DYSREGULATED B CELLS IN RELAPSING REMITTING MULTIPLE SCLEROSIS AND THEIR IMPACT ON T-CELL FUNCTION

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DEDICATION

Nancy, thank you for your mentorship and friendship. Beyond teaching me to become a scientist, you provided unwavering support and encouragement. The members of the Monson lab have been a source great support during my graduate career.

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DYSREGULATED B CELLS IN RELAPSING REMITTING MULTIPLE SCLEROSIS AND THEIR IMPACT ON T-CELL FUNCTION

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Relapsing-remitting multiple sclerosis (MS) is an autoimmune mediated inflammatory demyelinating disorder of the central nervous system. The role of B cells in the pathoetiology of MS is substantiated by cell depletion therapies but is not well understood. We hypothesized that B cells from MS patients would secrete more pro-inflammatory cytokines and support T cell responses to self-antigens and that the therapeutic agent glatiramer acetate (GA) could modulate these aspects of B cell function. To test this hypothesis we interrogated the ability of memory and naive B cells from healthy donors (HD), MS patients, and GA-treated MS patients (GA-MS) to proliferate and secrete cytokines *in vitro.* We identified a remarkable loss of IL-10 secretion by B cells from MS patients, but a marked increase in the

production of IL-6, particularly from naïve B cells. We also found that memory B cells from MS patients exhibited hyperactive proliferation compared to healthy donors and naïve B cells from MS patients. GA had no measurable impact on any of the B cell functions we tested; however, B cells from GA-treated MS patients had a restored ability to produce IL-10, greatly enhanced immunoglobulin production, altered proliferation capacity and a transient diminishment of IL-6 production for patients on therapy for less than 32 months.

To address whether memory or naïve B cells from MS patients supported neuro-antigen specific T cell responses, we co-cultured B and T cells in the absence or presence of foreign or neuro-antigens. We found that memory and naïve B cells from MS patients support more CD4+ T cell proliferation and TH1 and TH17 responses to neuro-antigens despite a similar frequency of neuroantigen specific T cells. Together, these data reveal that B cells from MS patients exhibit dysregulated proliferation and cytokine secretion that can be modulated by GA. Furthermore B cells from MS patients support neuroantigen-specific T cell proliferation and pro-inflammatory cytokine production in response to self neuro-antigens.

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MS – multiple sclerosis

HLA – human leukocyte antigen

DC – dendritic cells

APC– antigen presenting cells

GA – glatiramer acetate

CNS – central nervous system

RRMS – relapsing-remitting multiple sclerosis

SPMS – secondary progressive multiple sclerosis

PPMS – primary progressive multiple sclerosis

PLP – myelin proteolipid protein

MBP – myelin basic protein

MOG – myelin oligodendrocyte

SLE – systemic lupus erythematosus

SNPs – single-nucleotide polymorphisms

GWAS – genome-wide association study

EBV – Epstein Barr virus

OCB – oligoclonal bands

Ig – immunoglobulin

BAFF – B cell activating factor

APRIL – a proliferation-inducing ligand

EDSS – expanded disability status scale Term – Definition goes here

VH – variable heavy chain

EAE – Experimental autoimmune encephalomyelitis

BBB – blood brain barrier

Th – T helper

CD – cluster of differentiation

ELISpot – enzyme-linked immunospot

IFN – interferon

IL – interleukin

TNF – tumor necrosis factor

TEM – T effector memory cell

Tr1 – type 1 regulatory T cell

Treg – T regulatory cell

Foxp3 – forkhead box P3

CTL – cytotoxic T lymphocyte

BCR – B cell receptor

TI– thymus independent

TD – thymus dependent

TCR – T ell receptor

Tfh – T follicular helper cell

SHM – somatic hypermutation

NF-κB – nuclear factor kappa B

PI3K – phosphoinositide 3-kinase

TLR – Toll-like receptor

CpG – unmethylated CpG oligodeoxynucleotides

IRF7 – interferon regulatory factor 7

AP-1 – activator protein 1

LT-α – lymphotoxin alpha

B10 – a human B cell that produces IL-10 directly *ex vivo*

B10pro – a human B cell that produces IL-10 after stimulation *in vitro*

TT – tetanus toxoid

MHC – major histocompatibility complex

PBMC – peripheral blood mononuclear cells

BCDT– B cell depletion therapy

TACI – tumor necrosis factor receptor superfamily member 13B

STAT – signal transducer and activator of transcription

CHAMPS – controlled high-risk subjects Avonex multiple sclerosis prevention study

