Multiple sclerosis associated SNPs influencing B cell expression of co-stimulatory receptors and T cell responses

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I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other University. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. This dissertation contains less than 65,000 words including appendices, bibliography, footnotes, tables and equations and has less than 150 figures.

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Abstract

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) which has a complex aetiology that involves the interplay of both genetic and environmental factors. Although more than 200 susceptibility loci have been identified through genome-wide association studies, our understanding of the molecular mechanisms underlying these associations remains limited. My predecessor in the laboratory established that the MS associated variants rs9282641 and rs4810485 respectively influence the expression of CD86 and CD40 in freshly collected (ex-vivo) B cells. In my research, I sought to extend this work by investigating the impact of these variants in the context of activation in an effort to refine understanding of the molecular mechanisms underlying the effects of these genotypes on the risk of developing MS.

In the first phase of my work I processed venous blood samples from 108 healthy volunteers, who were recruited from the Cambridge BioResource (CBR) to enable a balanced representation of each possible genotype. I isolated peripheral blood mononuclear cells (PBMCs) from these samples and then activated these with CD40L-transfected L cells. In line with the ex vivo results, I found that the expression of CD86 and CD40 remained associated with the genotypes of the respective MS associated variants. I then went on to investigate the effects of these stimulated cells on co-cultured naïve T cells by studying a second cohort of 28 volunteers recruited in pairs from the CBR; the individuals in each pair carrying either the AA or the GG genotype at rs9282641. In these experiments I co-cultured naïve T cells with purified B cells that had been activated with CD40L-transfected L cells. In these 12-day co-cultures, the T cells from subjects homozygous for the risk allele (GG) at rs9282641 demonstrated significantly enhanced proliferation and displayed significantly higher IFN-γ and IL-17 production, and lower IL-10 production.

In my PhD I have therefore shown that the altered expression of co-stimulatory molecules on B cells induced by MS risk variants results in enhanced proliferation of pro-inflammatory T cells.
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Abbreviation

A Cooperative Clinical Study of Abatacept in Multiple Sclerosis, (ACCLAIM)

antigen presenting cell (APC)

autologous hematopoietic stem cell transplantation (aHSCT)

Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNE)

B cell depletion therapy (BCDT)

B cell receptor (BCR)

regulatory B cell (Breg)

carboxyfluorescein diacetate succinimidyl ester (CFSE)

clinically isolated syndrome (CIS)

central nervous system (CNS)

cerebrospinal fluid (CSF)

Cytotoxic T lymphocyte antigen-4 (CTLA-4)

experimental autoimmune encephalomyelitis (EAE)

Epstein-Barr virus (EBV)

enzyme linked immunosorbent assay (ELISA)

expression quantitative trait locus (eQTL)

fluorescence-activated cell sorting (FACS)

Forkhead box protein 3 (FoxP3)

forward scatter (FSC)

germinat centre (GC)

Graves’ disease (GD)

granulocyte macrophage – colony stimulating factor (GM-CSF)
genome-wide association study (GWAS)
human leukocyte antigen (HLA)
immunoglobulin (Ig)
linkage disequilibrium (LD)
mean fluorescence intensity (MFI)
major histocompatibility complex (MHC)
magnetic resonance imaging (MRI)
multiple sclerosis (MS)
next generation sequencing (NGS)
odds ratio (OR)
phosphate buffer saline (PBS)
Peripheral blood mononuclear cell (PBMC)
phorbol 12-myristate 13-acetate (PMA)
primary progressive multiple sclerosis (PPMS)
rheumatoid arthritis (RA)
radiologically isolated syndrome (RIS)
reactive oxygen species (ROS)
relapsing-remitting multiple sclerosis (RRMS)
small interfering RNA (siRNA)
systemic lupus erythematosus (SLE)
single-nucleotide polymorphism (SNP)
secondary progressive multiple sclerosis (SPMS)
side scatter (SSC)
T cell receptor (TCR)
T helper type 1 (Th1)
T helper type 2 (Th2)
T helper type 17 (Th17)
tumour necrosis factor (TNF)
T regulatory type 1 (Tr1)
regulatory T cell (Treg)
unique molecular identifier (UMI)
ultraviolet radiation (UVR)
zinc finger protein (ZFP)
Chapter 1  Introduction

1.1  Clinical features of Multiple sclerosis

1.1.1  Overview and classification

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disorder of the central nervous system (CNS), which results in multiple inflammatory lesions that are disseminated throughout the CNS in both time and space that cause a wide range of focal neurological symptoms and signs (Compston and Coles, 2008; Thompson et al., 2018b). It most commonly affects individuals during their early adult life, and frequently results in quality of life being severely compromised (Cree et al., 2016; Sanai et al., 2016) and shortened (Scalfari et al., 2013; Lunde et al., 2017). The disease runs a highly variable course in which it frequently affects mobility, sensation and cognition. Ultimately significant irreversible disability develops in approximately 80% of MS patients (Scalfari et al., 2011; Lublin et al., 2014).

The early disease course in MS is broadly classified into two major categories: primary progressive MS (PPMS) and the relapsing-remitting MS (RRMS) (Compston and Coles, 2008). The former, which accounts for 10-15% of cases, is characterized by the gradual accumulation of irreversible neurological disability, whereas the latter (85-90% of cases) is characterized by intermittent periods of relapses and remission; although the recovery from relapses often incomplete (Miller and Leary, 2007). Notably, in about 70% of RRMS patients relapse eventually give way to a phase of accumulating irreversible disability, or so called secondary progressive MS (SPMS) (Jokubaitis et al., 2016; Lorscheider et al., 2016). People with incidental magnetic resonance imaging (MRI) findings suggestive of MS but no clinical signs or symptoms, which is termed radiologically isolated syndrome (RIS), are at higher risk for developing into MS in the future, with one-third of patients diagnosed within 5 years (Okuda et al., 2014; Labiano-Fontcuberta and Benito-León, 2016). Therefore, appropriate follow-ups are necessary for the RIS population (Kantarci et al., 2016). From clinical and statistical perspectives, MS natural history data suggests that the condition is best regarded as one disease with a variety of different phenotypic dimensions rather than a syndrome encompassing a range of heterogeneous distinct diseases (Confavereux and Vukusic, 2006).
1.1.2 Clinical presentations and diagnosis

In general, the diagnosis of MS requires objective evidence of CNS lesions disseminated in time and space, with alternative explanations considered and excluded (Compston and Coles, 2008; Thompson et al., 2018b). The diagnostic process relies on the presenting neurological symptoms and signs supported by paraclinical testing, especially MRI findings (Karussis, 2013; Brownlee et al., 2017). Episodes of neurological dysfunction may be acute or subacute and spontaneous remit (Miller et al., 2005; Scalfari et al., 2010). By contrast, the onset of PPMS, as its name implies, is slowly progressive from the onset, with the symptoms continuously deteriorating over time (Kremenchutsky et al., 2006). The diagnosis of MS is clinical and using criteria that have developed over the years beginning with the Schumacher and Poser Criteria (Schumacher et al., 1965; Poser et al., 1983) and continue to evolve in the form of the various revisions of the McDonald criteria (McDonald et al., 2001; Polman et al., 2005; Polman et al., 2011; Thompson et al., 2018a). Of note, these criteria all necessitate the exclusion of other CNS demyelinating diseases, such as neuromyelitis optica spectrum disorder, neurosarcoïdosis, CNS vasculitis and connective tissue disorders, all of which can mimic MS (Solomon et al., 2016; Brownlee et al., 2017).

Given that brain MRI abnormality is found in nearly all established MS patients and over 80% of the CIS cases, and that MRI is necessary for differential diagnosis to exclude other demyelinating and non-demyelinating diseases, MRI remains the most crucial diagnostic tool for the disease (Fisniku et al., 2008; Wattjes et al., 2015). The MRI evidence suggestive of MS includes multifocal T2-hyperintensity, which is typically found in periventricular, juxtacortical and infratentorial regions of white matter (Polman et al., 2011; Rovira et al., 2015). Apart from brain MRI, since spinal cord lesions may also present in many MS patients, spinal cord MRI is recommended to confirm the dissemination in space and to exclude alternative spinal cord diseases, when brain MRI findings are not sufficient for the diagnosis of MS (Sombekke et al., 2013). Non-enhancing hypointensity on T1-weighted images are more common in progressive subtypes and patients with long disease duration and are indicative of chronic lesions of an established MS disease process (Filippi et al., 2016). Simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesion either on a single scan or on follow-up scan demonstrates dissemination in time and thereby facilitate the diagnosis, especially when there’s only one clinical attack (Milo and Miller, 2014).

In most cases, cerebrospinal fluid (CSF) examination is not necessary as a confident diagnosis can usually be made based on typical clinical and MRI findings (Tintore et al., 2013). Supportive CSF findings features such an elevated IgG index and the presence of oligoclonal bands, together with nearly normal white blood cell count, glucose and protein,
occur in most but not all patients. In many clinical diagnostic criteria CSF changes are essential to make the diagnosis of PPMS; although it is important to recognize that changes such as oligoclonal bands can be found in other neuro-inflammatory diseases; necessitating circumspect interpretation (Karussis, 2014).

1.1.3 Management

Over the past 20 years, a range of MS disease-modifying therapies have emerged, these agents have been shown to reduce the rate of relapses and probably also slow the rate of disability progression (Comi et al., 2017). The wide range of agents that are now available has made therapeutic choices increasingly complex (Montalban et al., 2018). The escalation strategy, one of the two therapeutic approaches available in current clinical settings, entails starting with a first-line treatment and escalating to a second-line treatment which is potentially more dangerous when relapses continue to occur (Thompson et al., 2018b). By contrast, in the highly active and rapidly evolving cases, an induction strategy, in which a highly effective treatment is used from the onset to achieve a persistent disease remission, is more often appropriate (Thompson et al., 2018b).

All the currently approved disease-modifying therapies for RRMS have inflammation related mechanisms of action. A summary of FDA-approved drugs for RRMS could be found in Table 1.1.3 (Havrdoa et al., 2009; Giovannoni et al., 2010; Leist et al., 2014; Cohen et al., 2015; Kalincik et al., 2015; Kalincik et al., 2017; Wiendl et al., 2017; Baecher-Allan et al., 2018). These agents have differing modes of action including reducing auto-reactive Th1/Th17 cells, inducing regulatory T cells, impeding the trafficking of immune cells, and modulating B cell activities. The anti-inflammatory efficacy and adverse effect burden vary between agents, with an inevitable inverse correlation (Bloomgren et al., 2012; Plavina et al., 2014; Gagne Brosseau et al., 2016), as shown in Figure 1.1.3, which is adapted from the presentation by Alastair Compston at the ECTRIMS 2018 conference.

<table>
<thead>
<tr>
<th>Drug (Date of Approval)</th>
<th>Category</th>
<th>Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon β-1b/1a (1993)</td>
<td>Injectable drug</td>
<td>Naturally occurring cytokine which reduces antigen presentation and T cell proliferation, alters cytokine expression, restores suppressor function</td>
</tr>
<tr>
<td>Glatiramer acetate (1997)</td>
<td>Injectable drug</td>
<td>A mixture of short polypeptides, which activates the anti-inflammatory glatiramer acetate-specific lymphocytes</td>
</tr>
<tr>
<td>Teriflunomide (2012)</td>
<td>Oral drug</td>
<td>The metabolite of leflunomide, which inhibits the proliferation of autoreactive B and T cells</td>
</tr>
<tr>
<td>Dimethyl fumarate (2013)</td>
<td>Oral drug</td>
<td>Inhibiting the transcription factor nuclear-factor-κB to reduce the release of inflammatory cytokines; activating the transcription</td>
</tr>
</tbody>
</table>
factor nuclear-factor-E2-related factor 2 to have an anti-oxidant effect

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fingolimod</strong></td>
<td>Oral drug</td>
<td>Acting as a functional antagonist of sphingosine 1-phosphate receptors, on which lymphocytes are dependent to egress from the lymphoid tissues, consequently resulting in a decrease of circulating lymphocyte number</td>
</tr>
<tr>
<td><strong>Cladribine</strong></td>
<td>Oral drug</td>
<td>Synthetic deoxyadenosine analogue, which depletes B and T cells</td>
</tr>
<tr>
<td><strong>Natalizumab</strong></td>
<td>Monoclonal antibodies</td>
<td>A humanized monoclonal antibody that acts by blocking α-4 integrin to prevent lymphocytes from entering the CNS across the blood–brain barrier</td>
</tr>
<tr>
<td><strong>Alemtuzumab</strong></td>
<td>Monoclonal antibodies</td>
<td>A humanized monoclonal antibody against CD52, which results in a long-lasting depletion of B and T cells</td>
</tr>
<tr>
<td><strong>Daclizumab</strong></td>
<td>Monoclonal antibodies</td>
<td>A monoclonal antibody acting as an interleukin-2 inhibitor (withdrawn in March 2018 due to reports of adverse events)</td>
</tr>
<tr>
<td><strong>Ocrelizumab</strong></td>
<td>Monoclonal antibodies</td>
<td>A humanized monoclonal antibody directed against CD20</td>
</tr>
</tbody>
</table>

Table 1.1.3. Drugs for RRMS.

**Figure 1.1.3.** Safety versus efficacy of currently available drugs for MS.

It should be noted that the predominance of inflammatory mechanisms seen during the initial stages of the disease tends to decline with time, which partly explains the variable declining efficacy of currently approved disease-modifying therapies among patients (Giovannoni et al., 2017). In the light of this, early treatment with therapies that can reshape the immune system has been shown to improve longevity and reduce the rate of evolving to secondary progressive MS (Grossman et al., 2016). Such variability in response to drugs and the risk of having serious adverse effects is also likely to be influenced by genetic variation (Pistono et
In contrast, the methods which evaluate how a person’s genomic makeup affects their response to therapies could in theory considerably facilitate the decision-making by clinicians, thereby helping patients receive the most optimal personalized treatment (Comi et al., 2017).

In contrast to the advances in treatment for RRMS, it remains the case that there is a lack of effective treatments for the progressive forms of the disease (Salvetti et al., 2015; Feinstein et al., 2015). The currently approved anti-inflammatory drugs, which target the adaptive immune system in the peripheral as opposed to the compartmentalized immune responses in CNS, have little effects on the progressive aspects of MS (Lorscheider et al., 2017). In various phase 2 and phase 3 clinical trials of interferon beta-1, glatiramer acetate, natalizumab, and rituximab, there has been a suggestion that these agents prevent the deterioration of disability (Salzer et al., 2016; Kapoor et al., 2018; Palace et al., 2018), although the benefits of oral fingolimod in slowing disease progression in PPMS seem modest and remain controversial (Ontaneda et al., 2015; Lublin et al., 2016). Currently Ocrelizumab is the only licensed agent for the treatment of primary progressive MS (Montalban et al., 2017), but the phase 3 results with siponimod are also promising (Kappos et al., 2017). The mechanisms by which these drugs influence MS progression are not well understood but are likely to reflect their anti-inflammatory effects rather than on effect on neurodegeneration itself (Beacher-Allan et al., 2018). The efficacy of re-myelinating drugs in MS patients has also been tested in one of the recent randomized trails (Green et al., 2017). Nevertheless, currently the aims of the management for progressive MS centre on minimizing symptoms and improving functions, which include weakness and fatigue, balance and mobility impairment, ataxia, spasticity, depression, pain, reduced cardiovascular fitness, cognitive deficits, bladder dysfunction, and pseudobulbar affect (Thompson et al., 2010; Amato et al., 2013; Gunn et al., 2015; Phe et al., 2016; Otero-Saint Paul et al., 2016; Peyro Motl et. al., 2017). Available data indicate that treatments for the progressive form of MS are likely to be most effective early in the disease, which affirms the importance of early diagnosis. A range of strategies have been suggested to address progressive aspects of the disease including immune-regulatory drugs, aimed at preventing the compartmentalized autoimmunity from developing in the CNS, in addition to approaches targeting innate immune responses and neurodegeneration (Beacher-Allan et al., 2018).

1.2 Epidemiology and evidence for environmental factors

1.2.1 Epidemiology of MS

The total number of people with MS worldwide is estimated to be around 2.3 million, however, this may well be underestimated given the relative lack of data from China and
African countries (Wasay et al., 2006; Thompson et al., 2018). The prevalence of MS is unevenly distributed globally, and generally displays a latitudinal gradient, ranging from 2.1/100,000 in sub-Saharan Africa and 2.2/100,000 in Eastern Asia, to 108/100,000 in northern/central Europe and 140/100,000 in North America (Koch-Henriksen and Sorensen, 2011; Browne et al., 2014). The prevalence also differs between distinct ethnic groups, with risk in Caucasians being relatively higher than in Afro-Caribbean and distinctly higher than in east Asians (Koch-Henriksen and Sorensen, 2010). The uneven distribution of the disease thus likely reflects the influence of both genetic and environmental factors, and the interplay between them.

In addition to the striking geographical distribution of the disease, it has also been noted that MS most often develops in young adults rather than in children or the elderly and affects females far more often than males (Leray et al., 2016). Patients with PPMS generally present at an older age (mean 40) than patients with RRMS (mean 30) and have less of a gender difference (Antel et al., 2012). The gender-preference most likely reflects the higher frequency of relapses found in female patients (Kalincik et al., 2013). Interestingly, it has been indicated by migration studies that the prevalence among immigrants from low-risk to high-risk regions in childhood tend to develop into similar rate as that of the indigenous population and vice versa (Ascherio and Munger, 2016), which support the roles of environmental factors contributing to the aetiology of MS. However, the notion of one single dominant environmental factor causing MS has now been largely disregarded (Marrie, 2004; Hempel et al., 2017). Several environmental factors have been suggested to contribute to the risk of developing MS; most notably childhood exposure to Epstein-Barr virus (EBV) (Ascherio et al., 2010), vitamin D deficiency (Ascherio et al., 2014) and cigarette smoking (Handel et al., 2011).

1.2.2 EBV infection, vitamin D deficiency and smoking

According to the hygiene hypothesis, exposure to infection during early childhood introduce by the order siblings should reduce the risk of developing allergic and autoimmune diseases (Bach, 2002). However, in a longitudinal, population-based cohort study, no relation was found between birth order and MS risk, and there was insufficient evidence supporting the hygiene hypothesis in MS (Sadovnick et al., 2005). On the other hand, it has been suggested that viral infection, particularly of the gastro-intestinal tract and upper respiratory tract, can increase the activity of the disease (Andersen et al., 1993; Buljevac et al., 2002). It has also been suggested that the gut microbiome could be involved in triggering autoimmunity (Baecher-Allan et al., 2018). Among the various proposed infectious agents that might be involved in the aetiology of MS the best supported is undoubtedly EBV. Notably, the
association of EBV infection with MS seems to have a critical time window, with infection during adolescence and early adulthood but not childhood being relevant to the disease predisposition (Olsson et al., 2017). In support of this, an age dependent association between the elevation of EBV antibody titters and the increased risk of MS has also been reported (Levin et al., 2005). Accordingly, the risk is about 15 times higher among individuals infected during childhood when compared with unaffected individuals, and approximately 30 times higher for those infected in adolescence or later (Ascherio, 2013). Primary EBV infection during childhood is typically asymptomatic but sometimes manifests as infectious mononucleosis when the infection occurs during early adulthood (Ascherio et al., 2010). Although the molecular basis is not completely clear, it has been suggested that the immune response against the virus might cross-reacts with myelin as a result of molecular mimicry (Lang et al., 2002). In a recent study, it has been shown that EBV infection can prevent a myelin oligodendrocyte glycoprotein epitope from being degraded, in a manner that involves cross-presentation of the disease-relevant epitopes to CD8+ CD56+ T cells (Morandi et al., 2017). However, evidence regarding whether EBV RNA or protein is present in the CNS of MS patients remains controversial (Sargsyan et al., 2010; Lossius et al., 2014), and a lack of suitable experimental models makes it difficult to confirm any causal role of EBV in triggering the disease. It has been suggested alternatively that, since EBV infection is also known to be correlated with other autoimmune diseases, instead of mimicking specific epitopes, EBV may rather have a more general role in the dysregulation of the immune system (Dendrou et al., 2015). It is clear that the exact role played by EBV in MS predisposition needs to be further clarified.

