. Z., Jo, J., Tan, A. T., et al. (2013). "IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells." J Immunol **190**(7): 3142-3152.

Targan, S. R., Hanauer, S. B., van Deventer, S. J., et al. (1997). "A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group." N Engl J Med **337**(15): 1029-1035.

Teitelbaum, D., Arnon, R., Sela, M., et al. (1989). "[Clinical trial of copolymer 1 in multiple sclerosis]." Harefuah **116**(9): 453-456.

Thornton, A. M. and Shevach, E. M. (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-296.

Trabattoni, D., Saresella, M., Pacei, M., et al. (2009). "Costimulatory pathways in multiple sclerosis: distinctive expression of PD-1 and PD-L1 in patients with different patterns of disease." J Immunol **183**(8): 4984-4993.

Trapani, J. A. and Smyth, M. J. (2002). "Functional significance of the perforin/granzyme cell death pathway." Nat Rev Immunol **2**(10): 735-747.

Trapp, B. D. and Nave, K. A. (2008). "Multiple sclerosis: an immune or neurodegenerative disorder?" Annu Rev Neurosci **31**: 247-269.

Trapp, B. D., Peterson, J., Ransohoff, R. M., et al. (1998). "Axonal transection in the lesions of multiple sclerosis." N Engl J Med **338**(5): 278-285.

Treiner, E., Duban, L., Bahram, S., et al. (2003). "Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1." Nature **422**(6928): 164-169.

Treiner, E., Duban, L., Moura, I. C., et al. (2005). "Mucosal-associated invariant T (MAIT) cells: an evolutionarily conserved T cell subset." Microbes Infect **7**(3): 552-559.

Tsuchida, P., Turner, MacFarland, Coligan, Biddison (1994). "Autoreactive CD8 T-cell responses to human myelin protein derived peptides." PNAS **91**: 10859-10863.

Tumes, D. J., Onodera, A., Suzuki, A., et al. (2013). "The polycomb protein Ezh2 regulates differentiation and plasticity of CD4(+) T helper type 1 and type 2 cells." Immunity **39**(5): 819-832.

Tzartos, J. S., Friese, M. A., Craner, M. J., et al. (2008). "Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis." Am J Pathol **172**(1): 146-155.

Ussher, J. E., Bilton, M., Attwod, E., et al. (2014). "CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner." Eur J Immunol **44**(1): 195-203.

van de Berg, P. J., van Leeuwen, E. M., ten Berge, I. J., et al. (2008). "Cytotoxic human CD4(+) T cells." Curr Opin Immunol **20**(3): 339-343.

van der Windt, G. J., Everts, B., Chang, C. H., et al. (2012). "Mitochondrial respiratory capacity is a critical regulator of CD8(+) T cell memory development." Immunity **36**(1): 68-78.

Vanderlugt, C. L. and Miller, S. D. (2002). "Epitope spreading in immune-mediated diseases: implications for immunotherapy." Nat Rev Immunol **2**(2): 85-95.

Veldhoen, M., Uyttenhove, C., van Snick, J., et al. (2008). "Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset." Nat Immunol **9**(12): 1341-1346.

Venken, K., Hellings, N., Hensen, K., et al. (2010). "Memory CD4+CD127high T cells from patients with multiple sclerosis produce IL-17 in response to myelin antigens." J Neuroimmunol **226**(1-2): 185-191.

Vergo, S., Craner, M. J., Etzensperger, R., et al. (2011). "Acid-sensing ion channel 1 is involved in both axonal injury and demyelination in multiple sclerosis and its animal model." Brain **134**(Pt 2): 571-584.

Viglietta, V., Baecher-Allan, C., Weiner, H. L., et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med **199**(7): 971-979.

Visscher, P. M., Brown, M. A., McCarthy, M. I., et al. (2012). "Five years of GWAS discovery." Am J Hum Genet **90**(1): 7-24.

Vogel, C. and Marcotte, E. M. (2012). "Insights into the regulation of protein abundance from proteomic and transcriptomic analyses." Nat Rev Genet **13**(4): 227-232.

von Boehmer, H. and Melchers, F. (2010). "Checkpoints in lymphocyte development and autoimmune disease." Nat Immunol **11**(1): 14-20.

Wajant, H., Pfizenmaier, K. and Scheurich, P. (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.

Wakim, L. M., Woodward-Davis, A., Liu, R., et al. (2012). "The molecular signature of tissue resident memory CD8 T cells isolated from the brain." J Immunol **189**(7): 3462-3471.