TN-MS – treatment naïve multiple sclerosis patients

GA-MS – multiple sclerosis patients receiving glatiramer acetate therapy

CFSE –carboxyfluorescein succinimidyl ester

DMSO – dimethyl sulfoxide

BCDF – B-cell depleted fraction

FACS – fluorescence-activated cell sorting

MACS – magnetic-activated cell sorting

ELISA – enzyme linked immunosorbent assay

OND – other neurological diseases

CHAPTER ONE
Introduction

The role of the immune system is to recognize pathogenic threats such as bacteria, viruses, and fungi and eliminate them with limited damage to the host. The immune response is a highly complex and coordinated system with numerous checks and balances to avoid aberrant activation. Yet these responses are imperfect and can lead to a brake in self-tolerance and the development of autoimmune disorders. The mechanisms by which self-tolerance is broken in certain individuals leading to the development of autoimmune disorders is not clear.

Relapsing-remitting multiple sclerosis (MS) is an autoimmune mediated neurological disorder characterized by inflammation and demyelination. While the etiology of MS is unknown, it is clear that CD4+ T cells are a central component of MS pathogenesis. Yet T cells do not act *in vaccuo,* they require presentation of cognate antigen in the context of HLA-DR, co-receptor stimulation, and direction from the local cytokine milieu for initial activation. Once licensed, T cells continue to need input from other cell types to shape the immune response. To this end, a critical role for B cells, macrophages, monocytes, dendritic cells (DC) and, other innate immune components in propagating T cell activation and contributing to pathology directly have been proposed, but have proven more difficult to characterize.

Recently B-cell depletion therapies have shown efficacy in reducing symptoms and clinical manifestations of MS. Aside from a role for secreting auto-reactive antibodies, it is unclear how B cells contribute to disease pathology. B cells are capable of pro-inflammatory and regulatory cytokine secretion and activating T cells by serving as antigen-presenting cells (APC). The work presented here is an investigation of B cell functions and the impact of B cells on antigen-specific T cell activation in the context of MS. We hypothesized that these antibody-independent B cell effector functions are dysregulated in MS patients and are modulated by glatiramer acetate.

CHAPTER TWO
Review of the Literature

2.1 Multiple sclerosis

2.1.1 Diagnostic Criteria

Multiple sclerosis was first described as a unique neurological disorder in 1826 by Jean-Martin Charcot based on findings from post-mortem central nervous system (CNS) tissues [1]. The modern clinical definition of MS began with Shumaker in 1965 [2]. Today the diagnosis of MS is based on the revised 2010 McDonald Criteria [3], which combines clinical evidence of symptoms, gadolinium (diethylenetriamine penta-acetic acid)-enhancing and/or nonenhancing lesions in MS such that characteristic locations in the CNS detected by magnetic resonance imagining (MRI) are disseminated in space (DIS) [4, 5] and/or disseminated in time (DIT) [6]. MS manifests as a wide range of neurological deficits including, but not limited to, cognition, numbness, weakness, mobility, and vision [7]. MS is the second most common inflammatory neurological condition in young adults, affecting over 400,000 individuals in the United States and 2.3 million individuals world-wide as estimated by the National Multiple Sclerosis Society [8]. Although the past two-decades have seen the advent of disease modifying therapies, MS remains an incurable disorder.