Besides viral infection, dietary and lifestyle-associated factors have also been shown to contribute to MS predisposition. It has been suggested that the differences in MS prevalence with geographical latitude might at least be partially explained by differences in the amount of sunlight exposure at different latitudes and the corresponding differences in vitamin D level (Simpson et al., 2011; Belbasis et al., 2015). In humans, the major natural source of vitamin D is via its synthesis in the skin from cholesterol, the chemical reaction of which depends on sun exposure. Since the conversion of vitamin D to its active metabolite relies on ultraviolet radiation (UVR), a lack of sunlight exposure would lead to a deficiency of vitamin D (Pantazou et al., 2015), however it turns out to be tricky to distinguish the relative importance of these two factors. Nonetheless, long term studies have suggested that higher serum 25(OH)D level is associated with decreased MS incidence and long-term disease activities (Ascherio et al., 2014); similarly, increased exposure to UVR is also associated with lower risk of MS (Simpson et al., 2011). Early life obesity is also associated with an increased risk of the disease particularly in women; a difference which could be due, at least in part, to the decreased bioavailability of vitamin D among obese individuals (Gianfrancesco and Barcellos, 2016). However, it should be noted that a direct link between the risk of MS and neonatal vitamin D deficiency is currently lacking (Ueda et al., 2014). It has been
suggested that vitamin D may have broad influences on immunity, such as suppressing lymphocyte proliferation and skewing T cells towards regulatory instead of inflammatory responses (Aranow, 2011). Another study has demonstrated that vitamin D could interact with HLA-DRB1*15 gene promoter via an MHC vitamin D response element (Ramagopalan et al., 2009). In the context of these data it has been suggested that correcting vitamin D insufficiency through dietary supplementation could be beneficial in preventing MS and could be easily achieved (Ascherio et al., 2012).

The association between MS and smoking has also been recognized, with smoking conferring a relatively modest influence on MS predisposition (risk ratio 1.48, 95% confidence interval 1.35-1.63) (Handel et al., 2011). The risk related to smoking is more prominent in male than female, and increases with intensity and duration (Belbasis et al., 2015). The speculative mechanisms that have been proposed to explain the association between smoking and MS include the activation of lung resident CD4+ antigen-specific autoimmune T cells or that smoking might compromise of the blood-brain barrier (van der Mei et al., 2011). Like other environmental risk factors discussed above, although the exact molecular mechanisms remain unclear, encouraging patients to quit smoking could be one of the most effective public health interventions to reduce the incidence of MS, and has some support from cross-sectional study on smoking cessation in MS (Ramanujam et al., 2015).

1.3 Genetic factors

1.3.1 Overview

Multiple sclerosis does not follow a Mendelian pattern of inheritance, but rather clusters within families (Goris et al., 2012). The familial recurrence rate falls geometrically with the degree of relatedness, ranging from roughly 25% among monozygotic twins, to 5-3% among dizygotic twins and other first-degree relatives and 1% in second-/third-degree relatives (Robertson et al., 1996; Robertson et al., 1996'; O’Gorman et al., 2013). The pattern of familial recurrence risk in MS suggests a polygenic model in which risk is determined by a single moderate-effect common allele [odds ratio (OR) near 3 or 4] and a great many other alleles of much smaller effect size (OR < 1.5) (Gourraud et al., 2012). It has also been suggested that uncommon genetic variants might make considerable contribution to the heritability of autoimmune diseases, i.e. that the susceptibility may be partly determined by rare variants of large effect size (Goris et al., 2012). However, a recent large multi-cohort study that genotyped over 150,000 coding variants found only a hand full of associated low-frequency variants and none of these had very large effect (International Multiple Sclerosis
Genetics Consortium, 2018). To date no Mendelian forms of MS have been identified (Hollenbach and Oksenberg, 2015), and it has been noted that such variants are unlikely to drive disease expression in complex diseases (Zenewicz et al., 2010). In summary, it is likely that both common and rare variants will influence the risk of MS, with some variants exerting larger effects than others but it is unlikely that any variant exerts a very large effect.

1.3.2 MHC class II molecules

The strongest genetic association with MS maps to the major histocompatibility complex (MHC) region on chromosome 6p21 and was first identified over 40 years ago (Jersild et al., 1972). This 3.6-Mb region contains over 250 genes including the highly polymorphic human leukocyte antigen (HLA) genes (Olerup and Hillert, 1991; Gutierrez et al., 2016). The extensive long-range linkage disequilibrium (LD) that exists across this region has made pinpointing which alleles are driving the association difficult (Gregersen et al., 2006). With the development of high-throughput genotyping technologies, fine mapping efforts have confirmed that the strongest susceptibility signal is almost certainly driven by the HLA-DRB1*15:01 allele (Moutsianas et al., 2015).

The frequencies of HLA-DRB1*15:01 is relatively higher in European and Asian populations (over 10%), but very low elsewhere (Hollenbach and Oksenberg, 2015). The partially dominant risk effect from this allele has been well characterized, with an estimated OR value of 3.92 (Moutsianas et al., 2015). Carriage of this haplotype has been suggested to be associated with disease severity markers, and with the presence of oligoclonal bands in the CSF (Okuda et al., 2009), which has not yet been confirmed. Except for the age at onset, no consistent association was found between any aspect of disease phenotype and HLA variants, suggesting that the later only has weak, if any, influence on MS disease features (Moutsianas et al., 2015).

Apart from HLA-DRB1*15:01, there are multiple additional independently associated HLA and non-HLA alleles in the MHC region. Some exert risk effects, such as the class II alleles HLA-DRB1*03:01, HLA-DRB1*13:03, HLA-DRB1*08:01 and HLA-DQB1*03:02, whereas others exert protective effects, including the class I alleles HLA-A*02:01, HLA-B*38:01 and HLA-B*55:01 (Sawcer et al., 2011; Moutsianas et al., 2015). In the most recent MS genome-wide association study (GWAS) meta-analysis, 32 independent associations in the extended region of MHC were suggested (International Multiple Sclerosis Genetics Consortium, 2017). However, this analysis failed to correct for dominance effects in HLA alleles and to date many of the alleles in this list remain unconfirmed. In addition to marginal effects, some of HLA alleles were also found to modulate the effects of others by the interaction, in particular HLA-DQA1*01:01–HLA-DRB1*15:01 and HLA-DQB1*03:01–HLA-DQB1*03:02 (Moutsianas et al., 2015). However, no evidence was found for
interactions between any of the HLA risk alleles and the non-MHC risk variants; the modulating effects of polygenic epistasis on major HLA allele risk for MS also turned out to be minimal (Moutsianas et al., 2015). Overall the HLA locus accounts for about 10.5% of the heritability of MS (Hollenbach and Oksenberg, 2015).

### 1.3.3 GWAS and non-HLA variants

In addition to HLA, GWAS, which involves genotyping several hundred thousand SNPs throughout the genome in large case-control cohorts, has led to the identification of over 200 common variants of modest effect sizes (International Multiple Sclerosis Genetics Consortium, 2017). These include variants related to IL-2RA and IL-7RA, the first two genes identified outside the MHC regions (Hafler et al., 2007; Gregory et al., 2007). However, it is important to remember that GWAS identify sets of tightly correlated variants rather than individual causal variants; sets of variants that often span a region containing many potential candidate genes (Beecham et al., 2013). As the genomic regions implicated by these risk loci are extensive, the task of identifying functionally relevant genes has turned out to be difficult (Sawcer et al., 2014).

In some complex traits screening for rare protein-coding variants has proven to be helpful in defining candidate genes within risk loci and has thereby provided insights into the mechanisms of the disease (Tasan et al., 2015; Gutierrez-Arcelus et al., 2016). In MS follow-up efforts such as the Immunochip (Beecham et al., 2013) and the Exomechip (International Multiple Sclerosis Genetics Consortium, 2018) have advanced fine mapping and refined understanding of the underlying allelic structure (Cortes and Brown, 2011; Swacer et al., 2014). Most recently, the meta-analysis has identified over 200 modest effect common autosomal susceptibility variants that lie outside MHC and one chromosome X variant (International Multiple Sclerosis Genetics Consortium, 2017; 2018). Most of these variants map to non-coding regulatory regions of genes with immunological function, and many of these variants are also associated with other autoimmune diseases (Farh et al., 2015). It has been suggested that the genetic burden of MS risk alleles might be helpful in a clinical setting (De Jager et al., 2009; Gourraud et al., 2011; Isobe et al., 2016). However, given the low prevalence and the modest familial clustering seen in MS, such approaches are unlikely to be useful in helping predict who will develop MS in large population; except perhaps in extreme circumstances (Sawcer et al., 2010; 2014).

The genetic architecture of MS susceptibility highlights the prominent role of immune system in disease predisposition (Sawcer et al., 2011), and thereby provides some of the strongest primary evidence supporting the view that MS is an immune-mediated disease. The genes implicated by the associated loci identified to date cluster in key immunological pathways...
involved in lymphocyte activation, receptor signalling, cytokine production and co-stimulation (Sawcer et al., 2011; Beecham et al., 2013). In the T cell activation assay analysing individuals with different ancestry, researchers observed an inter-individual variability in the activation of T helper cells, with clear biases according to their ethnic origins (Ye et al., 2014). It is notable that correlation between MS associated variants and regulatory epigenetic feature is almost exclusively found in immune cells (Roederer et al., 2013). In contrast to the overlap in risk variants seen between MS and other autoimmune diseases, there is very little such sharing with other neurodegenerative diseases such as Alzheimer’s or Parkinson’s disease (Baecher-Allan et al., 2018).

In conclusion, the available data suggests that MS susceptibility is determined polygenically, with some variants influencing vulnerability to autoimmunity in general and others defining a CNS specificity, perhaps by influencing the T cell repertoire (Dendrou et al., 2015). Collectively the associated variants identified to date account for about 25% of the heritability, with 20% attributable to common genetic variants and 5% to coding low-frequency variants which cannot be captured by GWAS (International Multiple Sclerosis Genetics Consortium, 2017; 2018). As in other complex genetic diseases, the so-called “missing heritability” most likely reflects as yet undiscovered risk variants with lesser effects and frequency but might also be due to the interactions with environmental factors (Koch et al., 2013; Olsson et al., 2017).

1.4 Pathogenesis

1.4.1 Inflammation

As noted above, the clinical presentation of MS can include a broad array of neurological signs and symptoms, depending upon both the location and extent of the CNS lesions. For example, it has been shown that cortical inflammatory lesion load is associated with the physical and cognitive disability progression over time (Calabrese et al., 2012). While the progressive form of MS involves diffuse immunological processes and neurodegeneration, the underlying pathological process in RRMS is multifocal and primarily inflammatory (Ciccarelli et al., 2014). In the early stages of the disease the pathology is characterized by perivenular infiltration of B and T lymphocytes, which migrate across the compromised blood-brain barrier (Lucchinetti et al., 2005). Whereas in the progressive stages, chronic demyelination and trans-synaptic degeneration develop, perhaps as results of microglial activation (Lucchinetti et al., 2011; Calabrese et al., 2012). The pathogenesis of MS is thus a gradual process, that involves inflammation, neurodegeneration and a failure of restoration, with inflammation being the trigger of the pathological cascade.
Given that the inflammation in MS affects the CNS, it has been suggested that the lymphocytes are selectively recruited by autoantigens specifically expressed in the CNS (Ransohoff and Engelhardt, 2012), although presently how the immune responses are initiated and maintained in MS is unclear, and the antigen specificity is unresolved. As no predominant exogenous risk factor has been identified so far, it remains debated whether MS is triggered in the periphery or centrally. According to the outside-in model, during the initial stages of lesion formation, autoreactive lymphocytes activated in the peripheral cross the blood-brain barrier and lead to an aberrant immune response against CNS (Hohlfeld and Wekerle, 2004). Under the alternate inside-out model it is hypothesised that some primary CNS infection or other neuronal disturbance leads to the release of CNS antigens which subsequently trigger an autoimmune response targeting CNS (Trapp and Nave, 2008). Both scenarios result in a deleterious cycle of CNS tissue damages, release of antigens to the peripheral, priming of peripheral immune responses, and invasion of autoreactive lymphocytes into CNS (Friese et al., 2014). As lymphocytes, plasma cells, and macrophages accumulate, the inflammatory cytokines they release further amplify the immune response, recruiting and activating naïve microglia and ultimately leading to neuronal dysfunction and cell death (Heneka et al., 2014). However, the association between inflammation and active lesions does not exclude the possibility that the inflammation activity is secondary, and the primary event is tissue damage. Nonetheless, both genetic analysis and pathological findings have pointed to the prominent roles played by immune system in MS pathogenesis.

### 1.4.2 Neurodegeneration

The inflammatory activities which predominate in the early stages are believed to trigger the cascade of events, including microglia activation, chronic oxidative injuries, mitochondrial damage and accumulation of toxic iron that drive progressive neurodegeneration (Mahad et al., 2015). In line with this, evidence suggests that the extent of active demyelination and axonal damage is positively related to the extent of lymphocyte infiltration (Frischer et al., 2009). During the past decade, our understanding of the molecular mechanisms behind neurodegeneration in MS has advanced considerably. It is now recognized that microglia in active MS lesions form close contact with myelin sheaths of the degenerating neurons, and express molecules associated with the production of reactive oxygen species (ROS) (Ontaneda et al., 2017). In cases of chronic oligodendrocyte damage and axonal demyelination, axons lose structural and trophic support, which exposes them to ROS and inflammatory mediators in the microenvironment (Lucchinetti et al., 2005). These also give rise to changes of sodium channels expressed along demyelinated axons (Craner et al., 2004; Friese et al., 2007), and consequently, saltatory impulse conduction is replaced by continuous conduction, resulting in accumulation of sodium in the cytoplasm. These changes lead to the
reverse operation of the sodium-calcium exchanger, resulting in calcium overload, activation of calpains and axonal cytoskeleton proteolysis (Trapp and Stys, 2009).

In addition to the changes noted above, the oxidative injury also results in mitochondrial dysfunction, which includes impaired mitochondrial transport, mitochondrial DNA mutation, and increased ROS production by mitochondrial (Haider et al., 2011). These lead to increased cellular energy demand, failure of energy production, imbalance of ionic homoeostasis and accumulation of various cytotoxic species (Lassmann et al., 2012). Given the geometry of the neuron-axon structure, which makes efficient distribution of ATP production units challenging, axons are particularly susceptible to mitochondrial dysfunction (Friese et al., 2014). The consequences of mitochondrial damage are twofold: firstly, it leads to energy deficiency and histotoxic hypoxia, which amplify the energy deficiency caused by inflammation, and result in axonal transection and cell death when the injury passes certain threshold; secondly, it disturbs respiratory chain function and liberates electrons, which react with oxygen, causing vicious cycles in the process of oxidative damage (Fischer et al., 2012; Mahad et al., 2015). In addition to the age-dependent accumulation of iron in brains, divalent cations (e.g. Fe$^{2+}$ and Cu$^{2+}$) liberated from damaged oligodendrocytes and myelin in MS lesions also act as an amplifier of oxidative injury by converting H$_2$O$_2$ into highly reactive hydroxyl molecules via the Fenton reaction (Mahad et al., 2015). Collectively, the increased energy demand and reduced ATP production induce a state of chronic hypoxia that results in the necrosis of demyelinated axons.

Ultimately apoptosis and DNA damage lead to extensive axonal and neuronal loss resulting in the progressive phase of MS (Kornek et al., 2000). The diffuse infiltration of lymphocytes and monocytes that occurs in the compartmentalised inflammatory lesions can also cause widespread white matter injury (Howell et al., 2011). In addition to the compartmentalized immune responses, dysregulation of astrocyte and microglial activation also contribute to the diffuse tissue injury (van Horssen et al., 2012; Schreiner et al., 2015). Although axons and neurons are generally preserved in the early stages, the gradual neuro-axonal loss ultimately results in brain atrophy and glial scars formed by astrocytes in white matter (Kutzelnigg et al., 2005), which contribute to the progression of MS at later stages.

### 1.4.3 Failures of re-myelination

Apart from neuro-axonal loss, astrocytic gliosis and demyelination, which are widely recognised as the hallmarks of MS pathology, the failure of re-myelination has also been recognized as a contributor to the disease progression (Popescu and Lucchinetti, 2012; Lemus et al., 2018). It has been demonstrated in mouse brain that prompt re-myelination could protect axons against axonal lost associated with demyelination (Irvine and Blakemore, 2008). However, although oligodendrocyte precursors are abundant in lesions, the presence
of inhibitory factors and a lack of pro-myelination factors result in incomplete re-myelination in human (Chang et al., 2002). The newly formed myelinated internodes tend to be shorter and thinner than the original ones, and the axonal membrane demonstrates an increase of sodium channels following demyelination (Franklin and Ffrench-constant, 2008). As a result, the partially restored nerve conduction is less saltatory and more continuous, which leads to an increased energy demand and consequent changes in mitochondria (Ontaneda et al., 2017). In support of this, symptoms such as physiological fatigue and distortions of sensation could also be explained by the partially demyelinated axons which are incapable of transmitting fast trains of impulse and discharge spontaneously (Mahad et al., 2015). Moreover, the increased mechanical sensitivity, spontaneous discharge in facial nerve neurons and the failure of conduction in partially demyelinated pathways may also explain Lhermitte’s symptom, myokymia and Uhthoff phenomenon (Trapp et al., 2009). Thus, although compensation may occur spontaneously in MS, cycles of demyelination and unsuccessful re-myelination exhaust the capacity for tissue repair, and thereby account for various manifestations of MS.

In summary, a series of immunological and neurodegenerative events, which act in concert and change in their relevance as the disease progresses, engender and sustain CNS damages during the development of MS. The acute inflammatory injuries mediated by infiltration of lymphocytes, the activation of microglia, the chronic degeneration of demyelinated axons, and a failure of re-myelination jointly contribute to the irreversible disability in MS. Therefore, a combination of anti-inflammatory, neuroprotective and regenerative strategies may all be beneficial in the treatment of MS in the future.

1.5 Immunopathology of MS

1.5.1 Overview

As outlined in previous sections, genetic and pathological studies have pointed to inflammation as the driving force in the pathogenesis of MS. It is clear that both innate and adaptive immunity, contribute to the pathogenesis of MS (Hemmer et al., 2015). The two major cellular components of the adaptive immune system, T cells and B cells, can be further classified into different immune cell subtypes by various surface markers. B cells develop in the bone marrow, whereas T cells mature in the thymus and interact with antigen presenting cells (APCs) for further activation (Crotty, 2015). Highly variable somatic rearrangements in α and β T cell receptor (TCR) chain genes enable T cells to recognize antigens and react to a wide range of antigens, and in turn the relevant T cells being activated when their cognate antigen is presented in conjunction with MHC class II molecules specialised APCs
This interaction is supported by a series of co-receptors such as CD3 and CD4 (Chen and Flies, 2013). In addition, the activation of CD4+ T cell also necessitates additional co-stimulation from CD80/CD86 expressed by APCs, which are the ligands for CD28 and CTLA4 on T cells (Chen and Flies, 2013; Yuseff et al., 2013). B and T cell responses directed against self-antigens are limited by a range of central and peripheral self-tolerance mechanisms such as the elimination or inactivation of self-reacting clones (Gonsette, 2012). During the initiation of MS and the progression of the disease, these self-tolerance immune mechanisms are compromised, and the activated microglia and macrophages induce autoreactive responses of T and B lymphocytes by secreting pro-inflammatory cytokines, chemokines and free radicals, which amplify the autoimmunity against CNS.

### 1.5.2 T cells

The long-appreciated associations of HLA with MS and the presence of T cells within CNS lesions even in early stages of the disease support the notion that T cells play a critical role in the susceptibility of MS. In accordance with this notion, an expression quantitative trait locus (eQTL) study using purified monocytes and CD4+ T cells has demonstrated that the T cell specific eQTLs among susceptibility alleles are over-represented in autoimmune diseases (Raj et al., 2014). Moreover, in animal MS models, the autoreactive myelin-specific CD4+ T cells has long been recognized as a key pathogenic effector and a major therapeutic target (Hohlfeld et al., 2016). It has been proposed that, once activated in the periphery by the myelin protein-derived antigens, the autoreactive CD4+ T helper type 1 (Th1) cells and T helper type 17 (Th17) cells, which are the main T cell subtypes implicated in the disease, would upregulate their expression of cytokine receptors and secretion of pro-inflammatory cytokines (Dendrou et al., 2015). The therapeutic mechanisms of some of the first-line disease-modifying therapies (IFN-β, glatiramer acetate and dimethyl fumarate) are also thought to be associated with skewing T cell differentiation away from these subsets and towards a T helper type 2 (Th2) cell phenotype (Dendrou et al., 2015), which highlights the pathological roles played by these T cell subtypes. However, conflicting results also exist, as antibodies targeting the p40 subunit shared by IL-12 and IL-23 (ustekinumab), which are involved in Th1 and Th17 cell differentiation respectively, didn’t pass the phase II clinical trial (Segal et al., 2008). Such failure might be due to the incompatibility of EAE models with human studies or the diminished importance of the two cytokines at later stages of the disease.