Walker, L. J., Kang, Y. H., Smith, M. O., et al. (2012). "Human MAIT and CD8alphaalpha cells develop from a pool of type-17 precommitted CD8+ T cells." Blood **119**(2): 422-433.

Walker, L. J., Tharmalingam, H. and Klenerman, P. (2014). "The rise and fall of MAIT cells with age." Scand J Immunol **80**(6): 462-463.

Watson, C. T., Disanto, G., Breden, F., et al. (2012). "Estimating the proportion of variation in susceptibility to multiple sclerosis captured by common SNPs." Scientific reports **2**: 770.

Waxman, S. G. (2006). "Axonal conduction and injury in multiple sclerosis: the role of sodium channels." Nat Rev Neurosci **7**(12): 932-941.

Westerlind, H., Ramanujam, R., Uvehag, D., et al. (2014). "Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden." Brain **137**(Pt 3): 770-778.

Westra, H. J., Peters, M. J., Esko, T., et al. (2013). "Systematic identification of trans eQTLs as putative drivers of known disease associations." Nat Genet **45**(10): 1238-1243.

Wherry, E. J. (2011). "T cell exhaustion." Nat Immunol **12**(6): 492-499.

Wherry, E. J., Ha, S. J., Kaech, S. M., et al. (2007). "Molecular signature of CD8+ T cell exhaustion during chronic viral infection." Immunity **27**(4): 670-684.

Wiberg, M., Friberg, E., Stenbeck, M., et al. (2015). "Sources and level of income among individuals with multiple sclerosis compared to the general population: A nationwide population-based study." Mult Scler **21**(13): 1730-1741.

Willing, A., Leach, O. A., Ufer, F., et al. (2014). "CD8(+) MAIT cells infiltrate into the CNS and alterations in their blood frequencies correlate with IL-18 serum levels in multiple sclerosis." Eur J Immunol **44**(10): 3119-3128.

Wucherpfennig, K. W. and Strominger, J. L. (1995). "Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein." Cell **80**(5): 695-705.

Yang, M., Sun, L., Wang, S., et al. (2010). "Novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells." J Immunol **184**(7): 3321-3325.

Yang, Q., Khoury, M. J., Friedman, J., et al. (2005). "How many genes underlie the occurrence of common complex diseases in the population?" Int J Epidemiol **34**(5): 1129-1137.

Yang, S., Liu, F., Wang, Q. J., et al. (2011). "The shedding of CD62L (L-selectin) regulates the acquisition of lytic activity in human tumor reactive T lymphocytes." PLoS One **6**(7): e22560.

Yang, Y., Xu, J., Niu, Y., et al. (2008). "T-bet and eomesodermin play critical roles in directing T cell differentiation to Th1 versus Th17." J Immunol **181**(12): 8700-8710.

Yates, R. L., Esiri, M. M., Palace, J., et al. (2015). "The influence of HLA-DRB1*15 on motor cortical pathology in multiple sclerosis." Neuropathol Appl Neurobiol **41**(3): 371-384.

Yu, D., Rao, S., Tsai, L. M., et al. (2009). "The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment." Immunity **31**(3): 457-468.

Yuan, Y., Tian, L., Lu, D., et al. (2015). "Analysis of genome-wide RNA-sequencing data suggests age of the CEPH/Utah (CEU) lymphoblastoid cell lines systematically biases gene expression profiles." Scientific reports **5**: 7960.

Zaguia, F., Saikali, P., Ludwin, S., et al. (2013). "Cytotoxic NKG2C+ CD4 T cells target oligodendrocytes in multiple sclerosis." J Immunol **190**(6): 2510-2518.

Zanin-Zhorov, A., Tal-Lapidot, G., Cahalon, L., et al. (2007). "Cutting edge: T cells respond to lipopolysaccharide innately via TLR4 signaling." J Immunol **179**(1): 41-44.

Zhang, B., Yamamura, T., Kondo, T., et al. (1997). "Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells." J Exp Med **186**(10): 1677-1687.

Zhang, S., Li, T., Zhang, B., et al. (2011). "Transcription factors engaged in development of NK cells are commonly expressed in nasal NK/T-cell lymphomas." Hum Pathol **42**(9): 1319-1328.

Zhou, X., Yu, S., Zhao, D. M., et al. (2010). "Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1." Immunity **33**(2): 229-240.