2.1.2 Forms of Multiple Sclerosis

There are several forms of MS [9]. Relapsing-remitting MS (RRMS, referred to after this section as MS) is the most common type of MS and accounts for approximately 80-85

percent of all MS cases at the time of diagnosis [10]. RRMS is characterized by acute periods of increased disability and symptoms (relapse/exacerbation) followed by a remission where neurologic dysfunction or disability is resolved to varying degrees. Over time, patients typically accrue disability during relapses leading to an increasing plateau of disability between relapses (Figure 2-01A). The majority of RRMS patients convert to secondary progressive MS (SPMS). In this form of MS, disability increases steadily in the absence of acute exacerbations (Figure 2-01B). Some patients convert to SPMS as early as three years, nearly fifty percent convert in 11-15 years [10] and the vast majority (90 percent) convert by 25 years [11] after diagnosis.

Other forms of MS include primary progressive MS (PPMS), which involves accumulation of disability independently of exacerbations (Figure 2-01C). While PPMS shares some diagnostic criteria and manifestations with RRMS, it is not clear that PPMS is autoimmune-driven. Several of the therapies that are efficacious for RRMS have been used to treat PPMS, but were unsuccessful in reducing disease [12].

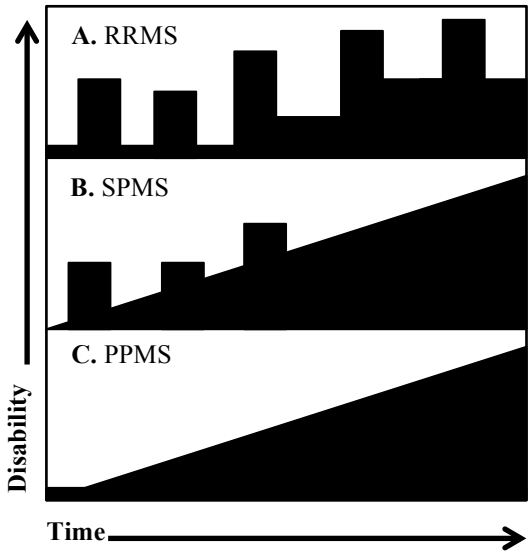


Figure 2-01. Graphical representation of the subtypes of MS based on disability over time for relapsing-remitting MS (**A**), primary progressive MS (**B**), and secondary progressive MS (**C**).

On a cellular level, RRMS symptoms arise largely from the destruction of the myelin sheath that wraps axons. The myelin sheath is generated by oligodendrocytes in the CNS, and it insulates the electrical potential of the axon when a neuron fires. The sheath itself is

comprised of a complex mixture of approximately 70% lipid and 30% protein [13]. Of the protein fraction, myelin proteolipid protein (PLP) accounts for ~50%, while a smaller but significant fraction ~30% is myelin basic protein (MBP). Myelin oligodendrocyte glycoprotein (MOG) makes up a very small fraction of the myelin sheath (~0.05%) but is of particular interest because MOG decorates the region of myelin that is exposed [14], whereas PLP and MBP are embedded under the surface of intact myelin. When the myelin sheath is degraded, the signals transmitted by neurons are disrupted, leading to many of the symptoms of RRMS. While oligodendrocytes can repair some damage to the myelin sheath (re-myelination), if the damage is severe enough axonal truncation and permanent loss occur. Indeed, there is extensive evidence for axonal loss in MS [15] and it is thought that axonal loss underlies the transition from RRMS to SPMS over time [16].

2.1.3. Etiology of Multiple Sclerosis

The exact cause of MS is unknown, but it is thought to arise from a complex interplay between genetic and environmental factors. MS affects women more commonly than men, with a ratio of 3.2:1 women to men and this female predominance appears to be increasing in recent years [17]. Female prevalence is not unique to MS and is observed in other autoimmune disorders such as SLE. Many plausible reasons for this female predominance have been put forward and include sex-linked genetic traits and the impact of hormones on the immune system [18]. Racial and ethnic differences have also contribute to the risk for developing MS [19].

Several geographic clusters of MS have been reported; however, no cluster has ever been linked to a clear environmental stimulus [20]. Moreover, adopted or step-siblings livinv in the same household with an affected non-biological sibling do not have an increased risk of developing MS [21]. This suggests that microenvironments are not a major contributing factor for MS. The most consistent evidence for an environmental MS risk is geographical latitude [22], although this, too, is disputed. For example, in the United States, individuals northern versus part of the country had a case to control ratio of 1.41 while those in the southern region had a ratio of 0.53 [23].