In comparison with CD4+ T helper cells, the precise contribution of CD8+ T cells is less clear. CD8+ T cells are more frequently found in the cortical demyelinating lesions of the white and grey matter than CD4+ T cells, and their numbers correlates with the degree of axonal damage (Frischer et al., 2009). The myelin-specific CD8+ T cells could be readily activated in CNS, a
quarter of which were found capable of producing IL-17 and IL-18 in the active lesions of MS patients (Willing et al., 2014). The long-lasting depletion of these autoreactive CD8+ T cells after autologous hematopoietic stem cell transplantation (aHSCT) implies that they might also play a crucial role in the disease pathogenesis, although the exact mechanisms remain unclear (Abrahamsson et al., 2013).

In addition, other relevant cell types might also participate in facilitating the infiltration and activation of T cells that cause damage in CNS. For instance, it has been shown that activated T cells could adhere to the endothelium and migrate across the blood-brain barrier by upregulating their expression of adhesion molecules and inducing reciprocal changes in endothelial cells (Dendrou et al., 2015). Once in the CNS, the autoreactive T cells could be further activated by local APCs such as microglia, which results in recruitment of inflammatory cells and activation of resident astrocytes and microglia (Kambayashi and Laufer, 2014). These events infer that complex multi-cellular and environmental interactions are critically important contributing to MS pathogenesis, among which the interaction with B cells seems most prominent.

1.5.3 B cells

Although historically T cells have been considered as the most critical pathological drivers in MS, the effectiveness of selective B cell depletion with anti-CD20 antibodies (rituximab and ocrelizumab) in limiting disease activity has also highlighted the as-yet incompletely understood roles played by B cells in MS pathogenesis. Since massive expansion of antigen-specific lymphocytes from the precursor cells in lymph nodes requires the presence of professional APCs, it is well-appreciated that complex interplay exists between T cells and B cells. It has been demonstrated that MS patients have an increased number of B cells, most of which are memory B cells and plasmablasts, and that the number of plasmablasts in CSF correlates with oligoclonal antibodies and inflammatory activities (Cepok et al., 2005). In the phase III clinical trials (OPERA I and II) of ocrelizumab, the humanized monoclonal antibody selectively depleting CD20+ B cells resulted in lower rates of relapse and lesion progression in patients as compared with IFN-β (Hauser et al., 2017). In addition, treatment with potent immunosuppressive therapy and autologous hematopoietic cell transplantation have also been found to be effective among patients who had failed to benefit from other disease-modifying therapies (Nash et al., 2017). Such evidence supports the notion of B cells’ involvement in MS and the necessity for more comprehensive understanding of the interactions between B cells and T cells.

Interestingly, most of the immune therapies for RRMS originally designed to target T cells are now known to impact B cell responses (Li and Bar-Or, 2018). Given that the
autoantibody-producing, terminally differentiated plasma cells are not depleted by anti-CD20 antibodies, and that the abnormal antibody profiles in the CSF remain largely unaffected after the depletion, the relevant mechanism is believed to be primarily dependent upon antibody-independent roles of B cells (Hauser et al., 2008; Barr et al., 2012). Studies have also found that memory B cells from some RRMS patients respond to myelin-associated protein by eliciting T cell proliferation, IFN-γ secretion and Th17 responses against neuro-antigens (Harp et al., 2010; Ireland et al., 2016). In addition, an increased frequency of the pro-inflammatory GM-CSF-expressing memory B cell subtype in MS patients has also been noted (Li et al., 2015). These imply the relevance of exploring the various B cell subtypes which are functionally distinct.

Given the limited effectiveness of currently available disease-modifying treatments, cell-based therapies have generated considerable interest as novel strategies for immune regulation in MS (Scolding et al., 2017). As mentioned previously, since the success of the B cell depletion therapy highlighted the immunomodulatory functions of B cells, it has been realised that the expression of MHC-II and the co-stimulatory molecules such as CD80 and CD86 maybe crucially involved in the aberrant activation of T cells in autoimmune diseases (Chen and Flies, 2013). The elimination of pro-inflammatory B cells could potentially lead to reduced Th1 and Th17 cell responses, thereby decreases disease activity (Dendrou et al., 2015). Thus, the abovementioned findings warrant further investigations on the mechanisms related to B cells, including effector and regulatory functions of B cells. Given that CD40 ligation is crucial for B cell activation, the following section focuses on this specific B cell activation pathway and the rationales for choosing it as the activation method in my study.

### 1.5.4 CD40-CD40L pathway

CD40, which belongs to the tumour necrosis factor (TNF) receptor superfamily, is constitutively expressed on APCs, such as B cells, microglia and macrophages, whereas CD40L is expressed on CD4+ T cells following TCR-MHC engagement (Elgueta et al., 2009). CD40-CD40L interaction, when in combination with B cell receptor (BCR) signalling, is a critical signal for B cell proliferation, activation, immunoglobulin production, isotype switching, memory B cell transformation and germinal centres formation (Guo et al., 2017). In addition, engagement of CD40 by CD40L can induce upregulation of CD80, CD86, MHC class I and class II, and promote the secretion of pro-inflammatory cytokines, which further drive T cell differentiation and activation (Summers deLuca and Gommerman, 2012). It has been reported that stimulating memory B cells via BCR and CD40 in vitro mimics T cell dependent antigen stimulation (Chen and Flies, 2013). Enhanced proliferation and over-production of IL-6, IFN-γ, IL-17 and TNF-α is seen in treatment-naïve RRMS patients as compared with that in healthy controls, whereas no difference was found in the expression of CD40 on B cells between MS patients and healthy controls (Duddy et al., 2004; Ireland et al.,
These imply that the CD40 pathway in B cells might be more sensitive to CD40 stimulation in MS patients.

The CD40 pathway has also been implicated in a variety of other autoimmune diseases and disease models and is indicated as a promising therapeutic target (Huber et al., 2012; Crotty, 2015). In the mouse model of MS, neutralizing CD40L with antibody was found to be effective in preventing experimental autoimmune encephalomyelitis (EAE), although its clinical potentials remain controversial due to the side effects (Gerritse et al., 1996; Kawai et al., 2000). Interestingly, although it is more often the case that the same variants influence the susceptibility of several autoimmune diseases in the same direction, some variants are protective for one disease but act as risk factors for others. For example, the allele of the CD40 SNP rs4810485 increases the risk of rheumatoid arthritis, but reduces the risk of MS (Raychaudhuri et al., 2008; Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene), 2009). These observations imply that the genetic basis of autoimmune diseases is more complex than merely enhancing the activation of immune cells, and that the relevant mechanisms are likely context-dependent.

In work completed in my supervisor’s lab prior to me joining the group it was shown that the risk allele of rs4810485 lowered the expression of CD40 on B cells (Smets et al., 2018). These findings imply that the dysregulation of CD40 signalling might contribute to the hyperresponsiveness of B cells when stimulated, and that CD40 might also be involved in regulatory mechanisms. In support of this, other researchers have shown that MS patients displayed considerably higher phosphorylated NF-kB level in memory and naïve B cells than healthy controls after CD40 stimulation, which could be partially modulated by glatiramer acetate therapy and IFN-β -1a/Cellcept combination therapy, and that MS risk loci are enriched for the binding sites of NF-κB (Chen et al., 2016). In line with these observations, it has also been found that two genetic variants are associated with enhanced NF-κB expression and signalling response after stimulation with TNF-α in MS patients (Housley et al., 2015). These findings highlight the relevance of investigating the genotypic influences on B cell when activated via CD40 under in vitro conditions.

1.5.5 Regulatory B cells

It is now widely accepted that, apart from producing antibodies upon activation, B cells also have antibody-independent functions, such as producing pro-/anti-inflammatory cytokines in response to stimuli (Bao and Cao, 2014; Li et al., 2017); for example, IL-10-producing B cells represent a major subset of regulatory B cells (Bregs) that suppress autoimmune responses (Filatreau et al., 2008; Rosser and Mauri, 2015). It has been suggested that the
regulatory capacity of B cells is dependent on their production of IL-10 and their ability to interact with pathogenic T cells. The clinical efficacy of B cell depletion therapy may be due in part to the repopulation of newly differentiated Bregs (Mauri and Ehrenstein, 2008; Mauri, 2010). Given the relevance of Bregs in the regulation of immune responses, it is plausible that associated genetic risk factors exert their effects primarily in this B cell subtype.

It is known that IL-10 has a central role in autoimmunity and infection by regulating the immune response to prevent excessive injuries to the host (Saraiva and O’Garra, 2010). In both human and mouse model, IL-10 has been found to attenuate the antigen presenting capacity of B cells by down-regulating CD86 expression, and thereby suppresses T cell proliferation (Matsumoto et al., 2014; Nova-Lamperti et al., 2016). The increase in CD86 expression in ex vivo B cells that we found in our previous analysis might be associated with IL-10 and IL-10-producing Bregs. Furthermore, Bregs have also been shown to induce the differentiation of regulatory T cells that produce IL-10 and TGF-β and suppress Th1 and Th17 responses (Flores-Borja et al., 2013). Unlike in mouse models, currently no consensus has been reached regarding the surface markers used to define the Breg population in humans, although a variety of cell surface markers have been suggested (Yanaba et al., 2008; Blair et al., 2010; Khoder et al., 2014). The role of CD24hiCD38hi transitional B cells have turned out to be most promising, given its reliability in capturing the human IL-10+ Breg populations, and were used in this study (Blair et al., 2010).

Lastly, it has been suggested that the induction of Bregs are also critically dependent on CD40-CD40L pathway (Vitale et al., 2010). Recent study has found that tolerant transplant patients exhibit higher IL-10 positivity among B cells after CD40 activation and reduced BCR signalling than healthy controls (Nova-Lamperti et al., 2017). By contrast, a reduced number of Bregs with impaired regulatory capacity were found in patients affected by autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) (Bankó et al., 2017). In the context of these data it seemed likely that activating B cells using CD40 will provide disease relevant information.
Chapter 2  Previous works and study design

2.1  Previous works  

The 2011 MS GWAS identified 57 non-MHC associated variants, three of which were identified within a 360kb region on chromosome 3q13 (Sawcer et al., 2011). This region contains several genes - IQCB1, EAF2, SLC15A2, ILDR1, CD86, CASR, CSTA and CCDC58. Given the critical role of CD86 within the adaptive immune system this seemed like a particularly strong candidate. It is notable that none of the lead associated SNPs in this region (rs9282641, rs4285028 and rs4308217) is correlated with any known missense coding variant in any of the nearby genes. Thus, like most other GWAS identified SNPs, it seemed more likely that these variants exert their influence by affecting gene expression rather than gene function (Sawcer et al., 2014).

Given that genetic effects on regulation are often cell type and cell state specific, and that CD86 is primarily expressed by APCs, Dr Fiddes (my predecessor) started his research by measuring CD86 expression in different subtypes of B cells and monocytes using flow cytometry and correlated these with the associated genotypes. In addition, he also assessed the soluble forms of CD86 in serum and the expression at RNA level in each of the cell types. His work was based on peripheral blood mononuclear cells (PBMCs) from 162 healthy volunteers which were collected from Cambridge BioResource based on their genotype to provide a balanced representation of the possible allele combinations across the three associated CD86 SNPs. The surface expression of CD86 was successfully measured in B cells from 145 subjects and in monocytes from 144 subjects. Overall, Dr Fiddes found a positive association between the carriage of rs9282641 risk allele (G) and the proportion of B cells expressing CD86 (p=0.0015), whereas neither of the other two SNPs were found associated with CD86 expression in B cells, and none of the three SNPs were associated with CD86 expression in monocytes. No statistically significant association with any of the three SNPs were found in the soluble CD86 in serum or mRNA expression experiments. After I genotyped the DNA samples collected by Dr Fiddes at the SNP rs4810485, we found a negative association between CD40 expression and the carriage of MS associated risk allele T in B cells, with naïve B cell subtype having the lowest p value, which would be addressed in detail in the results chapter. This is in line with a previous study, which has also found that the carriage of MS CD40 risk allele results in significantly lower expression level of CD40 on B cells (Field et al., 2015).
In addition, Dr Fiddes also examined CD86 surface expression in the main subtypes of B cells and monocytes, namely naïve (CD19^+CD27^-), class switched memory (CD19^+CD27^-IgD^+) and non-class switched memory (CD19^+CD27^-IgD^-) B cells, and classical (CD14^hiCD16^-), non-classical (CD14^loCD16^+) and intermediate (CD14^hiCD16^+) monocytes. No association between genotypes and the relative proportions of these subtypes within the B cell or monocyte populations was found for any of the three SNPs. However, among naïve B cell subtypes, it was found that the carriage of the risk allele at rs9282641 (G) is positively associated with the proportion of cells expressing CD86 (p=0.000056). In the other B cell subtypes, although a trend towards association between carriage of the risk allele of rs9282641 (G) and the proportion of cells expressing CD86 was found, which was nominally significant in class switched memory B cells, it did not survive the correction for multiple testing.

In summary, these previous efforts have shown that, across different B cell subtypes, naïve cells (CD19^+CD27^-) displayed the most significant associations between the expression of CD86/CD40 and their respective SNPs. These results have not only confirmed the relevance of the two MS GWAS SNPs in association with the expression of CD86 and CD40, but also refined our target cell type to naïve B cells as the population of most interest.

2.2 Overview of the research questions to be addressed

Despite the success of GWAS in expanding the number of identified loci associated with MS, a comprehensive model of disease pathogenesis and an understanding of the mechanisms by which genetic risk variants confer susceptibility to the disease is currently missing. In general, genetic variation may influence gene transcription, DNA methylation, histone modifications, mRNA translation/stability, and protein production (Maurano et al., 2012). These cellular processes may affect or be affected by immunophenotypes, which in turn can influence the manifestation of the disease. Thus, to better appreciate the complexity of the association between the GWAS-identified variants and the pathogenesis of MS, a few questions need to be further addressed.

As in cases of most disease-associated SNPs identified by GWAS, it is still unclear how the genes are affected by the variants and which are the causal variants (Tasan et al., 2015). Since over 95% of the GWAS-identified association signals are located within “gene deserts” that don’t contain any protein-coding genes, it is likely that most of the underlying causal variants influence regulatory elements, such as enhancers/repressors (Zenewicz et al., 2010; Maurano et al., 2015). This view is supported by the findings that GWAS SNPs, especially those with lowest P value, are more likely to be enriched in promoter-interacting fragments (Mifsud et
al., 2015). In addition, such elements can also exert long-range effects on very distant genes, and many have context-specific effects (Schoenfelder et al., 2015).

Thus, a critical follow-up step is to identify the in vivo and ex vivo cellular and molecular immune traits affected by the risk variants, including cell-type abundances, cell proliferation, signalling response, and cytokine production. As described above, previous efforts have shown that the two MS associated variants that respectively influence CD86 and CD40 expression, do so primarily in naïve B cells. To further investigate the questions raised, the following three issues need to be addressed: defining the relevant cell types and cell states that are implicated in the MS variants, identifying the molecular pathways involved, and elucidating the downstream effects of the genetic factors.

2.3 Relevant Cell types/states

The human immune system is composed of hundreds of cell subtypes, the functions of which reflect differences in intracellular gene regulation (Maecker et al., 2012). Due to the polygenic nature of MS, it has been hypothesized that most genetic risk/protective alleles exert their effect on a limited number of cell types, and influence gene regulation in a cell type specific manner (Gutierrez et al., 2016). Also, since each cell type may take on a variety of states in response to different stimuli, and that a considerable proportion of eQTLs are exclusively found in very specific stimulated states (Nica and Dermitzakis, 2013), it may be necessary to ascertain which cellular states are relevant to the genetic regulatory variants.

Notably, about two-third of the genes identified in MS GWAS are expressed by APCs, including MHC class II, CD86 and CD80 (Sawcer et al., 2011), whereas the association of their receptors/ligands such as TCR, CD28 and CTLA expressed by T cells are much underrepresented in various GWAS studies (Beecham et al., 2013). Such significant enrichment for cell type specific expression of genes in MS risk loci highlight the pertinence of defining the cell types involved. The correlated variants could either be local (cis eQTLs) or distant (trans eQTLs), typically defined as being more than 5Mb away or on a different chromosome (Brynedal et al., 2017). Therefore, to understand the mechanisms by which individual alleles influence gene expression, it would certainly be helpful, and maybe essential, to define the cellular subtypes and states that are the most relevant to the disease.

In order to more clearly define the B cell sub-types most strongly influenced by the MS associated SNPs I decided to include analysis of the CD24 and CD38 surface markers, which characterize the transitional B cell population that are believed to have regulatory capacity (Mauri and Bosma, 2012; Simon et al., 2016), and the CD24CD38+ plasmablast. In addition,
it has also been suggested that human CD25+ B cells may have some immunomodulatory properties and have higher expression of co-stimulatory molecules than CD25- B cells (Brisslert et al., 2006; Amu et al., 2007; de Andrés et al., 2014). In view of these considerations I also included CD25 in my analysis. Given that epigenetic regulation of expression is often both cell type and cell state specific I also hypothesised that perhaps the influence of genotype on expression might be increased (or reduced) in stimulated rather than resting cell. To explore this, I wanted to use a stimulation method that would perhaps most closely resemble the stimulation that might occur in vivo. I therefore used CD40L-transfected L cells to co-culture with PBMCs for 3 days, with un-transfected L cells used as negative controls. With such experimental designs, I reasoned that I would be able to, firstly, investigate a wider range of B cell sub types than had been considered by Dr Fiddes and secondly, to establish whether stimulation resulted in any substantial change the impact of associated variants on expression.

2.4 Molecular pathways involved

I also reasoned that relying exclusively on cell surface expression might miss important functional heterogeneity within B cells. Accumulating evidence has suggested that the dysregulated cytokines secreted by B cells play an important role in the pathogenesis of MS (Mauri and Bosma, 2012; Rosser et al., 2014), a disease in which the deficiency of regulatory cytokines and the exaggerated production of pro-inflammatory mediators have been well appreciated (Li et al., 2015). It therefore seemed logical include some degree of cytokine analysis.

Among the long list of potential cytokines that might be considered, the immunoregulatory cytokine IL-10 seemed particularly logical (Masanori et al., 2014). It has been described that tolerant kidney transplant patients exhibited higher percentages of IL-10-producing transitional B cells after dual-BCR/CD40L activation compared to healthy controls (Nova-Lamperti et al., 2016). Conversely, following stimulation with CD40, transitional B cells isolated from SLE patients have demonstrated lower positivity of IL-10 relative to healthy controls, and their regulatory capacity was functionally impaired (Blair et al., 2010). These might be due in part to the fact that the cytokine production and response of B cells after activation might be affected by their immediate microenvironment and overall immune propensity (Vazquez et al., 2015). Besides IL-10, other cytokines have also been suggested to be involved in MS. For example, it has been shown that combined CD40 and BCR stimulation can enhance human B cells’ secretion of TNF-α, which is implicated in inducing Th1 and Th17 responses and inhibiting Treg suppression (Duddy et al. 2007; Iwamoto et al., 2007). Furthermore, it has also been shown that B cells from MS patients produce significantly higher level of TNF-α upon such mode of activation (Duddy et al. 2007). In
addition, GM-CSF expressing B cells were found to be notably increased in MS patients, and co-express TNF-α and IL-6 but not IL-10 (Li et al., 2015). By contrast, IL-6 has been ascribed with both pro- and anti-inflammatory properties, and it has been shown that IL-10-producing B cells can be induced by IL-6 (Rosser et al., 2014), although in EAE IL-6 producing B cells have been shown to induce T cell polarization to Th17 (Vazquez et al., 2015). It has also been found that B cells secret higher level of IL-6 in MS patients than in healthy controls, which could be normalized after Rituximab treatment among reconstituting B cells (Barr et al., 2012), and that blocking IL-6/IL-6R signals could attenuate the secretion of antigen-specific auto-antibodies by B cells from SLE patients (Xiong and Lahita, 2011).

These studies implied that cytokines such as IL-10, TNF-α, GM-CSF and IL-6 are very likely be implicated in the molecular pathways relevant to MS pathogenesis, although whether these cytokines are produced by identical or different B cell subtypes is not clear. Moreover, these cytokines might also be involved in the modulation of the surface expression of CD86 and CD40. For example, it has been found that in both humans and in mouse model IL-10 produced by B cells can attenuate the antigen presentation capacity of B-cells by down-regulating CD86 expression on their surface (Mauri and Bosma, 2012). To better explain the genotypic difference of CD86 and CD40 expression under in vitro condition, it is necessary to include the relevant intracellular markers. Therefore, in addition to surface markers I also assayed the cytokines IL-10, TNF-α, IL-6 and GM-CSF.