More recently, a role for vitamin D3 supplementation has been linked with a lower incidence of MS [24] and associated symptoms [25] in women. Vitamin D is synthesized after sunlight exposure and fits well with the geographical distribution of MS being more common in Northern regions with less seasonal sunlight [26]. One study using monozygotic twin pairs showed that childhood sun exposure related to a lower risk of developing demyelinating disease [27]. However, other studies have demonstrated independent roles for sun exposure and vitamin D [28]. Indeed, while ultra violet (UV) radiation reduced the severity of disease in the mouse model of MS, the vitamin D levels induced by UV exposure were not sufficient to alter disease [29].

Genetics also contribute to MS susceptibility. However, twin studies demonstrate that genetics alone are not sufficient to cause MS. A monozygotic twin has a higher risk of developing MS if the other twin is affected, particularly in female twins; however, dizygotic twins and non-twin siblings have similar risk [30]. The first genetic contributor to MS, HLA-DRB1-1501, was identified in the 1970s and remains the most highly linked MS gene.

However, single-nucleotide polymorphisms (SNPs) in multiple other genes have been identified since the advent of high-throughput genome wide association studies (GWAS). In one such study, over 9,000 patients were characterized, a total of 52 loci were identified, including the the 26 loci that had been identified in prior studies [31]. Other studies have verified these results and identified a few additional genes [32]. However, the majority of the newly identified loci have only moderate odds ratios. The overwhelming majority of the loci identified have a role in immune cell function, differentiation and/or activation. Despite the increased relative risk of developing MS, it remains to be seen how to the development of MS in certain individuals based on genetic susceptibility, alone [33].

Many suggestions have been made surrounding the possibility that bacterial, fungal, or viral infection directly contribute to individuals developing MS. For example, pathogens could initiate breakdown of the blood brain barrier, which could facilitate the infiltration of lymphocytes into the CNS or cause antigen-specific responses due to molecular similarities of pathogen components and CNS tissues. One of the most well studied infectious agents studied in the context of MS is Epstein Barr virus (EBV). Individuals that have had infectious EBV (mononucleosis) have a higher rate of developing MS while individuals with no evidence for EBV infection have a lower rate of MS [34, 35]. It is however, worth noting that approximately 95% of all individuals have been infected to EBV and multiple other disorders have been associated with EBV [36]. In one study, B cells harboring EBV were identified in ectopic follicles in the meninges of SPMS patients [37]. Yet other observations in RRMS found that EBV transcripts are not present in the CNS lesions of MS patients [38]. Thus, the impact of EBV on MS is still hotly debated.

It is clear from the studies highlighted here that MS is, indeed, a disorder not caused by a single identifiable factor. Rather, it is likely a complex interplay among factors including genetic susceptibility, environmental exposures and, perhaps, infectious agents that act in concert to initiate MS. While studies are still ongoing to identify risk factors as a means to prevent MS, others are underway to determine how to treat or eradicate MS once it has taken hold.

2.2. RRMS as an Autoimmune Disorder

2.2.1. Oligoclonal Bands

The first evidence for immune cell involvement in RRMS was the presence of two or more enriched immunoglobulin G (IgG) bands termed "oligoclonal bands" (OCB) present in cerebrospinal fluid (CSF) of MS patients, which were visualized by isoelectric focusing [39]. OCB are specific to the CSF and are not present in the peripheral blood and are thought to be a result of intrathecal IgG production by short and long-lived plasmablasts. While OCB are not specific to MS, they are found in approximately 80-95% of MS patients [40] and, until the most recent update, was a feature of the McDonald criteria for diagnosing MS [3]. OCB are predominantly IgG1 and IgG3, but some patients have enrichments of IgG2 and IgG4 [41]. Despite the presence of IgG in the CSF, it is not clear if these antibodies are pathogenic. In addition, the CSF has elevated levels of B cell and plasma cell survival and differentiation factors including CXCL12, BAFF and APRIL [42]. The B cell chemoattractant CXCL13 is elevated in the CSF of MS patients and is correlated with OCB, relapse rate, and the

expanded disability status scale (EDSS), which is used to assess the severity of disability in MS patients [43].

Several studies have used CSF fluid, CSF B cells, and CNS tissue to identify antibodies against MOG, and MBP [44], among others [45]. In one study, serum from approximately one-third of MS patients contained Ig capable of mediating demyelination in a complement-dependent fashion *in vitro* [46]. Because OCB are segregated to the CSF, many have used sorted B cells from the CSF of MS patients to identify the antigenic targets. B cells from MS CSF are enriched for variable heavy chain 4 (VH4) [47], clonally expanded and extensively mutated [48, 49], and are the products of germinal center reactions [50].

2.2.2. Immune Cell Infiltration in the MS Lesion

Areas of demyelination are found in white matter, but also extend to grey matter [51]. MS lesions can be categorized by the extent of myelin loss and immune cell infiltrates. Henderson *et al.* identified three stages of lesion development in which, in the earliest stage undergoes oligodendrocyte loss and an increase in microglia, the resident CNS APC (pre-phagocytic) [52]. This is consistent with evidence that in some newly forming lesions, oligodendrocyte loss occurs prior to the influx of immune cells [53]. This is followed by infiltration of monocytes and macrophages accompanied by partial myelin loss with infiltration of B and T cells in perivascular spaces, but limited infiltration in the lesion [52]. Finally, areas completely devoid of myelin contained foamy macrophages that had phagocytized myelin lipids and T cells [52]. Perivascular cuffing, which is localization of lymphocytes in close proximity to blood vessels, occurs near such lesions. These cuffs

contained a mixture or CD4+ and CD8+ T cells, B cells and plasma cells, but were heterogeneous in the cell make-up [52]. One study found that macrophages in these lesions expressed CD163, a marker of alternatively activated macrophages that may be protective, in the brain tissue in MS patients [54].

MS lesions have also been categorized based on the type of immune cell infiltrate of T cells, macrophages and deposition of antibody and complement [55]. Deposition of antibody was recognized as an early feature of MS plaques [56] and is prevalent in active phagocytic lesions containing macrophages [57]. Complement deposition has also been reported in lesions [58].

2.2.3 Experimental Autoimmune Encephalomyelitis

While the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) does not manifest with the same symptoms or course as MS, it has been instrumental for MS research. EAE was developed in order to understand disseminated myelitis responses to vaccines more than 70 years ago [59, 60]. Today EAE is carried out using purified myelin proteins with adjuvant and has emerged as an instrumental model to study MS. There are numerous models of EAE utilizing different myelin antigens and mouse strains [61]. Induction of EAE results in breakdown of the blood brain barrier (BBB), infiltration of immune cells to the CNS (predominantly in the spinal cord), demyelination and ascending paralysis. One of the most critical observations made in EAE is that encephalitogenic CD4+ T cells from a mouse with active EAE can adoptively transfer disease to a naïve mouse [62-64].

2.3 T cells in in Multiple Sclerosis

2.3.1 Introduction

Like EAE, MS is largely considered a T cell mediated condition [65]. Human CD4+ T helper (Th) cells that secrete IFN-γ and TNF-α (Th1) or IL-17, IL-21, and IL-22 (Th17) are considered pathogenic in MS. The CD4+ and CD8+ T cells present in MS lesions are clonally expanded [66]. T cells that are CD45RO+CCR7- T effector memory cells (TEM) and IFN-γ-secreting are enriched in the CSF of MS patients [67]. Although greatly outnumbered by IFN-γ+ cells, Th17 cells are significantly expanded in the CSF during MS relapse [68] and IL-17 is present in MS lesions [69]. These results suggest selective extravasation of antigen-experienced effector memory T cells into the CNS of MS patients.