2.5 How T cells may respond to these genetic factors

Traditionally MS has been considered as a T cell driven autoimmune disease (Dendrou et al., 2015), however, as discussed above, evidence is now emerging which suggests that B cells may also be important, or even primary, perhaps by orchestrating T cells differentiation in a pro-inflammatory and self-reactive direction (Dendrou et al., 2015; Jackson et al., 2015). It has been suggested that dysregulated signalling of B cells could be sufficient to induce the initial break in tolerance and promote CD4+ T cell activation (Jackson et al., 2015). In this context, any genotypic effects on B cell expression of CD86 and CD40 should influence T cell expansion and differentiation.

Contrary to the late involvement of T cells in immune responses, B cells could rapidly capture antigens via BCR without cleaving them into peptides, which enable them to function at an early phase and facilitate the recruitment of regulatory T cells (Tregs) till they become functional (Berthelot et al., 2012). A variety of cytokines produced by activated B cells could influence T cell differentiation. For instance, it has been found that the balance of T cell differentiation into Th17 cells and Tregs depends on IL-6 signalling, overproduction or
dysregulation of which could lead to autoimmune diseases including MS and RA (Kimura and Kishimoto, 2010). Breg production of IL-10 has been shown to inhibit T-cell proliferation and differentiation into Th1 and Th17 cells, and induce regulatory T-cell differentiation, thereby reducing the production and release of pro-inflammatory cytokines and repress the autoreactive responses (Flores-Borja et al., 2013).

Furthermore, CD40-activated B cells have been found to be more efficient at inducing Foxp3+ regulatory T cells than other APCs; interaction between T cells and B cells being dependent on cellular contact via CD28 on T cells and CD86/CD80 on B cells (Chen and Jensen, 2007). In a study using EAE, researchers found that B cell deficiency results in delayed regulatory T cell response in the CNS, and that reconstitution with wild-type B cells restores the expression of IL-10 and Foxp3, whereas CD86/CD80 deficient B cells do not (Mann et al., 2007). It has been suggested that regulatory T cells can be induced by the interaction with CD40-activated naïve B cells via CD80 and CD86 (Mauri, 2010; Lemoine et al., 2011). These findings suggested that the interaction between B cells and T cells are highly dependent on these co-activation molecules.
Chapter 3  General methodology

3.1  Human subjects and sample collection

In total 136 healthy volunteers were recruited from the Cambridge BioResource. The study was approved by the research ethic committee (15/SC/0087) and all subjects given valid written informed consent. 108 samples were first collected, with the date of attendance randomized regarding the genotypes at rs9282641, rs4810485 and rs1131265, the combination of which had been balanced by the Cambridge BioResource. Since the minor allele at rs9282641 has very low frequency among general population (5%), 28 homozygous subjects were recruited based on their genotype at rs9282641 and analysed as matched pairs (14 pairs). All data collection was undertaken fully blinded to genotype group. 50ml of peripheral venous blood was collected from each volunteer and was kept in EDTA- and lithium heparin- containing tubes for DNA genotype analysis and cell culturing respectively. 5ml serum was collected in clotting activator serum tubes (S-Monovette, Sarstedt, Germany) and isolated by centrifugation for the first 108 subjects and stored at -80°C for batched ELISA analysis. All samples were collected in the morning and fully processed within four hours of collection.

3.2  Mouse fibroblast cell line

Mouse fibroblast L cells stably transfected with human CD40L (CD154) (kindly provided by Dr. D. Hodson) were grown in a humidified atmosphere containing 5% CO₂ at 37°C in T25 culture flasks. The medium used was Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, Life Science Ltd., St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (p/s, 100 U/mL; 0.1 mg) (both from Sigma-Aldrich). The efficacy of transfection was confirmed by mycoplasmid test, with the CD40L expression routinely checked by flow cytometry after staining with CD154-PE mAb. CD40L expressing and un-transfected control L cells were irradiated for 40 minutes (10,646 rads) prior to the co-culturing with PBMCs or B cells to prevent overgrowth. Repeated passaging of L cells using Trypsin/EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, Life Science Ltd., St. Louis, USA) was performed when the confluence of cells reached 90%.
3.3 PBMC isolation and activation

Human peripheral blood mononuclear cells (PBMCs) were isolated freshly by Ficoll-PaquePLUS (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Blood collected in lithium heparin tubes were transferred in a sterile vessel and diluted with phosphate buffer saline (PBS) (1:1 dilution). 15ml of Ficoll medium was pipetted in 50ml falcon tubes. The diluted blood was carefully layered over the Ficoll medium to obtain a clear separation between the two. The falcon tubes were centrifuged at 300 x g for 30 min at 4°C with maximum acceleration and the break function switched off. The PBMC layer located at the Ficoll-serum interphase was aspirated under visual control and transferred into a new 50ml falcon using a 10ml sterile plastic pipet. PBMCs were washed twice with PBS (centrifuged at 350 x g for 10 min) before being counted.

Isolated PBMCs were divided for ex vivo and in vitro flow cytometry analysis. For the in vitro analysis, all cells were cultured in RPMI-1640 (Sigma-Aldrich) medium supplemented with L-Glutamine (2 mM), p/s (100 U/mL; 0.1 mg) and 10% FBS (Sigma-Aldrich). PBMCs were mixed with irradiated CD40L-transfected/un-transfected L cells at a ratio of 20:1, and were cultured in 96-well U-bottom plates (1x10⁶ PBMCs/well) for 72 h in a humidified atmosphere containing 5% CO₂ at 37°C.

For the paired samples, 4x10⁶ freshly isolated PBMCs from both individuals were plated in U-bottom 96-well plate at 2x10⁶ per well, with each well containing 150ul of the above mentioned RPMI medium, supplemented with Cell Stimulation Cocktail (phorbol 12-myristate 13-acetate (PMA) and ionomycin cocktail, 2ul/ml, eBioscience Thermo Fisher Scientific), and Protein Transport Inhibitor Cocktail (Brefeldin A and Monensin cocktail, 2ul/ml, eBioscience Thermo Fisher Scientific) to assess intracellular staining, and incubated for 5 h at 37°C prior to the staining.

3.4 B/T cell separation and activation

The rest of the PBMCs from the paired samples were stained with the separation panel for 30 minutes, washed with sterile PBS and kept in 5ml Polypropylene tubes on ice till being sorted. The panel includes CD25-PE (Miltenyi Biotec), CD14-APC, CD45RA-BV786 (BD Biosciences), CD45RO-PerCP-Cy5.5, CD3-APC-Cy7, CD4-BV650 (all from BioLegend) and CD19-eFluor450 (eBioscience Thermo Fisher Scientific), as summarised in Table 3.4. CD19⁺CD14 B cells and CD3⁺ CD4⁺CD25 CD45RA⁺CD45RO naïve T cells were sorted using BD Influx cell sorter, with confirmed purities of >99%.
Table 3.4. The panel for isolating B cells and naïve T cells.

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CellTrace™ Far Red and CFSE cell proliferation kits (Thermo Fisher Scientific) were used, as per the manufacturer’s protocol, to quantify naïve T cell and B cell proliferation respectively. Briefly, isolated naïve T cells and B cells were diluted in pre-warmed serum-free RPMI medium at 1x10⁶ cells/ml with 1 mM CFSE slowly added into the B cell suspension and 1 mM Far Red into the T cell suspension. Cells were then incubated at 37 °C in a water bath for 20 min, washed twice with RPMI medium containing FBS. T cells were plated in the anti-CD3 (UCHT1, 1 mg/ml; eBioscience Thermo Fisher Scientific) –bounded U-bottom 96-well plates at 1x10⁶ cells per well.

Reagents used to activate B cells included soluble goat anti-human BCR F(ab’)₂ fragment Ab (10 mg/ml; Jackson ImmunoResearch Laboratories) and IL-4 (20 ng/ml; R&D Systems), in addition to the CD40L-transfected L cells as described previously. B cells were co-cultured with L cells at the ratio of 15:1 in the U-bottom 96-well plates for 24h and were then added to T cell plate in at 2x10⁵ cells per well in a total volume of 200 ml of medium and cultured for another 11 d. To measure the cytokine production profiles of B and T cells under ex vivo and in vitro conditions, cells were stimulated with Cell Stimulation and Protein Transport Inhibitor Cocktail 5 hours prior to cell surface and intracellular staining, which activated cytokine production and enabled the accumulation of cytokines within cells for detection by flow cytometry.

### 3.5 Flow cytometry immunophenotyping

Flow cytometry phenotyping of B cell and T cell subsets and their intracellular cytokine staining, besides B/T cell proliferation as mentioned above, was accomplished with antibodies targeting CD3 (APC-Cy7; BV570), CD4 (BV650), CD24 (PerCP), CD86 (BV650), CD40 (BV605), IL-10 (PE-Dazzle 594), GM-CSF (PE-Dazzle 594), TNF-a
(AF700) (all from BioLegend); CD19 (eFluor 450), CD27 (PE-Cy7), IgD (FITC), CD38 (APC), CD80 (PE) (all from eBioscience Thermo Fisher Scientific); IL-10 (BV786), IL-6 (APC), IFN-γ (BV395), IL-17 (PE), CD25 (BV737) (all from BD Biosciences). The purchase information of the panels used for phenotyping B cells in cultured PBMCs (Table 3.5.1), and phenotyping B cells (Table 3.5.2) and T cells (Table 3.5.3) in co-culture experiments are as summarised below.

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**Table 3.5.1.** The panel for phenotyping B cells in cultured PBMCs.

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Table 3.5.2. The panel for phenotyping B cells in co-culture experiments.

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Table 3.5.3. The panel for phenotyping T cells in co-culture experiments.

For general staining procedures, cells were first stained with Live/Dead marker Fixable Viability Dye eFluor506 (eBioscience Thermo Fisher Scientific) for 30 min at 4 °C and washed with FACS buffer (PBS, 0.1% BSA), after which cells were incubated in FACS buffer containing 2% mouse serum for 10 minutes at 4 °C to block unspecific binding sites, and stained with surface Abs for 20 min. Cells were then fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience Thermo Fisher Scientific). Intracellular Abs were added and incubated for 30 min at room temperature. Samples were then washed and analysed by LSRFortessa (BD Biosciences) and the corresponding FACSDiva software.

Flow Cytometry Compensation Beads (Thermo Fisher Scientific) were used to set up compensation for all flow analysis. Data was analysed using FlowJo (Tree Star Inc.). Of note, for APC, AF700, PE-Dazzle and BV786, no distinct positive populations could be captured when the above-mentioned beads were used, resulting in a failure of compensation calculation. Such issue might be due to the incompatibility of intracellular antibodies and the beads. Therefore, antibodies with identical fluorochromes but targeting cell surface molecules were tested and used for setting up compensation (IgD-FITC, CD45RA-AF700, CD38-APC, CD45RA-BV786 and CD25-PE-Dazzle594). Notably, CD25-PE-Dazzle594 antibody couldn’t attached to the BD™ CompBeads properly as the other three, thus the alternative OneComp eBeads™ Compensation Beads were used instead for this single-color control.

However, for APC, AF700, PE-Dazzle and BV786, no distinct positive populations could be captured, resulting in a failure of compensation calculation. Such issue might be due to the incompatibility of intracellular antibodies and the beads. Therefore, antibodies with identical fluorochromes but targeting cell surface molecules were tested and used for setting up compensation (IgD-FITC, CD45RA-AF700, CD38-APC, CD45RA-BV786 and CD25-PE-
Dazzle594). Notably, CD25-PE-Dazzle594 antibody couldn’t attached to the BD™ CompBeads properly as the other three, thus the alternative OneComp eBeads™ Compensation Beads were used instead for this single-color control.

3.6 Culture supernatant collection and storage

PBMC culture supernatant was collected after three/twelve days’ culture and stored at -80°C for batched ELISA analysis in the future. For the collection of the co-cultured cells’ supernatant, 50ml/well of the supernatant from each condition was carefully collected without disturbing cell pallets prior to adding the activation cocktail at the end of the twelve days’ culture, and stored at -80°C.

3.7 DNA purification and genotyping of MS and CBR samples

DNA previously extracted by Dr Fiddes from PBMCs using TRIzol® Reagent (Life Technologies) was purified with the QIAamp DNA micro kit following the manufacturer’s “Clean-up of Genomic DNA” protocol. Samples were first lysed in the presence of proteinase K and Buffer ATL. Buffer AL was then added to the lysate, which were transferred onto QIAamp MinElute columns. DNA was washed with Buffer AW1 and Buffer AW2, before being eluted from the column using Buffer AE.

DNA of the newly recruited 136 CBR samples were extracted from blood collected in EDTA tubes following the DNA Extraction Protocol. Blood collected in EDTA tubes were transferred to 50ml falcon tubes and incubated with lysis buffer (MgCl₂ 0.005M, TrisHCL pH7.5 0.02M) for 15 min on ice. The tubes were centrifuged for 7 min (3800 rpm, 4°C) to remove lysed red blood cells, with the pallet washed using lysis buffer till the pellet turned white. 5ml SE buffer, 50ul of proteinase K (20mg/ml) and 500ul of SDS (10%) were added to the pellet with vortex, and incubated overnight at 37°C. Afterwards, 2ml 5M NaCl was added into the tubes, which were shaken vigorously until foamed. The tubes were centrifuged for 15 min (5000 rpm, 4°C) twice, with the pellets discarded and supernatants transferred into 20ml falcon tubes. 100% ethanol was added into the tubes slowly, which were inverted several times until a tight string of DNA became visible. The tubes were then centrifuged for 15 min (5000 rpm, 4°C) and the supernatants were carefully poured off. The DNA pellets were washed with 70% ethanol (centrifuged for 10 min at 5000 rpm) and dried for two hours with the tubes turned upside down onto tissue. DNA was resuspended in 500ul sterile water, with the DNA purity checked and concentration quantified using a Nanodrop 1000.
Genotyping was completed using Taqman methodology according to the manufacturer’s standard protocol. For genotyping DNA samples dried on one optical 384-well plate, a master mix containing 10ul TaqMan genotyping assay, 840ul MilliQ and 850ul of Universal PCR master mix was prepared. 4ul of master mix was dispensed into each well of the 384-plate using a multichannel pipette, and the plate was sealed with an optical plate cover and spun briefly. PCR reaction was done on the ABI 7900 machine using the following cycling conditions: 10 min at 95°C; 40 cycles (15 sec at 95°C, 1 min at 60°C).

3 SNPs were genotyped (Table 3.7) for the correlation with B cell surface expression of CD86, CD80 and CD40. Genotypes were determined via the Quantstudio 7K Flex System using predesigned genotyping assays (Thermo Scientific). To check for genotyping consistency, duplicates were included for each subject.

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<td>Chr.3: 119503609</td>
<td>MS Risk Allele (C)</td>
<td>CD80</td>
</tr>
<tr>
<td>rs4810485</td>
<td>C__1260190_10</td>
<td>Chr.20: 46119308</td>
<td>MS Risk Allele (T)</td>
<td>CD40</td>
</tr>
</tbody>
</table>

Table 3.7. Genotyping at rs4810485, rs9282641 and rs1131265 using Taqman technology

### 3.8 Statistical analysis

Student’s unpaired t test was used for statistical comparisons between two groups, and paired t test was used for comparisons between the 14 genotype-paired samples. One-way ANOVA was used for statistical comparisons among more than two groups. All statistical tests have been indicated in the figure legends. P value <0.05 was considered significant.
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Chapter 4  B cell stimulation using PBMCs

4.1  Introduction

In his PhD my predecessor Dr Fiddes showed that the MS associated variant rs9282641 influences the expression of the co-stimulatory molecule CD86 on ex-vivo B cells (Smets et al., 2018). In my PhD I wanted to expand this on this work by assessing the influence of genotype in the context of stimulation, considering a wider range of B cell sub-types and establishing whether these observed expression changes had any effects on T cell proliferation. In his work Dr Fiddes had also shown that a second MS associated variant (rs4810485) influenced the expression of another co-stimulatory molecule CD40 in ex vivo B cell. It therefore seemed logical to explore the potential of using CD40 activation as a means of stimulating B cells in my experiments; the association with rs4810485 also suggest that this might be an MS relevant pathway. To establish the stimulation method, I tested and confirm the efficacy of using CD40L-transfected L cells to activate B cells, and optimised culture duration. To evaluate the genotype dependent effects on activated/un-activated B cells in vitro, I collected PBMCs from 108 healthy volunteers (recruited via the Cambridge BioResource on the basis of genotype) and 20 MS patients (recruited from our local clinic without the benefit of prior genotyping). Cells were then cultured with transfected/non-transfected cells for three days, and the impact of genotypes on CD86, CD80 and CD40 expression was assessed.

4.2  Correlating the genotype at rs4810485 with CD40 expression

In his PhD Dr Fiddes had measured CD40 expression in a range of B cell sub-types from a large number of people but had not genotyped all of these subjects for the MS associated variant rs4810485 (which lies within the CD40 gene on chromosome 20). I therefore began my efforts to expand on his work by completing this genotyping in the 156 healthy volunteers previously studied by Dr Fiddes where the rs4810485 genotype was missing. These healthy individuals had all been collected from the Cambridge BioResource, FAC sorted and had CD40 expression quantified in a range of B cell sub-types. Dr Fiddes had extracted DNA from all these subjects using Trizol® Reagent (Life Technologies) but had not cleaned these samples. I therefore first cleaned these samples using QIAamp DNA micro kit’s “Clean-up of
Genomic DNA” protocol. I then measured the concentration and purity of the DNA using the Nanodrop 1000 and normalised aliquots from each sample to a concentration of 1ng/ul. Genotyping was then completed using Taqman methodology with a predesigned genotyping assay supplied by Thermo Scientific™. The genotype of each subject was determined via the Quantstudio 7K Flex software System. To confirm the genotyping consistency, duplicates were included for all subjects.

Genotyping was successful in 149/156 study participants (>95%) with 100% concordance between duplicates. The genotype frequencies were 61.8%, 29.5% and 8.7% for the GG homozygotes, GT heterozygotes and TT homozygotes respectively; frequencies which are in accordance with those seen in the EUR population from the 1000 Genomes Project (Phase 3) (Auton et al., 2015). I then tested for association between rs4810485 and the expression of CD40 as measured by Dr Fiddes in each of the B cell sub-types he assessed and found statistically significant evidence that carrying the rs4810485 risk allele (T) reduces the surface expression of CD40 in B cells, replicating the previously reported finding (Smets et al., 2018).

4.3 Flow cytometry panel defining different B cell subtypes

In order to define transitional B cells and plasmablasts, as well as the class-switched memory, non-class-switched memory and naïve B cell subtypes studied by Dr Fiddes, I designed and optimised a flow cytometry panel. I included a Live/Dead Fixable Blue Dead Cell Stain in this panel in order to be able to exclude debris and dead cells, which frequently confound the phenotyping of cultured cells (Perfetto et al., 2010). The photomultiplier tube (PMT) voltage of each channel was optimized to minimize spill-over between different channels during compensation (Dr Natalia Savinykh guided me in these efforts) (Adan et al., 2017). The PMT voltage for each channel is as shown in Table 4.3. These voltages were established in preparatory experiments based on 4 samples in two experiments and were maintained constant throughout the study. I used a BD™ CompBeads set stained with the antibodies conjugated with their respective fluorochromes as single colour controls for setting up the compensation, and PBMCs were used for the unstained control and Live/dead staining control.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Fluorochromes</th>
<th>Detector name in the base configuration</th>
<th>Voltages</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IgD</em></td>
<td>FITC</td>
<td>blue laser-530/30</td>
<td>442</td>
</tr>
<tr>
<td><em>CD24</em></td>
<td>PerCP</td>
<td>blue laser-695/40</td>
<td>630</td>
</tr>
<tr>
<td><em>CD38</em></td>
<td>APC</td>
<td>red laser-670/14</td>
<td>650</td>
</tr>
<tr>
<td><em>CD3</em></td>
<td>APC-Cy7</td>
<td>red laser-780/60</td>
<td>646</td>
</tr>
</tbody>
</table>
CD19 | eFluo450 | violet laser-450/50 | 472
Viability dye  
CD40 | BV605 | violet laser-605/12 | 600
CD86 | BV650 | violet laser-655/8 | 610
CD80 | PE | yellow laser-582/15 | 600
CD27 | PE-Cy7 | yellow laser-780/60 | 699

Table 4.3. Flow panel used for defining different B cell subtypes and their expression of CD86, CD80 and CD40, with the conjugated fluorochromes and the voltages for their respective detectors as displayed.