2.3.2 Frequency of antigen-specific T cells

The majority of studies find reactivity to neuro-antigens both in HD and MS patients with some studies reporting instances of elevated responses to particular antigens in MS patients. The frequency of antigen-specific T cells has been quantified using several techniques including bulk PBMC proliferation or limiting dilution assays, IL-2 sensitivity, HLA-DR tetramer staining, clonotypic analysis, and ELISpot. The frequency of MBP specific T cells is similar in HD and MS patients [70, 71]. Detection of MBP-specific T cells by IFN-γ ELISpot showed similar frequencies [72]. Using IL-2 activation as a means to elicit proliferation by recently activated T cells, MBP-reactive T cells were enriched in the CSF of MS patients [73, 74]. Using clonotypic analysis from single-sorted peripheral T cells showed that as many as 1 in 300 T cells could be reactive to MBP in MS patients, while limiting

dilutions assays that yield approximately 2 in $2x10^6$ cells [75]. In peripheral blood, MBP-reactive clones are predominately Th1 INF-γ+ with very few Th2 IL-4+ cells reported by ELISpot [76].

Sun *et al* used ELISpot to determine the frequency of MOG-specific T cells is $13.7/1x10^6$ PBMC in MS patients [77]. While Kerlero de Rosbo *et al* used proliferation-based assays to study MOG reactivity [71], both of these studies found that MOG-specific T were enriched in MS peripheral blood compared to HD and other neurological diseases (OND). Several others have identified similar responses to MOG between HD and MS patients such as Lindert and colleagues, who also identified a predominantly Th1 IFN-γ secretion by MOG-specific clones [72, 78].

In addition, MBP-specific T cells from MS patients require less co-stimulation through CD28 to become activated suggesting that they were memory T cells [79]. In another study, 13 percent of MBP-specific cells from HD were memory T cells while 84 percent from MS patients were a memory phenotype [80]. This is not true for all neuro-antigens. For instance, PLP-reactive T cells from HD are distributed equally in memory and naïve compartments [81]. These studies highlight the differences in antigen-specific T cells using different methodologies. Indeed, myelin-specific T cells can be generated from both HD and MS patients [82].

2.3.3 Regulatory T cells

The evaluation of regulatory T cells in MS has been long-standing interest in the field [83]. Studies using techniques such as intracellular staining have allowed for the identification of

T cells that produce IL-10 (Tr1). However, Tr1 cells that produce IL-10 in a Stat3-dependent manner early upon activation do not fall into a single phenotypic category (reviewed in [84-86]). The suppressive capabilities of Tr1 by IL-10 are accompanied by other soluble molecules, such as transforming growth factor beta (TGF-β), and are cell contact-independent unlike CD4+,CD25+/hi,FoxP3+ T regulatory cells (Treg) [87]. To determine whether the induction of Tr1 cells was altered in MS patients, T cells were stimulated with anti-CD3 and CD46. The resulting Tr1 cells derived from MS patients secreted less IL-10 compared to HD [88]. Similarly, stimulation with anti-CD28, -3 and -46 resulted in a lower frequency of IL-10+ T cells and secretion of IL-10 by T cells [89]. Together these studies showed that *in vitro* differentiation of Tr1 is defective and Tr1 cells fail to secrete high levels of IL-10 in MS patients. In addition, Treg are found at similar frequencies in MS patients compared HD [90], although some differences in Foxp3 expression [91] and functional defects have been described [92-95]. It remains to be seen whether these defects are reflected in impaired Tr1 or Treg functions *in vivo*.