The gating strategies I used to define the different B cell subtypes are as illustrated in Figure 4.3. Briefly, lymphocytes were defined in the forward scatter-area (FSC-A) versus side scatter-area (SSC-A) plot and were then further gated for single cells by the ratio of area to width in forward scatter. The amine-reactive Live/Dead Fixable Blue Dead Cell Stain was used to gate out dead cells. Live B cells were gated as CD19⁺CD3⁻ cells. The customarily used IgD/CD27 plot classified peripheral blood B cells into naïve (IgD⁺CD27⁻), non-class-switched memory (IgD⁺CD27⁺) and class-switched memory (IgD⁻CD27⁺). Transitional B cells were gated as CD24hiCD38hi, and plasmablast as CD24⁻CD38hi. The same gating strategies defining different cellular subtypes were applied to all samples. Of note, considerable decrease of the CD24 and CD38 expression was found after 3 days’ culture, which made it difficult to define distinct populations of transitional B cells and plasmablasts reliably after this duration of culturing.
Figure 4.3. Gating strategies for defining class-switched memory, non-class-switched memory, naïve, transitional B cells and plasmablasts. The images were taken from ex vivo PBMCs.

4.4 Stimulation method development

To establish the optimal way of using CD40 to stimulate B cells, I compared both soluble CD40L (1μg/ml; Enzo Life Science) (Li et al., 2017) and CD40L-transfected L cells (at a PBMC to L cell ratio of 20:1) (Duddy et al., 2007). In these experiments for each of two subjects I collected PBMCs and established three parallel cultures; the first supplemented with soluble CD40L, the second co-cultured with CD40L-transfected cells and the third cultured without any supplementation (negative controls). I then compared the expression of CD86 and CD80 after three days (Figure 4.4). In the negative control cultures there was only minimal expression of the activation markers after three days’ culture. In contrast, both soluble CD40L and CD40L-transfected cells substantially increased the expression of CD86 and to a lesser extent CD80, the increase being greatest with transfected L cells.
Figure 4.4. The expression of CD86 and CD80 in B cells after 3 days’ culture. 2x10^6 PBMCs were either cultured alone, supplemented with soluble CD40L or co-cultured with CD40L-transfected L cells (n=2).

Given the higher activation efficiency of CD40L-transfected cells and under the assumption that the stimulation provided by cell-cell interaction was likely more physiologically relevant, I decided to use transfected L cells to activate B cells in my studies. To minimize the potential confounding effects resulting from the activation produced by L cells via some other form of signalling, non-transfected L cells were used instead for comparison in the studies. Based on the results from all collected samples, the effects of L cells per se in activating B cells were minimal, as shown in the following sections.

4.5 L cells irradiation test and CD40L expression confirmation

To prevent fibroblasts overgrowth, L cells were irradiated for 40 minutes (10,646 rads) prior to the co-culture. The growth rate of irradiated and un-irradiated cells was compared after three days. Images were taken using a CX41 Olympus microscope. The growth of irradiated L cells was visually reduced compared with the negative control, which had the same initial
number of cells (Figure 4.5.1). In the co-culture experiments irradiated L cells were mixed with PBMCs at the ratio of 1:20 and cultured in RPMI medium for three days.

![Figure 4.5.1](image1.png)

**Figure 4.5.1.** Cell growth after three days in unirradiated mouse fibroblasts (A) and cells irradiated for 40 minutes (B). Bars indicate 50um.

I then evaluated the surface expression of CD40L in transfected and un-transfected L cells using flow cytometry, in order to confirm that the transfected cells did indeed express CD40. In these experiments cells were dissociated with Accutase solution prior to the staining, rather than separated using Trypsin, to avoid any possible enzymatic cleavage of CD40L. Cells were then stained with anti-CD154-PE (CD40L). Representative results are shown in Figure 4.5.2. Cells were gated as single cell using the FSC-A and FSC-W plot. A notably increased percentage of CD40L⁺ cell was found in the transfected population, with 69.6% of these cells positive for CD40L compared with just 1.09% in the un-transfected cells; these experiments thereby confirming the reliable transfection of CD40L in to these L cells.

![Figure 4.5.2](image2.png)

**Figure 4.5.2.** The expression of CD154 (CD40L) in transfected and un-transfected L cells. Cells had been gated as single cell using FSC-A and FSC-W prior to gating CD40L positivity.
4.6 Optimisation of culture duration

To confirm whether the conventionally used 3 days’ co-culture of PBMCs with CD40L-transfected L cells could sufficiently activate B cells, I undertook a time course experiment in which (in each of two individuals) I co-cultured PBMCs for 12h, 24h, 48h and 72h, and then measured the expression of CD86, CD80 and CD40 in naïve B cells. I used co-cultures with un-transfected L cells as negative controls (Figure 4.6). The results demonstrated that the surface expression of CD86 and CD80 reached their peak expression at 24h but decreased very little out to 72h. By contrast, in the unstimulated conditions, the positivity of these CD86 and CD80 were markedly lower as compared with the stimulated cells; the positivity of CD40 was over 95% at all time points in both conditions.
Figure 4.6. The expression of CD86, CD80 and CD40 on naïve B cells in co-cultures with stimulated (left column) and un-stimulated L cells (right column).

The experiments showed that B cells were maximally activated by L cells after 24 hours of co-culture and that the expression of CD86 and CD80 remain relative stable for the following 48 hours. These data suggest that the standard approach of co-culture for three days was reasonable.

4.7 Different B cell subtypes displayed distinct phenotypes

Next, I assessed the expression of CD86, CD80 and CD40 in each of different B cell subtypes, namely class-switched memory B cells, non-class-switched memory B cells, naïve B cells, transitional B cells and plasmablasts, as defined by the gating strategies described previously. To do this I collected and processed venous blood samples from 108 healthy individuals recruited from the Cambridge BioResource (see methods). The gates defining the positive populations were established using Fluorescence Minus One (FMO) controls for each marker, as illustrated in Figure 4.7.1. To reduce batch effects, gates were always set to ensure that the positive population in each FMO control was 0.5% of total events.
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使用“开始”选项卡将 Heading 2 应用于要在此处显示的文字。
In ex vivo condition, CD86 was expressed by 4.01±0.25 % of B cells, rising to 12.81±0.63 % after un-activated culture and 45.74±1.59 % when co-culture with activated L cells. Similarly, CD80 went from 5.87±0.47 % in ex-vivo conditions to 14.05±0.84 % after culture and 22.6±1.30 % in co-culture with activated L cells. In contrast the expression of CD40
decreased modestly from 95.15±0.38 % in ex-vivo cells to 91.55±0.77 % after culture and 91.81±0.70 % in co-culture with activated L cells (see Figure 4.7.2).

Figure 4.7.2. Expression of CD86, CD80 and CD40 in ex vivo and cultured B cells (unstimulated and stimulated). Figure is based on data from 108 healthy subjects (see methods).

The Ex vivo expression of these surface markers on the five B cell subtypes, namely class-switched memory, non-class-switched memory, naïve, transitional B cells and plasmablasts, is shown in Figure 4.7.3A. In the ex vivo condition, cell expression of CD86 and CD80 was highest in class-switched memory B cells and plasmablasts (P<0.0001), while CD40 was lowest in the transitional B cells and plasmablasts (P<0.0001). Notable variation was found in plasmablasts’ expression of CD86, CD80 and CD40, with about half of the cells being CD86 positive, one-fifth being CD80 positive and three quarters being CD40 positive on average.
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Figure 4.7.3. Expression of CD86, CD80 and CD40 in B cell subtypes in ex vivo (A), unstimulated (B) and stimulated (C) cultures (n=108).

For cultured cells, the observed changes are summarised in Table 4.7. Compared with ex vivo cells, except for plasmablasts, the unstimulated B cell subtypes demonstrated significantly higher expression of both CD86 and CD80. When stimulated by CD40, these four B cell subtypes further increased their expression of CD86 and CD80, except for the expression of CD80 in transitional B cells, the increase of which is not statistically significant. Notably, the greatest increase of CD86 in response to CD40 ligation was found in naïve and transitional B cells, whereas the greatest increase of CD80 was found in class-switched memory B cells and non-class-switched memory B cells. By contrast, a decrease of CD86 and CD80 expression was found in plasmablasts when cultured for three days without stimulation, and only CD86 increased significantly after stimulation. The expression of CD40 increased moderately in class-switched memory B cells and transitional B cells but decreased slightly in non-class-switched memory B cells and naïve B cells after three days’ culture without stimulation. For CD40 no difference was found between unstimulated and stimulated conditions in any B cell subtypes.
Table 4.7. The changes of CD86, CD80 and CD40 expression in class-switched memory B cells, non-class-switched memory B cells, naïve B cells, transitional B cells and plasmasblasts after three days’ culture. Comparison was made between unstimulated and ex vivo conditions, and between stimulated and unstimulated conditions. The mean and standard deviation of the difference is shown in the table. *p<0.05, **p<0.01, ****p<0.0001.

In conclusion, I have shown that naïve B cells demonstrate the greatest response to culturing and CD40L stimulation. Whether the increased CD86 expression and/or decreased CD40 expression in cultured conditions are associated with the previous findings using ex vivo cells which implied that the risk genotypes can increase the expression of CD86 and decreased the expression of CD40 respectively will be further analysed in the following sections.

4.8 CD86, CD80 and CD40 expression at the individual cell level

On inspecting Figure 4.7.3 I noticed that, across the different B cell subtypes and the different conditions, there seemed to be a degree of correlation between the expression of CD86 and CD80 and anticorrelation between CD86 and CD40. Given that each data point in these plots represents the proportion of positive cells of that subtype that are expressing the marker, I wondered whether the expression of these molecules occurred in the same or different individual cells. To explore this question, I compared the observed proportion of double positive cells with the expected proportion for each pair of surface molecules in each of the
three main cell subtypes; class-switched memory, non-class-switched memory and naïve B cells (the low numbers of transitional B cells and plasmablasts prevented such analysis in these groups).

The figures below summarise the pairwise comparison among CD86, CD80 and CD40 in the three B cell subtypes in ex vivo (Figure 4.8.1), unstimulated (Figure 4.8.2) and stimulated (Figure 4.8.3) conditions. Given the low positivity of CD86 and CD80, especially cells at resting state, variation among subjects was notable. For CD86 with CD80, within each cellular subtype and in each condition, I found that the observed proportion of double positive cells is significantly higher than the expected proportion. For CD40 with CD86, and CD40 with CD80, the observed proportion of double positive cell is significantly higher than that expected under random assortment for each cell type and in each condition (except for ex vivo class-switched memory B cells where the reverse is seen).
Figure 4.8.1. Pairwise comparison of the proportion of observed and expected double positive cells among CD86, CD80 and CD40 in class-switched memory B cells, non-class-switched memory B cells and naïve B cells under ex vivo condition. The comparison was made using paired t-test. **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4.8.2. Pairwise comparison of the proportion of observed and expected double positive cells among CD86, CD80 and CD40 in class-switched memory B cells, non-class-switched memory B cells and naïve B cells under unstimulated condition. The comparison was made using paired t-test. ****p<0.0001.
**Figure 4.8.3.** Pairwise comparison of the proportion of observed and expected double positive cells among CD86, CD80 and CD40 in class-switched memory B cells, non-class-switched memory B cells and naïve B cells under stimulated condition. The comparison was made using paired t-test. ****p<0.0001.

### 4.9 Genotypic effects on B cell expression

For each of the subjects I studied the Cambridge BioResource provided the genotype for three MS associated SNPs - rs9282641 (which maps close to CD86), rs4810485 (which maps within CD40) and rs1131265 (another MS-associated SNP reported by GWAS studies mapping close to CD80 (Sawcer et al., 2011)). Genotype counts are shown in Table 4.9.1. These data allowed me to test for the influence of genotype on B cell subtype surface expression.
SNPs | rs9282641 | rs4810485 | rs1131265
---|---|---|---
Genotypes | AA | AG | GG | GG | GT | TT | CC | CG | GG
No. of subjects | 20 | 19 | 68 | 43 | 27 | 37 | 39 | 19 | 48

Table 4.9.1. The genotype counts at rs9282641, rs4810485 and rs1131265 in the 108 healthy controls provided by the Cambridge BioResource (for each SNP genotype data was missing in one/two subjects; no subject had more than one missing genotype).

rs9282641

Despite our earlier work I found no statistically significant evidence for an influence of this SNP on the expression of CD86 in any of the ex-vivo B cell subtypes (Figure 4.9.1A, D, G, J, M). This failure to replicate is likely to be a consequence of the smaller sample size in this study (lower power) and the inevitable regression to the mean. In contrast, statistically significant effects of genotype, consistent with those we previously reported (Smets et al., 2018), were found in cultured cells. Carrying the risk allele (G) leading to greater expression of CD86 in naïve, class-switched memory and non-class-switched memory B cell subtypes in both unstimulated and stimulated cells; these differences being statistically significant in all but the unstimulated class-switched memory B cells (Figure 4.9.1).
Figure 4.9.1. Percentage of CD86⁺ cells according to rs9282641 genotype, in naïve B cells (A-C), class-switched memory B cells (D-F) and non-class-switched memory B cells (G-I). For each cell type results are shown in ex vivo (left), unstimulated (centre) and stimulated (right) conditions. In each panel the risk allele G is plotted on the right.

Considering medium fluorescence intensity (MFI) rather than the percentage of positivity revealed significant changes only in the stimulated cells (Figure 4.9.2). In line with the results obtained considering the percentage of positive cells, the MFI of CD86⁺ cells were higher in individuals with the GG genotype in the stimulated condition (Figure 4.9.2C, F, I). In the ex vivo (Figure 4.9.2A, D, G) and unstimulated (Figure 4.9.2B, E, H) conditions, we found no significant association with genotype. The consistency of results between MFI and positivity indicates reliability in the gating strategies used.
Mean expression in each genotype groups are shown in Table 4.9.2 for percentage positivity and MFI. In the stimulated condition, for both the percentage positivity and MFI, the risk allele homozygotes (GG) is significantly higher than the heterozygous (AG) individuals in all three B cell subtypes, with naïve B cells having the most significant difference.

<table>
<thead>
<tr>
<th></th>
<th>Ex vivo</th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
</tr>
<tr>
<td>Naïve B cells</td>
<td>%</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Class-switched memory B cells</td>
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<tr>
<td>Non-class-switched memory B cells</td>
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</table>

**Figure 4.9.2.** MFI in CD86+ cells according to rs9282641 genotype in naïve B cells (A-C), class-switched memory B cells (D-F) and non-class-switched memory B cells (G-I).
Table 4.9.2. Genotype specific expression of CD86 positivity and MFI in naïve B cells, class-switched memory B cells and non-class-switched memory B cells in ex vivo, unstimulated and stimulated conditions.

rs4810485

Similarly, results concordant with those we observed previously (Smets et al., 2018) were seen for the effects of rs4810485 genotype on the expression of CD40. The risk allele reduces the expression with statistical significance in the majority of the cell types amongst the unstimulated and stimulated cells for both CD40 positivity and MFI. (Figure 4.9.3 and 4.9.4). Table 4.9.3 lists the mean expression level in each genotype group.
Figure 4.9.3. Positivity of CD40 according to rs4810485 genotype; in naïve B cells (A-C), class-switched memory B cells (D-F) and class-switched memory B cells (G-I) in ex vivo. For each cell type results are shown in ex vivo (left), unstimulated (centre) and stimulated (right) conditions. In each panel the risk allele T is plotted on the right.
Figure 4.9.4. MFI of CD40+ cells according to rs4810485 genotype; in naïve B cells (A-C), class-switched memory B cells (D-F) and class-switched memory B cells (G-I). The risk allele T is plotted on the right.

<table>
<thead>
<tr>
<th></th>
<th>Ex vivo</th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>TT</td>
</tr>
<tr>
<td>Naïve B cells</td>
<td>%</td>
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<tr>
<td>Class-switched</td>
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<tr>
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**Table 4.9.3.** Genotype specific expression of CD40 positivity and MFI in naïve B cells, class-switched memory B cells and non-class-switched memory B cells in ex vivo, unstimulated and stimulated conditions.

**rs1131265**

I found no significant association between CD80 expression and the genotype at rs1131265 (Figure 4.9.5), suggesting that the effects of rs1131265 on MS risk are unlikely to be related to any effect on CD80 expression. Although it is still possible that such an effect exists, it is too modest to be detectable with the number of subjects tested in this study.
Figure 4.9.5. CD86 expression in naïve, class-switched and non-class-switched B cells and according to rs9282641 genotype.

The genotypic effects on the expression of CD86, CD80 and CD40 were tested in transitional B cells and plasmablasts, but no statistically significant effects were observed (Figure 4.9.6, 4.9.7 and 4.9.8). The reduced number of cells in these two subtypes present after three days will undoubtedly have limited the power to see effects in these sub-types.

The genotypic effects of these three SNPs on the composition of B cell subtypes were also analysed. Although remarkable variation in the relative proportions of B cell sub-types existed between individuals, no significant result was found suggesting that any of these three SNPs affect the relative proportions of the different cell types. In this context it therefore seems unlikely that the genotypic effects we observed in naïve B cells have resulted from changes in the percentage of CD24<sup>hi</sup>CD38<sup>hi</sup> or CD24<sup>-</sup>CD38<sup>hi</sup> subpopulation, although these B cell subtypes displayed markedly different phenotypes in terms of CD86, CD80 and CD40.
expression. Whether the variation in B cell composition is dependent on other un-typed MS associated SNPs that affect B cell development and maturation was not addressed in this study.

**Figure 4.9.6.** CD86 expression in transitional B cells and plasmablasts according to rs9282641 genotype.
Figure 4.9.7. CD40 expression in transitional B cells and plasmablasts according to rs4810485 genotype.

Figure 4.9.8. CD80 expression in transitional B cells and plasmablasts according to rs1131265 genotype.
In an effort to explore the genotypic effects found in healthy controls in the context of disease, I also collected PBMCs from 20 MS patients (excluding those taking fingolimod or other B cell depletion therapy). Unfortunately, the low frequency of the minor alleles for the SNPs of interest in the European population, together with the modest number of patient available for study meant that the majority of patients I studied (18/20) where in fact homozygotes for the major allele (GG), the other two subjects being heterozygotes (AG) and none of the subjects being homozygous for the other allele. The low numbers of patients with other genotypes limited the power to look for genotype effects within patients. I found no case control difference in the positivity of CD86, CD80 and CD40 in total B cells (Figure 4.10) - even when data was restricted to just those cases and controls that were homozygous for the common allele (GG).
4.11 Summaries and conclusions

By studying PBMCs from 108 healthy volunteers selected on the basis of genotype (to ensure adequate representation of each possible genotype), I have shown that, across various B cell developmental stages (i.e. transitional, naïve, non-class-switched, class-switched B cells and plasmablasts), the expression of CD86, CD80 and CD40 varies considerably in ex vivo condition. I also shown that the response of B cells to stimulation via CD40 differ markedly between these subtypes, with naïve B cells, transitional B cells and plasmablasts more readily increasing CD86 positivity, and non-class-switched and class-switched memory B cells tending to enhance CD80 positivity preferentially. Interestingly using a single cell approach, I was also able to show that within B cells CD86 expression is positively correlated with the expression of CD80, a coupling that was strongest in ex vivo naïve B cells.

Although I was unable to replicate the established association of rs9282641 and rs4810485 genotype on the expression of CD86 and CD40 respectively in the ex-vivo cells from my study subjects, which is almost certainly due to the limited power in this study, I was able to confirm effects concordant with those we described previously (Smets et al., 2018) in many of the B cell subtypes after culture; the effects of rs9282641 and rs4810485 genotype being strongest in CD40 activated naïve B cells. The scale of changes in expression resulting from carrying MS risk alleles were substantially larger in the cultured cells, particularly after stimulation. As in the ex-vivo cell analysis the absence of statistically significant genotypic effects in the transitional B cells and plasmablasts most likely relates to limitations of power. Comparisons between MS and controls samples revealed no major difference suggesting that the effects of these SNPs observed in the healthy subjects I studied are likely to also apply in the context of disease.
Chapter 5  T/B cells co-culture

5.1  Introduction

Having demonstrated that the effects of the MS associated genotypes on CD86 and CD40 expression are amplified in B cells that are first activated by CD40L, I hypothesized that one consequence of this increased expression might be an increased proliferation and perhaps proinflammatory differentiation of T cells. Since PBMCs contain a variety of cell types and cellular subtypes, and the relative proportion of B cells and T cells varies between individuals, I reasoned that to test this hypothesis, it would be more informative to explore this question by combining purified B cells and purified naïve T cells and then co-culturing these in isolation from other cell types. In addition, I wanted to further elucidate the potential underlying molecular mechanisms that might drive any effect on T cells that resulted from changes in B cell expression. The available literature suggests several relevant intracellular cytokines could be involved (Duddy et al., 2007; Barr et al., 2012; Li et al., 2015), which I therefore wanted to include in a new extended panel. To undertake an evaluation of these hypotheses I collected PBMCs from 28 healthy subjects (recruited via the Cambridge BioResource on the basis of genotype) and 13 MS patents (recruited from our local clinic without the benefit of prior genotyping). From each sample I separated B cells and naïve T cells and labelled these with CellTrace™ CFSE, and CellTrace™ Violet respectively. I then set up parallel one-day cultures of the B cells, the first with CD40L transfected L cells and the second with un-transfected L cells. In parallel I set up three T cell cultures all in plates with bound CD3. After the initial one-day B cell activation phase, I then co-culture each set of B cells with the naïve T cells from the same individual for 11 days; the third T cell culture had no added B cells. All these cultures were supplemented with IL-4 and goat anti-human IgM BCR cross-linking antibody. At the end of the 12th day from each individual I therefore had one T cell and activated B cell co-culture, one T cell and un-activated B cell culture and one T cell alone culture. Within the context of each individual I was therefore able to assess the impact of the increased expression of the co-stimulatory molecules (CD80 and CD86) induced by activation of CD40. While comparison between individuals enabled me to assess the impact of genotype and disease state in each of the conditions.
5.2 Optimising the voltages for B/T cell panels

In the context of the fluorochrome-conjugated antibodies that were already available to me, to avoid compromising flow cytometer’s compensation capacity, I would need to define and optimise two separate panels: one panel focusing on B cell phenotypes and the other on T cell phenotypes. In addition to viability dyes, I also used CellTrace CFSE/Violet in the proliferation assay. Ultimately the B cell panel included 8 surface and 4 intracellular makers, whereas the T cell panel had 3 surface and 3 intracellular markers. With the kind assistance from Dr. Mukanthu Nyirenda, I optimised the voltages for each channel to minimize the compensation parameters. Table 5.2.1 and 5.2.2 summarize the target molecules, the conjugated fluorochrome, the laser detectors and the finalized voltage values in both panels.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Fluorochromes</th>
<th>Detector name in the base configuration</th>
<th>Voltages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation assay</td>
<td>CellTrace™ CFSE</td>
<td>blue laser-530/30</td>
<td>445</td>
</tr>
<tr>
<td>IL-6</td>
<td>APC</td>
<td>red laser-670/14</td>
<td>540</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AF700</td>
<td>red laser-735/45</td>
<td>520</td>
</tr>
<tr>
<td>CD19</td>
<td>BV450</td>
<td>violet laser-450/50</td>
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</tr>
<tr>
<td>L/D</td>
<td>BV506</td>
<td>violet laser-525/50</td>
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</tr>
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<td>CD3</td>
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<td>CD40</td>
<td>BV605</td>
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</tr>
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<td>violet laser-655/8</td>
<td>600</td>
</tr>
<tr>
<td>IL-10</td>
<td>BV786</td>
<td>violet laser-780/60</td>
<td>561</td>
</tr>
<tr>
<td>CD80</td>
<td>PE</td>
<td>yellow laser-582/15</td>
<td>579</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>PE-Dazzle 594</td>
<td>yellow laser-610/20</td>
<td>510</td>
</tr>
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<td>CD27</td>
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<td>550</td>
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</tr>
<tr>
<td>CD25</td>
<td>BUV737</td>
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</tr>
</tbody>
</table>

Table 5.2.1. B cell panel showing fluorochrome and voltages.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Fluorochromes</th>
<th>Detector name in the base configuration</th>
<th>Voltages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation assay</td>
<td>CellTrace™ Violet</td>
<td>red laser-670/14</td>
<td>540</td>
</tr>
<tr>
<td>CD19</td>
<td>BV450</td>
<td>violet laser-450/50</td>
<td>429</td>
</tr>
<tr>
<td>L/D</td>
<td>BV506</td>
<td>violet laser-525/50</td>
<td>496</td>
</tr>
</tbody>
</table>

Table 5.2.2. T cell panel showing fluorochrome and voltages.
5.3 Establishing the baseline state of surface and intracellular markers in ex vivo cells

Figure 5.3.1 demonstrates the gating strategies from B cell and T cell panel, which allow for characterizing CD4⁺ T cell, memory B cell and naïve B cell populations. After 5 hours’ culture with PMA/ionomycin and GolgiStop, the morphology of cells, their viability and percentage of various cellular subtypes remained largely the same as the freshly isolated PBMCs described in the previous chapter.

![Gating strategies for defining CD4⁺ T cell, memory B cell and naïve B cell populations.](image)

**Figure 5.3.1.** Gating strategies for defining CD4⁺ T cell, memory B cell and naïve B cell populations. Freshly isolated PBMCs were activated with PMA/ionomycin and GolgiStop mix for five hours before being analysed by flow cytometry.

<table>
<thead>
<tr>
<th>CD3</th>
<th>BV570</th>
<th>violet laser-585/15</th>
<th>520</th>
</tr>
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<tbody>
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<td>violet laser-655/8</td>
<td>600</td>
</tr>
<tr>
<td>IL-17</td>
<td>PE</td>
<td>yellow laser-582/15</td>
<td>579</td>
</tr>
<tr>
<td>IL-10</td>
<td>PE-Dazzle 594</td>
<td>yellow laser-610/20</td>
<td>510</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>BUV395</td>
<td>UV laser-379/20</td>
<td>371</td>
</tr>
</tbody>
</table>

**Table 5.2.2.** T cell panel showing fluorochrome and voltages
This experiment was based on $4 \times 10^6$ PBMCs from each of the 28 healthy CBR subjects and 13 MS. First, the expression of CD86, CD80, CD40 and CD25 was compared between memory and naïve B cells (see Figure 5.3.2 and Table 5.3). In accordance with the results using ex vivo cells, the expression of CD86 and CD80 by naïve B cells was minimal and was significantly lower than memory B cells. CD40 and CD25 positivity was also lower in naïve B cells than in memory B cells. Similarly, the expression of the four intracellular markers was also significantly lower in the naïve population than in the memory population.
Figure 5.3.2. Positivity of surface (CD86, CD80, CD40 and CD25) and intracellular (IL-6, IL-10, GM-CSF and TNF-α) markers in memory and naïve B cells. Cells were activated with PMA/ ionomycin and GolgiStop mix for 5 hours before staining fixation. Memory B cells had significantly higher expression of these markers than naïve population. ****p<0.0001.

<table>
<thead>
<tr>
<th></th>
<th>Memory B cells</th>
<th>Naïve B cells</th>
<th>Mean of differences</th>
<th>SD of differences</th>
</tr>
</thead>
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<tr>
<td>CD86</td>
<td>9.05</td>
<td>5.56</td>
<td>3.49</td>
<td>1.94</td>
</tr>
<tr>
<td>CD80</td>
<td>15.91</td>
<td>1.10</td>
<td>14.81</td>
<td>6.76</td>
</tr>
<tr>
<td>CD40</td>
<td>96.87</td>
<td>93.18</td>
<td>3.69</td>
<td>4.17</td>
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<tr>
<td>CD25</td>
<td>37.33</td>
<td>8.13</td>
<td>29.19</td>
<td>11.32</td>
</tr>
<tr>
<td>IL-6</td>
<td>3.15</td>
<td>1.09</td>
<td>2.06</td>
<td>1.89</td>
</tr>
<tr>
<td>IL-10</td>
<td>9.19</td>
<td>2.99</td>
<td>6.20</td>
<td>3.14</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7.44</td>
<td>4.19</td>
<td>3.25</td>
<td>2.82</td>
</tr>
<tr>
<td>TNF-α</td>
<td>51.63</td>
<td>24.08</td>
<td>27.55</td>
<td>10.84</td>
</tr>
</tbody>
</table>
Table 5.3. Mean and SD of differences between memory and naïve B cells’ expression of CD86, CD80, CD40, CD25, IL-6, IL-10, GM-CSF and TNF-α.

Next, I compared the expression of these markers between CD25+ and CD25- B cells (Figure 5.3.3). Like CD27+ memory B cells, cells that are CD25 positive had significantly higher expression of the surface and intracellular makers. In general, the phenotypes of CD25+ B cells overlapped with those of memory B cells.

Figure 5.3.3. Positivity of surface (CD86, CD80, CD40) and intracellular (IL-6, IL-10, GM-CSF ad TNF-α) markers in CD25+ and CD25- B cells. **p<0.01, ***p<0.001, ****p<0.0001.
5.4 B cells and naïve T cells isolation

In the previous chapter, PBMCs were cultured with L cells for three days. However, in order to avoid any confounding influence on the interaction between B cells and T cells attributable to other cell types included within PBMCs, I only included T and B cell in these second round of experiments. For example, since co-stimulatory molecules such as CD86 and CD40 are also expressed on monocytes, it would be impossible to infer that any difference seen in T cell responses had resulted from genotypic effects on B cells, unless monocytes had been excluded from the co-culture. Thus, to test for effects on T cells resulting from changes in B cells during co-culture, I wanted to use a cell sorting strategy to isolate B cells and naïve helper T cells (Th0) with high purity, so that I could combined only these cells in these experiments. This more restricted co-culture also allowed me to set the relative proportion of T and B cells based on existing literature (Li et al., 2017).

I initially tested the suitability of the Human B Cell and naïve CD4⁺ T Cell Isolation Kits available from Miltenyi Biotec. Processing the samples using these kits manually in a hood (to reduce the risk of contamination) gave high levels of purity (>95%) but inadequate numbers of cells. Using the autoMACS Pro Separator available in the lab (which could only be operated in a non-sterile environment) improved the yield but gave inadequate levels of purity (<90%).

I next tested the BD Influx™ Cell Sorter available at NIHR BRC-Cambridge Phenotyping Hub. According to the regulations of the facility, the Influx machine needs to be operated by their own specialized staff. Therefore, after I have finished isolating and staining the PBMCs with the designed separation panel, the operation of the machine was performed by the staff at the facility. Figure 5.4 illustrates the gating strategies used for isolating B cells (CD3⁻CD19⁺CD14⁻) and naïve T cells (CD3⁺CD19⁻CD4⁺CD45RO⁻CD25⁻CD45RA⁺). Briefly, B cell and T cell populations were defined using the standard CD3 and CD19 makers, with B cells further gated to be CD14⁻ to exclude any CD19⁺ monocytes. Since CD4⁺ responder T cell population is expected to have both naïve and memory T cells, CD45RO and CD45RA were used to exclude memory responder T cells, and CD25⁺ regulatory T cells were also excluded from the naïve population. An example of the purities routinely checked by flow cytometry for B cells and naïve T cells are shown in the figure.
Figure 5.4. Sorting strategies to isolate B cells and naïve T cells from PBMCs. Typical purities are shown in the panel.

5.5 Minimizing gating strategy bias

Samples were provided by the Cambridge BioResource in pairs, one sample from a risk allele homozygote and one from a wild type allele homozygote (I was blind to the genotypes until all samples had been processed and the data finalised). In Chapter 4, in order to maintain the consistency of gating strategies across all samples that were processed, I used FMO controls for each of the three surface markers. However, given the larger number of markers being analysed simultaneously, it was technically and financially infeasible to set up individual FMO for all markers. I therefore used unstained control instead to help decide the positive populations for each marker. Since proliferating cells tend to have greater forward scatter (FSC) and side scatter (SSC) than the original un-proliferated population (which implies that these cells are larger in size and more granular) (Adan et al., 2017), I used the same gates for defining lymphocytes in each pair of samples; thereby avoiding the tendency for any confounding related to the changes in FSC/SSC induced by proliferation.

In most cases, a distinct lymphocyte population separated from the cell debris could be easily identified, so that lymphocytes could be defined with confidence (Figure 5.5A). To test how the gate boundaries might affect the measurement of proliferation in these samples, the
boundary of the gate was set at six random position within the range of the two red lines shown in figure 5.5A. Adjusting the gate in the interval between the two lines only resulted in very modest, and highly correlated, changes in the percentage of proliferated cells in the two samples (Figure 5.5B), confirming the precise, and essentially arbitrary, positioning of the gate had little to no consequence on the comparison between the two samples. Across the 14 pairs of samples, there were two pairs in which a clear boundary couldn’t be seen, as shown in Figure 5.5C. This was most likely due to the relatively lower number of cells acquired after 12 days’ culture. In such cases, it is less convincing that the gating strategies for these two pairs can be as reliable as the other and were therefore excluded from the proliferation analysis.

Figure 5.5. Example FSC-H/SSC-H plots used for defining lymphocytes (A and C). The right-hand plots show the relationship between the percentage of proliferating T cells in the two samples (X and Y axis) as the position of the gate is varied (B and D).
5.6 **Surface and intracellular markers in co-cultured cells**

In order to be able to distinguish the T or B cell origin of the cells present after co-culture, B cells were labelled with CellTrace™ CFSE, and naïve T cells with CellTrace™ Violet. To establish the influence of pre-activation via CD40 I set up parallel one-day cultures of B cells, one with un-transfected L cells and the other with CD40L-transfected L cells. In my earlier time-course experiment (Figure 4.6) I showed that this duration of exposure is sufficient to boost B cells’ expression of the co-stimulatory molecules CD86 and CD80. After this first 24-hour pre-activation, each set of B cells were then co-cultured with naïve T cells for a further 11 days. At day 12, each co-culture was subjected to a 5-hour activation with PMA/ionomycin and GolgiStop mix. The cellular proliferation together with the expression of costimulatory molecules and intracellular cytokines were then measured in both B and T cells (Figure 5.6.1).

![Figure 5.6.1](image-url) **Figure 5.6.1.** Gating strategies for in vitro cells after 12 days’ culture. Purified B cells were first activated by CD40L-transfected L cells for 1 day, and then co-cultured with naïve T cells for another 11 days.

Due to the limited number of cells available for co-culture in some cases, I was unable to have the un-activated condition for 5 pairs of samples, ending up with 24 activated samples and 14 un-activated samples. As anticipated I saw significantly higher proliferation in the co-culture where the B cells had been pre-activated by exposure to CD40L-transfected L cells than in those where the B cells had only been pre-exposed to un-transfected L cells.
The CD40L pre-activated B cells had higher CD86 and CD25 positivity, although this increase was not statistically significant (Figure 5.6.2B, E). Likewise, there was no statistically significant difference in the expression of CD40 itself on these B cells (Figure 5.6.2D). By contrast, CD80 expression was significantly higher on the activated B cells (p=0.0059) (Figure 5.6.2C). The positivity of TNF-α was significantly lower in the activated cells (p=0.0077) (Figure 5.6.2F) but there was no difference in GM-CSF positivity (Figure 5.6.2H). In accordance with literature, B cells activated with CD40L had significantly lower expression of IL-10 (p<0.0001) (Figure 5.6.2G) and higher expression of IL-6 (p=0.0057) (Figure 5.6.2I).
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Figure 5.6.2. B cell proliferation and phenotypes after 12 days of co-culture with naïve T cells. Human B cells were labelled with CFSE and were either pre-activated with CD40L-transfected L cells, BCR and IL-4, or un-transfected L cells, before co-culture with naïve T cells. **p<0.01, ***p<0.001, ****p<0.0001.
To analyse whether activated B cells can induce the proliferation of naïve T cell, T cells were cultured in CD3-plated wells for 12 days, either co-cultured with pre-activated B cells, un-activated B cells or without B cells. Co-culturing un-activated B cells with T cells strongly promoted T cells proliferation (p<0.0001) (Figure 5.6.3A) and IFN-γ production (p=0.0018) (Figure 5.6.3B) as compared with culturing T cells alone. Compared with un-activated B cell, activated B cells further promoted T cell proliferation (p<0.0001) and IFN-γ production (p=0.034). Co-culturing B cells also increased IL-17 production, which didn’t seem to be dependent on activation with CD40L and BCR (Figure 5.6.3C). By contrast, IL-10 positivity in T cells was significantly lower when co-cultured with activated B cells (p=0.0002), and co-culturing un-activated B cells didn’t affect IL-10 production as compared with culturing T cells alone (Figure 5.6.3D).
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使用“开始”选项卡将Heading 2应用于要在此处显示的文字。
Figure 5.6.3. T cell proliferation and phenotypes after 12 days’ culture. Human T cells were labelled with CellTrace Violet and were either co-cultured with activated B cells and un-activated B cells or cultured without B cells. Proliferation (A), IFN-γ (B), IL-17 (C) and IL-10 (D) expression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Although CD86 is predominantly expressed on APCs, I also found that within the T cell population there is a distinct CD86 positive sub-population (Figure 5.6.4A). Compared with T cells co-cultured with pre-activated B cells, T cells cultured with un-activated B cells displayed significantly higher percentage of CD86 positive cells (p=0.0006); in contrast to the findings in B cells. T cells also displayed modest positivity of CD40 and CD80 (Figure 5.6.4B and C), with CD80 significantly higher (p=0.0018) and CD40 (p<0.0001) significantly lower in the activated condition. Although CD25+ T cells had been excluded from the naïve T cell population, after 12 days’ co-culture with B cells, a proportion of T cells were once again CD25+, with no statistical difference between the two experimental conditions (Figure 5.6.4D). By contrast, in the activated condition, the positivity of GM-CSF was significantly higher in the activated condition (p=0.0003) (Figure 5.6.4E). Although considerable number of T cells were TNF-α positive, activating B cells did not result in any difference between the two (Figure 5.6.4F).
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错误！使用“开始”选项卡将Heading 1应用于要在此处显示的文字。
5.7 Genotypic effects on B cells and T cells

Having demonstrated that co-culturing with B cells results in proliferation of the T cells, and that this proliferation is increased when B cells have been pre-activated via CD40, I went on to assess the effects of genotype on the observed changes. Once again, the Cambridge Bioresource (CBR) provided the genotype for all three of the SNPs of interest. These genotypes were only provided after all sample collection and processing was complete, so that I was blind to the genotype during the experiments. Samples were provided by CBR in pairs based on their genotype (one major allele homozygote and one minor allele homozygote) to reduce any confounding related to batch effects. Given the low minor allele frequency of rs9282641, this massively increased the efficiency of the experiment compared to random recruitment. Ultimately CBR provided 14 pairs samples (28 individuals). As expected, in such a modest number I saw no significant association of genotype with CD86 positivity in the ex vivo cells B cells, B cell subtypes or T cells (Figure 5.7.1A). In the activated condition (Figure 5.7.1B), however, the proliferation of T cells was significantly higher in individuals carrying GG risk allele (p=0.0125). No statistically significant difference was found in B cell proliferation or CD86 expression by B/T cells. There were no statistically significance associations with genotype in the un-activated condition (Figure 5.7.1C). As noted in Chapter 5.5, in 2 pairs of the samples there were insufficient cells available after co-culture, hence the total number of samples included in the activation analysis was reduced to 24 (12 pairs).
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Figure 5.7.1. Genotype specific expression of CD86. Row A demonstrates the positivity of CD86 in ex vivo total B cells, memory B cells, naïve B cells and T cells. Row B and C show after 12 days’ co-culture the proliferation of T cells and B cells and their CD86 positivity in activated and un-activated in vitro conditions. *p<0.05.

Although not statistically significant, the expression of CD86 was higher in risk allele homozygotes as expected. The modest sample size employed in this complex, time consuming and costly follow up experiment has limited power, and it is clear that more samples would need to be assessed to confirm the results suggested here, i.e. that the increased expression of CD86 on B cells resulting from carrying the risk allele at rs9282641 leads to increased proliferation of T cells, particularly in the context of immune stimulation.

In addition, I also looked for association with rs4810485 (the CD40 related SNP) and rs2293370 (the CD80 related SNP). Data from a different CD80 related SNP was provided by the Cambridge BioResource as they had not genotyped rs1131265 in many of their samples. Recruits were therefore selected on the basis of rs2293370 genotype; this SNP being a perfect proxy for rs1131265 (the two SNPs having a D’ and $r^2$ of 1.0). A summary of the genotypic information is shown in Table 5.7.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>rs4810485</th>
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<tbody>
<tr>
<td>Genotypes</td>
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<tr>
<td>No. of subjects</td>
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Table 5.7. The genotype information at rs4810485 and rs2293370 (risk allele: G) for the 28 healthy controls was collected from Cambridge BioResource.

No statistically significant associations were seen with either rs4810485 or rs2293370. Again, the modest sample size of this experiment limits its power, even to detect the previously confirmed associations. Given the previous suggestion that the genotype at rs4810485 might influence the downstream expression of IL-10 (Smet et al., 2018), the positivity of IL-10 was also examined. In Figure 5.7.2A ex vivo CD40 positivity was compared between genotypes in B cells, in particular the memory and naïve subtypes. Individuals carrying homozygous risk allele (TT genotype) demonstrated slightly lower IL-10 positivity in B cells. After 12 days’ culture, both activated and un-activated B cells showed decreased CD40 positivity, from over 95% in ex vivo cells to around 60% (Figure 5.7.2B). The carriage of the risk allele T in the heterozygous state also seemed to be associated with a trend of decreased CD40 and IL-10 positivity. These findings are thus in accordance with our previous data suggesting that CD40 expression and the plasma levels of IL-10 correlated with the CD40 SNP in untreated MS patients (Smets et al., 2018). If I had had sufficient time I would like to have measured the level of IL-10 in culture supernatant and tested this for association with genotype.

The expression of CD80 was compared between different genotypes at rs2293370 (Figure 5.7.3), however, with only one AA homozygous individual available among the 28 subjects this analysis was primarily a comparison between heterozygotes and risk allele homozygotes. Similar to the results from the 3 days’ culture, no statistically significant association between CD80 expression and genotype was observed.
Figure 5.7.3. Expression of CD80 by genotypes at rs2293370. Row A demonstrates the positivity of CD80 in ex vivo total B cells, memory B cells, naïve B cells (n=28), and Row B demonstrates after 12 days’ co-culture the positivity of CD80 in activated (n=14) and un-activated conditions.

5.8 Comparison between proliferated and un-proliferated cells

To assess the impact of the proliferation induced by the initial activation for 24 hours via CD40 on the eventual phenotype of the B cells after 12 days of culture, I compared the expression of the four surface makers and four intracellular markers between the proliferated and non-proliferated B cells in the activated cultures (Figure 5.8.1). The B cells that had proliferated showed lower expression of CD86, CD80, CD40 and CD25. This might be because only the original B cell population was activated by CD40L on day 1, and the T cells in the co-culture system were less potent than CD40L-transfected L cells in further stimulating the newly proliferated B cells. In addition, IL-10 and TNF-α positive B cells were also lower in the proliferated population. These could partly explain the findings described above in which the activated B cells had significantly lower IL-10 and TNF-α than the un-activated B cells after 12 days (Figure 5.6.2F and G): the activated B cells demonstrated higher proliferation, with the proliferated cells having lower positivity for these two cytokines. In contrast, no significant difference was found in GM-CSF and IL-6 positivity between the proliferated and un-proliferated population. Since activated B cells have significantly higher IL-6 positivity (Figure 5.6.2I), it could be argued that the CD40
Ligation might have had a prolonged effect on B cell expression of IL-6, not only increasing the positivity of IL-6 among the original populations, but also among the proliferated cells.
To assess the immunological phenotype of the proliferated T cells I compared the positivity of intracellular cytokines IL-17, IL-10 and IFN-γ in the proliferated and un-proliferated CD4+ T cells (Figure 5.8.2). The production of IL-17 and IFN-γ were significantly increased in the proliferated cells (both p<0.0001), whereas the production of IL-10 was significantly decreased as compared with the un-proliferated cells (p=0.0002). These data imply that the activated B cells not only promoted naïve T cell proliferation, but also induced T cell differentiation towards Th1 and Th17 responses, indicating that in subjects carrying the risk allele at rs9282641, CD40 activated B cells are likely to result in a higher proliferation in naïve T cells, with the cells produced having more pro-inflammatory and less immune regulatory phenotype.
5.9 Comparison between MS and healthy subjects

To compare and contrast these observed effects in the context of disease I went on to repeat the analysis described above in 9 MS patients. Given the low minor allele frequency of rs9282641 in the general population, it is unsurprising that none of the studied individuals was minor allele homozygotes (AA). However, as the rs9282641 SNP had been genotyped in many of the patients attending our local MS clinic, I was able to balance to some extent recruitment of AG and GG genotypes (see appendix for clinical details and current treatment for these subjects). As with samples from the CBR, I was blind to the genotype of study subjects until after all sample processing had been completed.

In contrast to the results described in the previous chapter, MS patients showed lower positivity of the pro-inflammatory surface markers CD86, CD80 and CD25, and higher positivity of the immune regulatory CD40, and also showed lower positivity for the pro-inflammatory GM-CSF, IL-6 and TNF-α but no statistically significant difference in IL-10 positivity (see Figures 5.9.1 and 5.9.2). While these differences might be disease specific, it is also possible that they are a consequence of the immune-modulatory drugs being taken by these patients.
Figure 5.9.1. Surface marker and cytokine expression in B cells from treated MS patients and healthy controls. *p<0.05, **p<0.001

Figure 5.9.2. Cytokine expression in CD4⁺ T cells from treated MS patients and healthy controls.

Comparing the proliferation of B cells and CD4⁺ T cells in treated MS patients and healthy controls revealed that in the activated condition B cells and T cells demonstrated higher proliferation in healthy controls (Figure 5.9.3). There was no statistically significant difference in the expression of surface and intracellular markers in B cells from MS patients and healthy controls (Figure 5.9.4). Similarly, B cells from treated MS patients demonstrated slightly lower CD86 positivity and higher CD40 positivity in both activated and un-activated conditions than B cells from healthy controls. The positivity of IL-17 and IL-10 was
considerably lower in MS samples in the three in vitro conditions (Figure 5.9.5). In addition, the difference of IL-10 positivity in the activated and un-activated conditions reached nominally significant level (P=0.04 and P=0.02 respectively). No statistically significant difference was found in T cells’ positivity of IFN-γ.

Figure 5.9.3. Proliferation of B cells and CD4+ T cells from treated MS patients and healthy controls.
Figure 5.9.4. Surface marker and cytokine expression in B cells from treated MS patients and healthy controls after 12 days of co-culture, with and without pre-activation.

Figure 5.9.5. Expression of intracellular cytokine markers by CD4+ T cells from treated MS patients and healthy controls after 12 days of culture alone and co-culture, with and without pre-activated B cells.

In short, the data from treated MS samples shows that the expression of surface markers and cytokines is similar to that seen in healthy controls, with the few differences observed which may be related to treatments being received by the subjects in the patient group. To confirm these differences as disease related, a large number of untreated patients would need to be studied in future experiments.
5.10 Summaries and conclusions

In this chapter, I have described how I established B cell and T cell panels to assess the phenotypes of naïve T cells and B cells. In line with the results from the previous chapter, memory B cells demonstrated significantly higher expression of surface and intracellular markers than naïve B cells. Preliminary experiments comparing alternate methods for isolating naïve T cells and B cells showed that the Influx Cell Sorter was the most suitable approach for this study. Since even minimal changes of lymphocyte gating could in principle influence the measurement of proliferation, the gating strategies were adjusted accordingly in order to minimize the potential bias, and stringent quality control measures were employed to exclude data of inadequate quality from analysis.

In my B and T cell co-culture system, I found that stimulated B cells displayed significantly higher proliferation, CD80 and IL-6 expression, but lower IL-10 and TNF-α expression than unstimulated B cells. In accordance with this, T cells co-cultured with activated B cells showed significantly higher proliferation and expression of IFN-γ but lower IL-10 expression than T cells co-cultured with un-activated B cells or T cells cultured alone. These results confirmed the efficacy of the co-culturing of B cells and T cells I employed in this study. Of note, a distinct CD86 positive T cell population was found after 12 days’ co-culture. In contrast to B cells, which displayed significantly higher CD86 positivity when stimulated, T cells co-cultured with stimulated B cells demonstrated lower percentage of CD86+ cells than those cultured with unstimulated B cells.

The genotypic effects on T cells were investigated using 14 pairs of homozygous healthy controls carrying either AA or GG genotypes at rs9282641. Despite the fact that the differences in CD86 expression on B cells were not statistically significant in this modest samples size, the T cells from GG individuals demonstrated significantly higher proliferation when cultured with the CD40L-activated B cells from the same individuals, with the proliferated cells producing higher level of IFN-γ and IL-17 but lower IL-10 level.

Finally, in keeping with the results from previous chapters, I found that the samples from treated MS behaved very much like those from healthy controls under in vitro conditions. Although no statistical difference was found between the two groups, there was a trend of lower expression of pro-inflammatory CD86, CD80 and CD25, but higher CD40 expression in B cells from treated MS samples than health controls, in addition to a general decreased cytokine production by B cells and T cells. To confirm these trends, more MS samples from untreated patients would need to be included in future studies.
Chapter 6  Discussion

6.1  Correlating phenotypes with gene expression

In my research, I have shown that the genotype at rs9282641 influences the expression of CD86 in cultured lymphocytes. According to Gencode Release 21 (GRCh38), the CD86 gene contains eight exons and spans 65kb on chromosome 3q21. To date nine alternate transcripts have been described; the full-length transcript coding for the membrane bound protein CD86 (also known as B7-2A). Among these different transcripts, five start with exon 1 and four with exon 2. Four transcripts lack the transmembrane domain encoded by exon 6 and are therefore soluble form of CD86 (sCD86). As well as the full-length transcript three alternate isoforms have been observed in humans, the two soluble isoforms, B7-2B and CD86ΔEC (Jeannin et al. 2000; Magistrelli et al. 2001), and one membrane bound form which lacks the IgV-like counter-receptor binding domain (B7-2C) (Kapsogeorgou et al. 2008). The proportion of the full length functional CD86 is relatively low in resting APCs, but increases upon activation, whereas the truncated isoforms, which are unable to provide co-stimulation for T cells, were found to behave in the opposite manner (Jeannin et al. 2000). It therefore follows that the efficacy of the interaction between CD86 and its cognate receptors on T cells is likely to be partially determined by the relative proportion of these isoforms (Kapsogeorgou et al. 2008). Unfortunately, the antibody I used to detect CD86 in my research does not distinguish between the different surface-bound isoforms, so it was not possible for me to explore the extent to which genotype influences the balance of alternate transcripts/isoforms. It remains possible that the effects of genotype I observed on cultured cells are primarily driven by altered balance in isoform production rather than changes in absolute surface expression of CD86, although clearly there are changes in this absolute surface expression. Since rs9282641 is located within the 5’UTR of exon 2 of CD86, it had been hypothesized that the genotype may alter the relative proportion of the alternate soluble forms of CD86 (Smets et al., 2018). However, in the ex vivo cells, they found no effect of rs9282641 genotype on the serum level of sCD86 or on the relative proportion of transcripts with different starting exons or transmembrane domains. I did not explore these issues in the activated cultures, so it remains possible that the rs9282641 genotype might influence these factors after activation.

It has previously been shown that the soluble isoforms of CD40 (sCD40) produced by the transcripts lacking either exon 5 or exon 6 exert antagonistic effects on membranous CD40L
(Eshel et al., 2008), and that during activation stages there is a shift in pre-CD40 RNA splicing away from signal-transducible full length mRNA towards signal-nontransducible mRNAs, thereby reducing the functional form of CD40 available on the cell surface (Tone et al., 2001). In the context of these known negative feedback systems, it was unsurprising that the percentage of CD40+ B cells decreased significantly after 3 day’s activation with CD40L. The results showing that subjects carrying the MS risk allele (rs4810485*T) had even lower CD40 surface expression after activation implies that those individuals carrying this allele are more sensitive to this regulatory post-transcriptional splicing feedback mechanism, which is in line with earlier observations confirming that carrying the MS risk allele rs4810485*T increases the proportion of exon 5-lacking isoform and decreases the proportion of exon 6-lacking isoform (Smets et al., 2018).

In order to further explore the extent to which altered soluble isoform balance plays a role mediating the observed effects of MS risk alleles I collected and stored the 3-day and 12-day culture medium from the 136 healthy controls I studied. This material is available and could be quantified using the enzyme linked immunosorbent assay (ELISA) in the future. I was unable to undertake this additional work due to limited research budget.

6.2 How B cells may influence T cell responses

6.2.1 Implications from genetic studies

A considerable proportion of the genes implicated by GWAS relate to intercellular signalling between APCs and T lymphocytes (Sawcer et al., 2014) and show a notable bias in favour of the ligands/receptors expressed on the APCs side of the interaction. For example, the MHC class II molecules show strong association, but no significant association has emerged in T cell receptor (TCR) gene regions (Sawcer et al., 2014). The GWAS undertaken by my supervisor identified three common variants that all lie close to the CD86 gene on chromosome 3q21 and independently influenced susceptibility to MS (Sawcer et al., 2011). Following up these discoveries my predecessor, Dr. Fiddes, showed that the carriage of the risk allele from one of these variants, rs9282641, increases the proportion of CD86+ naïve B cells in ex vivo condition, and therefore suggested that this variant might exerted its effect on MS risk by promoting the activation of T cells (Smets et al., 2018). It seemed likely that this effect would primarily be relevant in the context of immune stimulation and therefore interesting that variants close to CD40 were also associated with MS risk and influenced the expression of this B cell receptor. In my research, I wanted to test and explore these suggestions and reasoned that stimulation of CD40 would likely be a disease-relevant manner in which to activate the B cells. Using co-culture experiments, I have shown that the proliferation of T cells is indeed promoted by CD40L-activated B cells, and also that carrying
the risk allele at rs9282641 increases the resulting proliferation of T cells. A range of molecular mechanisms might underlie this genotype-dependent interplay between B cells and T cells.

6.2.2 Roles played by B7 molecules

In recently published work it was shown that the auto-proliferation of peripheral Th1 cells is enhanced in MS, and that the extent of this auto-proliferation is increased in individuals carrying the MS associated allele HLA-DR15 (Jelcic et al., 2018). These researchers co-cultured T and B cells (both activated and un-activated) and, in line with my data, saw significantly higher proliferation of T cells in these co-cultures compared with when T cells auto-proliferated in lone cultures. In considering the role of the B7 molecules (CD86 and CD80) in these processes, it is important to remember that although interaction with the CD28 co-receptor has stimulatory effects, interaction with the alternative co-receptor CTLA-4 (CD152), which is constitutively expressed by Tregs, has inhibitory effect (Wing et al., 2014; Crotty, 2015). The impact of increased CD86 is thus dependent upon the context and partner cells encountered.

The importance of B7 molecules in the interaction between APCs and T cells has been noted in both murine and human studies. In the EAE model, it was found that the conversion of effector T cells to Tregs relied on the interaction with CD80 and CD86 (Mann et al., 2007). In human studies, it has been reported in a case-control study that polymorphisms in CD28/CTLA-4–CD80/CD86 interaction could influence the risk of MS and the age of onset (Wagner et al., 2015). In line with this, an MRI study has shown that B cells from MS patients with high neurodegeneration had significantly higher expression of both CD86 and CD80 (Comebella et al., 2015). Conversely, Foxp3+CD4+ follicular regulatory T cells expressing CXCR5 and Bcl6 can down-regulate B cell expression of CD80 and CD86, thereby impeding the interactions between B cells and T cells (Wing and Sakaguchi, 2010).

In accordance with the data presented by Jelcic et al., who found that T cell proliferation is primarily dependent on memory B cells, other researchers have shown that both class-switched and non-class-switched memory B cells were able to elicit CD4+ T cell proliferative responses in vitro, whereas naïve B cells failed to induce T cell activation irrespective of the number of B cells cultured with T cells (Good et al., 2009). Since blocking CD86 and CD80 would significantly reduce T cell proliferation, it was argued that the T cell responses were induced via the expression of CD86 and CD80 by B cells (Good et al., 2009). Therefore, observed differences in naïve and memory B cells’ capacity in inducing T cell responses observed by Jelcic et al. might simply relate to the low expression of CD86 and CD80 on the surface of ex vivo naïve B cells as compared with memory B cells (a situation which changes...
after stimulation). Certainly, one of the most noteworthy phenotypic differences between naïve and memory B cells that I observed was the expression level of CD86 and CD80, with ex vivo memory B cells having significantly higher CD86 and CD80 positivity. This difference in the expression of these molecules by naïve and memory B cells might underlie the difference in T cell auto-proliferation seen by Jelcic et al. In keeping with earlier work (Bar-Or et al., 2001), I have shown that the expression of CD86 and CD80 on naïve cells increased to a level similar to that of resting state memory B cells when these cells were activated by CD40L. In the B/T cell co-culture experiments, T cells demonstrated significantly higher proliferation when co-cultured with activated B cells as compared with un-activated B cells, which had much lower CD86 and CD80 expression. It has been suggested that the dysregulated B cell signalling might be sufficient to induce the initial breaks in T cell tolerance and induce CD4+ T cell expansion and activation during autoimmunity (Jackson et al., 2015). Therefore, the T cell responses relevant to the pathogenesis of MS might be highly dependent on the co-stimulatory interaction with B cells.

6.2.3 T cells’ expression of pro-/anti-inflammatory cytokines

As well as showing that co-culturing CD4+ T cells with autologous B cells increases their proliferation, I have also shown that the cells generated in this way have higher positivity for pro-inflammatory cytokines such as IFN-γ and IL-17, and lower positivity for anti-inflammatory cytokines such as IL-10 (see Figures 5.6.3 and 5.8.2). The increase of these pro-inflammatory cytokines is likely to be disease relevant, given the correlation of IFN-γ with disability in MS (Moldovan et al., 2003), the adverse effects of exogenous IFN-γ (Panitch et al., 1987) and the likely role of IL-17-secreting CD4 T cells (Th17 cells) and γδ T cells in the pathogenesis of MS (Gold and Luher, 2008; McGinley et al., 2018). Although how these cytokines initiate and augment the disease remains incompletely understood, these studies strongly implicate IFN-γ and IL-17-secreting CD4 T cells as pathogenic drivers of MS.

Although the suppressive effects of Forkhead box protein 3 (FoxP3) expressing regulatory T (Treg) cells are primarily mediated via cell-contact mechanisms (Tao et al., 2017), it has recently been recognized that the so-called type 1 regulatory T (Tr1) exert many of their effects via their secretion of IL-10 (Gregori et al., 2012). Inadequacy in the production of this potent anti-inflammatory cytokine is believed to play a crucial role in the development of many autoimmune diseases (Iyer and Cheng, 2012). It is also been shown that the FoxP3 effector T cells can acquire the capacity of producing IL-10 (Korn and Kallies, 2017). In keeping with these data, a recent high-throughput transcription analysis has shown that IL-10 expression is suppressed in MS and negatively correlated with disease activity (Hu et al., 2017).
In my research I have thus been able to confirm that the genotype dependent increase in CD86 expression on B cells observed by Dr Fiddes in his PhD do indeed correlate with increased proliferation and pro-inflammatory differentiation of T cells; a process which could be a therapeutic target in MS. However, its notable that the clinical experience with the CTLA4-Ig fusion protein Abatacept, which blocks the CD28-B7 costimulatory pathway was disappointing; the clinical trial, A Cooperative Clinical Study of Abatacept in Multiple Sclerosis, (ACCLAIM) (Khoury et al., 2017) being negative despite the drug being approved for the treatment of RA and juvenile idiopathic arthritis. The possible explanations behind this failure are worthy of further consideration.

6.3 CD86 and the failure of Abatacept in MS

6.3.1 Unspecific target cell types

Given that CD86 and CD80 can result in anti-inflammatory as well as pro-inflammatory signalling, it is possible that the lack of any cell type specificity in the blockade of co-stimulatory signalling resulting from Abatacept might have little overall effect, with its wanted blockade of pro-inflammatory effects being balanced (or even outweighed) by its blockade of anti-inflammatory effects on other cell types. It is clear that cellular subtypes differ in their expression of co-stimulatory surface (Pieper et al., 2013).

In my research I confirmed that CD86 is not restricted to B cells but is also found on T cells (see Figure 5.6.4A), indicating that Abatacept might as well have effects on signalling coming from both of these cell types (and in principle any other cell type expressing CD86). Although the exact function of CD86 on T cells remains unclear, it has been suggested that CD86+ T cells can also serve as APCs, enabling sustained T cell proliferation (Lal et al., 1996; Menezes et al., 2014). In line with this, another human study has shown that CD86+ memory effector T cells can enhance naïve T cells proliferation and their production of IFN-γ (Jeannin et al., 1999). Since the consequences of blocking B7 costimulatory signalling in T cells is unknown, this again might offset the anticipated anti-inflammatory functions of Abatacept. Aggravation of disease activity induced by blocking B7 medicated signalling has been reported by others (Kuchroo et al., 1995; Menezes et al., 2014), so perhaps the negative results of the Abatacept trial were not so unexpected.
6.3.2 Roles played by CD80

The fact that Abatacept blocks CD80 as well as CD86 could be another source of unwanted and unexpected effect that offset the benefits anticipated in the Abatacept trial. Despite their mostly overlapping roles as co-stimulatory molecules, CD86 and CD80 also have some unique modulatory roles. For example, although the ligation of CD86 and CD80 with CD28 leads to shared early signalling transduction events, it has been suggested that they induce distinct signalling pathways, and that the tyrosine phosphorylation of CD28 initiated by these ligands is quantitatively different (Slavik et al., 1999). In line with this, different outcomes have been observed when blocking either CD86 or CD80 using antibodies with greater specificity. In a mouse transplantation study, researchers found that silencing CD86 in dendritic cells via siRNA transfection significantly reduced the IL-2 and IFN-γ production while increasing the production of IL-10 and TGF-β, thereby inducing immune tolerance, whereas silencing CD80 in the same manner had no such effects (Ke et al., 2016). It has also been found in a virus infection model that the expression of CD86, but not CD80, by B cells is crucial to the formation of follicular T helper cells, which are involved in germinal centre development (Salek-Ardakani et al., 2011). By contrast, in acute viral myocarditis models, treating the mice with anti-CD80 monoclonal antibodies, but not anti-CD86 antibodies, will markedly suppress ROR-γt mRNA expression and Th17 cell differentiation (Huang et al., 2018). These data confirm that the effects of CD86 and CD80 on T cells are not identical or redundant.

In addition to their distinct effects on T cells, the dynamic regulation of their expression also differs. It has been suggested that CD86 has a dominant role during the initiation of immune reactions, whereas CD80 plays a more prominent role in chronic inflammatory conditions (Sharpe and Freeman, 2002). This is perhaps due to the fact that upon B cell activation, CD86 levels typically increase more rapidly and to a higher level than CD80 (Bar-Or et al., 2001). In agreement with these findings, I saw notable differences in the time course of CD86 and CD80 expression during my cultures, with increased expression of CD86 occurring much earlier than increased expression of CD80. It has been found that inhibiting the expression of Dicer, which is involved in the biogenesis of microRNAs, leads to increased expression of CD80, but not CD86 (Aung and Balashov, 2015).

6.4 CD40 and its complex roles in B cell activation

My predecessor Dr Fiddes showed that in resting cells the MS associated variants rs9282641 and rs4810485 exert their strongest respective effects on the expression of CD86 and CD40 in naïve B cells. These data therefore suggest that this cellular subtype is perhaps the most implicated cell type in MS, and also that activation via CD40 might be the most disease-
relevant mechanism for stimulating B cells. In keeping with these results, the expression of CD86 has previously been shown to be increased in B cells in the context of MS (Bar-Or et al., 2001), again with the greatest increase being seen in naïve B cells when activated in vitro (Kinnunen et al., 2013). In my research I wanted to determine the effects of these genotypes in the context of stimulation and optimized an experimental design that activated B cells via CD40, a critical co-stimulatory molecule in APCs and innate immune cells (Elgueta et al., 2009).

Engagement of CD40 with CD40L promotes both canonical and non-canonical NF-kB pathways in B cells, which are required for germinal centre (GC) formation, immunoglobulin (Ig) class-switching and affinity maturation, as well as memory B cells and plasma cells differentiation (Mauri et al., 2003, Lemoine et al., 2011, Hostager and Bishop, 2013). The importance of CD40 in immune regulation is also suggested by the association of rs4810485 with a range of autoimmune diseases in addition to MS, including RA (Raychaudhuri et al., 2008), Graves’ disease (GD) (Tomer et al., 2002; Li et al., 2012), SLE (Vazgiourakis et al., 2011), Crohn’s disease (Blanco-Kelly et al., 2010; Jostins et al., 2012) and Kawasaki disease (Onouchi et al., 2012). As with many other overlapping GWAS risk variants, the effects of the MS risk allele (T) (Sawcer et al., 2011; Beecham et al., 2013) vary between diseases, with the T allele increasing risk of inflammatory bowel disease (IBD) (Jostins et al., 2012), but reducing the risk of GD (Tomer et al., 2002), SLE (Vazgiourakis et al., 2011), RA (Raychaudhuri et al., 2008) and Kawasaki disease (Onouchi et al., 2012). These differences presumably reflect varying importance of the complex and pleiotropic effects of CD40 signalling. For example, the risk allele for GD (Jacobson et al., 2005) at rs1883832 (a perfect proxy for rs4810485) is associated with increased CD40 expression (C, major allele), whereas the same risk allele for MS is associated with decreased expression of CD40 (T, minor allele) (Gandhi et al., 2010; Field et al., 2015).

It has been reported that dual stimulation (sequential BCR engagement followed by CD40 signalling) results in lower production of IL-10 and higher production of TNF-α, than CD40 signalling alone (Duddy et al., 2007), and also that activated T cells can act as a major source CD40L stimulation to B cells (Vazquez et al., 2015). Furthermore, in the context of disease it has been observed that B cells can respond differently to CD40 activation. For example, transitional B cells isolated from SLE patients have been found to be refractory to CD40 stimulation (Blair et al., 2010). It should also be noted that although CD40L has been suggested as the primary stimuli for B cell activation, other co-stimulatory signals provided by activated CD4+ T cells might also play a part in the development of regulatory B cells (Mauri et al., 2008; Mauri, 2010).
Since activating B cells with CD40L-transfected L cells can significantly increase the expression of CD86 and CD80 (Nova-Lamperti et al., 2016), intuitively, it might be expected that genetic variants reducing CD40 expression would be associated with reduced MS risk. In fact, the reverse is seen, with variants reducing the level of CD40 resulting in increased risk of MS (Sawcer et al., 2011). In addition, no association between the CD40 SNP and the expression of CD86 and CD80 was found in the samples that I have processed. It is clear therefore that the reduced expression of CD40 resulting from carrying the risk allele (T) at rs4810485 must have more effects that just reducing CD86 and CD80 expression (effects which would be expected to reduce rather than increase MS risk). In keeping with these GWAS findings, it has been noted that blocking the CD40-CD40L pathway has beneficial effects in EAE models (Howard et al., 1999), although the effects of such blocking in humans are less clear. Intriguingly, it has been shown that at the mRNA level the expression of CD40 is higher in MS patients than in healthy controls, which is contrary to the decreased CD40 positivity on cell surface seen in individuals at higher risk (Huang et al., 2000). As noted previously, such inconsistency between RNA and protein expression might be due to alterations in the balance of CD40 isoforms: an increased transcription of the truncated CD40 mRNA and simultaneous decrease of cell surface CD40 protein in MS patients as compared to healthy individuals.

### 6.5 From B cell subtypes to single cell transcriptional analysis

As mentioned in Chapter 1, the notion of MS being an autoimmune disease driven solely by myelin-reactive T cells has been challenged by recent progress in the understanding of B cells. It is now well recognized that B cells are able to function as professional APCs, and indeed are much more potent in this role than dendritic cells and macrophages, especially when the concentration of antigens is low (Rahmanzadeh et al., 2018). It is also clear that B cells can polarize the differentiation of T cell towards Th1, Th2 or Th17 via their production of a wide spectrum of cytokines (Jackson et al., 2015). B cell depleting therapy (BCDT) that targets CD20 has emerged as a highly effective treatment for controlling inflammatory activity in various autoimmune diseases, including MS (Franciotta et al., 2008). Contrary to CD19, which is expressed on all circulating B cell lineage and regulates the signalling threshold for B cell activation, CD20 is only expressed by B cells from the pre-B cell stage till the mature naïve and memory B cell stage, and its precise function remains unknown (Baecher-Allan et al., 2018). Therefore, the depletion of CD20+ B cells targets naïve and memory B cells while sparing antibody producing plasma cells. So far, three anti-CD20 antibodies targeting different CD20 epitopes have been approved or are under investigation in clinical trials for MS, namely rituximab (chimeric human/mouse Ig G1), ocrelizumab (humanized Ig G1), and ofatumumab (full-length human Ig G1) (Rahmanzadeh et al., 2018).
It is possible that the anti-CD20 drugs could result in long term disease regulation benefits in MS perhaps by reducing pro-inflammatory memory B cells, and/or restoring regulatory B cell populations in circulation. However, in some cases B cell deficiency or B cell depletion therapies have been shown to paradoxically exacerbate rather than ameliorate the inflammatory condition (Dass et al., 2007; Goetz et al., 2007). For example, the recombinant fusion protein atacicept, which was designed to block B cell activities via the BLyS/April receptor, has turned out to worsen CNS inflammation in a phase II clinical trial of MS (Kappos et al., 2014). Such paradoxical outcomes might be due in part to the heterogeneity of B cells, which could be further divided into various cellular subtypes having completely different phenotypes and cytokine secretion profile. Thus, it could be postulated that the pathogenesis of MS might result from the dysfunction of a relatively small subset of B cells, within which we might expect more prominent effects of MS associated risk alleles.

Cell type specific differences have been reported in studies comparing MS patients with healthy controls (Niino et al., 2009), and those comparing patients in the relapse and remission phases of the disease (Fraussen et al., 2016). It is worth noting that most existing disease-modifying therapies for MS modulate B cell immunity to some extent (Longbrake et al., 2016). In general, these therapies lead to a relative decrease of memory B cells and a concomitant expansion of immature and naïve B cells, which can further increase the production of IL-10 and concurrently suppress the secretion of pro-inflammatory cytokines (Fillatreau et al., 2002; Mauri et al, 2003; Claes et al., 2014; Matsumoto et al., 2015). Moreover, the auto-proliferation of CD4 T cells was found to be positively associated with memory B cells and negatively with naïve B cells (Jelcic et al., 2018), so it is possible that not all of the changes in B cells induced by existing therapies are helpful. Given the overwhelming evidence supporting cellular subtype specific effects, including the work of my predecessor Dr Fiddes, it was clear that in my research I needs to explore the effects of genotype in a cell type specific manner, following this logic to its conclusion it would seem logical to infer that ultimately single cell resolution analysis would likely be optimal. Unfortunately, although this is now possible, the techniques currently available are prohibitively expensive.

I used several surface markers to characterize the B cell types and assess activation. In line with existing literature (Amu et al., 2007), I found that in ex vivo cells CD25 positivity was significantly higher in memory B cells than in naïve B cells. However, after 12 days’ culture, there was no statistically significant evidence for association of CD25 positivity with rs9282641 in any of the B cells subtypes. Given the pivotal roles of IL-10 secreting regulatory B cells (Breg) in murine models of autoimmune diseases, I was keen to also assess these cells in humans. However, as there are currently no transcription factors or surface markers that can robustly define these cells, it was only possible to explore the impact of
genotype on these cells indirectly, via the production of IL-10 (Sims et al., 2005). It has previously been observed that the regulatory capacity of CD19+CD24hiCD38hi transitional B cells was impaired in patients affected by autoimmune diseases (Blair et al., 2010; Lemoine et al., 2011; Oka et al., 2014). Unfortunately, primarily due to the small numbers of transitional B cells available after culturing, I was unable to reliably assess IL-10 expression in this subtype in my experiments. The low number is unsurprising given the short half-life of the transitional B cells (Vitale et al., 2010; Bemark, 2015). Assessment of IL-10 in plasmablasts was similarly limited by low cell number. In addition, I found no evidence to suggest that any of the three SNPs I investigated influence the composition of B cells under any of the study conditions in my analysis, although it is well established that other genetic variants alter the relative proportion of immune cells (Orru et al., 2013; Astel et al., 2016; Lagou et al., 2018).

Given that the function of B cell is reflected by its cytokine profile as well as its surface markers (Li et al., 2016), I included assessment of TNF-α, IL-10, IL-6 and GM-CSF in my flow panels. Others have found evidence suggesting that dysregulated B cell cytokine production plays a crucial part in MS (Vazquez et al., 2015), in particular that a deficiency in the production of anti-inflammatory IL-10 by B cells is associated with a more aggressive disease course (Duddy et al., 2007; Bar et al., 2012; Li et al., 2015). The regulatory capacity of B cells has been recognized in both murine autoimmunity models and human clinical trials (Berthelot et al., 2012). It has been reported that in the EAE model transplanting IL-10-producing B cells into B cell deficient mice reduces the production of pro-inflammatory cytokines and the proliferation of auto-reactive CD4+ T cell, and that transplanting B cells that are CD40 deficient cannot restore the balance of immunity (Yoshizaki et al., 2012). In keeping with this suggestion that IL-10 production might be dependent on CD40 signalling, I have found that activated B cells had significantly lower CD40 positivity at day 3 and lower IL-10 positivity at day 12. In our previously published work, we found nominally significant association between IL-10 levels and the MS risk allele rs4810485*T (Smets et al., 2018). Despite these data suggesting the importance of cytokine production in B cell function, I found no statistically significant evidence for association of these intracellular markers with any of the three key variants I tested.

In terms of T cell responses, it has been suggested that IL-10 secreted by transitional B cells can prevent the production of pro-inflammatory cytokines by CD4+ T cells (Blair et al., 2010; Lemoine et al., 2011), and that transitional B cells produce higher levels of IL-10 as compared to other B cell subtypes after CD40 engagement (Nova-Lamperti et al., 2016). Transitional B cells can also inhibit the differentiation of naïve T cells into Th17 and suppress their secretion of TNF-α and IFN-γ, in addition to promoting their conversion into Tregs (Flores-Borja et al., 2013; Wang et al., 2015). It has therefore been hypothesized that regulatory B cells, which might be a heterogeneous population of cells at various stages of differentiation, act at early stages to facilitate Tregs recruitment and become less active once
Tregs function become optimal (Berthelot et al., 2012). However, using the functional definition to characterize the population of regulatory B cells makes it difficult to determine whether the genotype/disease associated differences in their activity are the results of numerical or functional defects of this population, and whether such suppressive functions are fixed or only transient (Baecher-Alan et al., 2018).

Although our knowledge of human B cell development has improved considerably during the past two decades, most of our understanding of B cell differentiation is based on the presence or absence of certain cellular markers, and unavoidably, a great deal of cellular diversity involved is often underappreciated (Bemark, 2015). For instance, to separate naïve B cells from total B cells, the most straightforward way is to remove cells that are CD27 positive. However, such an approach would not differentiate naïve B cells from the cells staying at the transitional stages, which have been suggested to be associated with potential immunosuppressive role (Li et al., 2015). Moreover, it has also been demonstrated that substantial numbers of class-switched memory B cells do not express CD27 (Fecteau et al., 2006). Therefore, the cells gated within the naïve group could be a mixture of immature B cells at various developmental stages. For these reasons, it needs to be remembered that the B cell subtypes defined using flow cytometry are likely to be heterogeneous rather than inevitably functionally or phenotypically identical. Focusing on cell populations broadly defined by surface markers unavoidably averages out such heterogeneity within those specific cellular subtypes.

Although flow cytometry is one of the most frequently used methods of characterizing immune cells it is only able to measure a relatively few selected cell properties at any one time. This limitation means that the method can often only provide a crude guide to the functional nature of the analysed cells and inevitably misses much of the heterogeneity within those defined cellular subtypes. Given that cells are the most fundamental units of the immune system, the establishment of the whole gene expression profile within individual cells has the potential to overcome these limitations.

### 6.6 Where next: Single cell expression profiling

Ultimately, the behaviour of a cell is determined by the pattern of protein expression within the cell, which has been suggested to be primarily determined by transcription rather than translation or protein degradation (Battle et al., 2015). In turn, gene expression is determined by the particular set of transcription factors and cis-regulatory elements that are active in the cell (Ostuni et al., 2013). Genetic factors profoundly influence expression and many of the variants identified in GWAS lie in regulatory regions of the genome and likely exert their
effects by altering gene expression. However, deciphering the biological consequences of these noncoding variants has turned out to be extremely challenging (Farh et al., 2015).

Given that the regulatory effects of genetic variation are frequently cell type and activation state specific, it is to be expected that identifying the disease relevant effects of the variants suggested by GWAS will require the analysis of expression in the relevant cell types under appropriate conditions. As CD86 and CD40 are expressed on B cells and are involved in immune signalling, it seemed logical to extend the work conducted by my predecessor and look at the expression of these receptors on B cells in the context of relevant stimulation.

Although the number of regulatory elements in the human genome is enormous, the functional clusters of transcriptional enhancers, which drive the expression of genes that define the identity of the given cells, are more limited in number, and those variants associated with diseases are particularly enriched in these super-enhancers, especially in the cell types relevant to the diseases (Hnisz et al., 2013). However, identifying cell type specific eQTLs can be challenging when the cells of interest only makes up a small fraction of those tested (Wills et al., 2013). In contrast, single cell analysis has the potential to character each cell individually, rather than considering the average properties of a group of cells defined in terms of a restricted number of canonically used surface makers.

Next generation sequencing (NGS) technologies have enabled the efficient and robust characterization of the transcriptome at the single cell level in thousands of cells and have thereby enabled the detailed analysis of extremely rare cell types (Wu et al., 2017). Thus, exploring the effects of MS associated expression in cell types such as transitional B cells and plasmablasts would likely have been possible using this approach rather than FAC sorting. Furthermore, through single cell expression analysis it would be possible to group together cells with similar functional activity (as reflected by their gene expression profile) rather than on the basis of pre-determined surface markers, and thereby potentially identify previously un-recognised sub-types of immune cells.

In NGS experiments, the most basic measurement unit is the read, which represents a fragment of cDNA being reverse transcribed and amplified (Wu et al., 2017). For single-cell RNA sequencing using mammalian cells, it has been recommended that 1 to 2 million reads per cell should be performed for capturing genes with confidence (Grün et al., 2014). As a consequence, these methods are currently primarily limited by their considerable computational and financial costs. These costs could be reduced by pre-sorting cells in those of interest (Drissen et al., 2016), but this inevitably limits the potential for the discovery of novel cell types. The approach is extremely sensitive and great care has to be taken to limit the impact of experimental variations and batch effects (Munro et al., 2014).

If I had had the time and financial means to extend my research, I would have undertaken a single cell expression analysis in both MS patients and controls, with the control samples
selected so as to ensure a balance of genotype at the CD86 and CD40 SNPs. I would have used FACS to focus the sequencing efforts on to B cells but would have allowed the observed expression patterns to characterize the B cell sub-types. Variation in technical performance and low efficiency in the conversion from mRNA to cDNA are major problems with existing single cell transcription methods. The use of exogenous RNA molecules as spike-in positive controls, such as the synthetic transcripts External RNA Controls Consortium RNA standards that have known sequence and abundance, can help to reduce the impact of such experimental variation (Munro et al., 2014), as can the incorporation of random barcode sequence of nucleotides, which function as unique molecular identifier (UMI) that effectively allows the counting of mRNA molecules (Islam et al., 2014). I anticipate that the resulting high-resolution single cell RNA-sequencing data set would greatly refine our understanding on the effects of MS associated variation in MS and might even identify previously un-appreciated B cell sub-types.

6.7 Limitations of this study

There are several limitations inherent in the research I completed during my PhD. Firstly, like most other human based MS studies, I obtained my study samples from venous blood rather than from the brain itself or its draining lymphoid tissue. Given that the immunological phenotypes of cells in the periphery may not genuinely reflect the conditions behind the blood-brain barrier, it is possible that important effects relevant within the CNS might not be apparent in studies based on circulating immune cells. Cells acquired from CSF might have more closely represented those in the CNS, but it was infeasible to access large numbers of CSF samples from a cohort of healthy volunteers for ethical and practical reasons. Another limitation of my research is that although I have confirmed that the MS associated genotypes are associated with the surface expression level of CD86 and CD40 on B cells and that these changes result in increased proliferation of T cells, I have not established the underlying mechanisms by which these changes occur. The extensive LD associated with each of the associated SNPs means that the causal variants underlying these association have yet to be identified. If these associations could be fine-mapped, then genomic editing techniques could be employed to explore these mechanisms. Moreover, although there is compelling logic behind my use of CD40L-transfected L cells as the means to stimulate B cells, this is only one of many physiologically relevant modes of stimulation, and these alternatives remain unexplored. Finally, my work has focused on B cells and has not considered alternative APCs such as dendritic cells and macrophage (Korn and Kallies, 2017). Similarly, although CD4 T cells are considered the primary pathogenic drivers of MS, CD8 T cells might also play a role but were not specifically assessed in my cultures. In a recently published study of brain
infiltrating lymphocytes, the predominant cells identified were CD8+ T cells and CD20+ B cells irrespective of MS disease course or lesion stages, whereas CD4+ T cells were scarce (Machado-Santos et al., 2018). Expanding the work to include analysis of all potentially relevant cell types would be a logical extension to my research.
Concluding remarks

In conclusion, in my PhD studies I assessed the influence of MS associated genotypes on B cells and T cells in both healthy volunteers and MS patients. My analysis confirmed that the MS risk alleles at rs9282641 and rs4810485 respectively lead to higher expression of CD86 and lower expression of CD40 in the context of stimulation. Moreover, I was able to show that the expression changes induced by carrying the risk allele at rs9282641 result in increased proliferation of pro-inflammatory T cells. Overall, the results of my PhD studies have improved our understanding of the relationship between genotype and the development of MS and understanding that has the potential to promote the development of novel therapeutic strategies.
References


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## Appendix

### Treatment information of the recruited MS patients

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