2.3.4 CD8+ T cells

CD8+ cytotoxic T lymphocytes (CTLs) recognize peptides presented on MHC I and can directly target and lyse cells that present their cognate antigen. CD8+ T cells outnumber CD4+ T cells in lesions [66]; however, the role of CD8+ T cells in MS less clear [96]. Antigen-specific murine CD8+ T cells are capable of transecting neurite outgrowths *in vitro* [97] and MHC I-deficient mice with EAE show demyelination but less neurological defects [98]. Based on expression of CD45RA and CD62L CD8+ effector memory T cells are

decreased in MS patients [99]. In addition, a role for CD8+ T cells in restraining auto-reactive CD4+ T cells has been uncovered in the recent past [100] this function appears to be deficient during exacerbations [101]. CD8+ T cells were capable of producing IL-10 [102] and have been described in the peripheral blood and CSF of MS patients. CD8+ IL-10 producing cells have a similar phenotype as CD4+ Treg, characterized by the expression of CD25+ FoxP3+ and low levels of CD127 and also secrete TGF-beta [103]. The frequency of IL-10+ CD8+ T cells is increased in the periphery of MS patients [104, 105]. Although CD8+ Treg secrete IL-10, their suppressive capacity in MS is thought to be carried out predominately through cell contact dependent mechanisms [103]. Thus CD8+ T cells may contribute to MS pathology but also play a role in controlling auto-reactive T cells.

2.4 Involvement of B Cells in Multiple Sclerosis

2.4.1 Introduction

The primary role of B cells in the immune system is to secrete antibodies. The potential for secreted neuroantigen-specific antibodies to serve as a diagnostic marker [106] and contribute to demyelination is well documented. Aside from antibody secretion, B cells possess a variety of capabilities that may contribute to MS. B cells are capable of secreting a wide array of cytokines, express co-stimulatory molecules, and act as antigen presenting cells (APC).

2.4.2 B Lymphocyte Development and Tolerance

Antibodies and the BCR are resultant of a unique feature of B cells in their ability to rearrange immunoglobulin genes, yielding millions of possible permutations of rearrangement. B cells arise in the bone marrow from common lymphoid progenitors. During development, B cells undergo positive and negative selection in the bone marrow (central tolerance) to ensure expression of a functional B cell receptor (BCR) composed of a heavy and light chain that is capable of signaling (positive selection) [107]. However, if the BCR signal is too strong to self-antigen, the B cell with undergo clonal deletion, although B cells are able to undergo receptor editing before being negatively selected [108].

Despite the central tolerance mechanisms, the majority of B cells newly emerged from the bone marrow display some level of reactivity to self-antigens [109]. Once in the periphery, the auto-reactive repertoire is refined by peripheral checkpoints that include anergy for high avidity auto-reactive B cells, inhibitory receptors, requirement for T cell help and B cell survival factors (e.g. BAFF). In MS patients, a defect in the peripheral, but not central tolerance, has been described. Transitional naïve B cells from MS patients were poly-reactive, and showed elevated binding to HEP2-lysates and were reactive to white matter [110]. The authors noted that these defects were similar to those in patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome who lack normal T regulatory cells (Treg) due to dysfunctional FOXP3 and present with a variety of autoimmune disorders. Interestingly, in the mouse model of IPEX syndrome, scurfy mice, unrestrained auto-antibody production by B cells leads to severe pathology [111]. Whether the dysregulated peripheral tolerance in MS is due to altered Treg remains to be seen but would fit with the alterations in Treg function. There are many other aspects of B cell

development that contribute to the development of auto-reactive B cells in the mature repertoire including perturbations in intrinsic signaling pathways for BCR or Toll-like receptors (TLR), and extrinsic factors (i.e. T cell help) [112].

2.4.3 Memory and Naïve B cells

Naïve B cells can undergo two types of activation. Thymus-independent (TI) B cell activation can occur by TLR stimulation or extensive BCR crosslinking induced by multivalent antigens. The predominant form of B cell activation is antigen specific and requires help from T cells (thymus-dependent, TD) involving HLA-DR/TCR interaction, co-stimulation and cytokines.

In TD activation, B cells interact with a Th to acquire a memory phenotype but these memory B cells bear relatively germline BCR because they do not require germinal center reactions [113]. Alternatively, B cells will interact with T follicular helper (Tfh) cells in the context of a germinal center where B cells undergo class switching and extensive somatic hypermutation (SHM) that refines antigen specificity and antibody effector function [114]. During germinal center reactions, short-lived plasma blasts and plasma cells are generated [115]. Plasma cells are terminally differentiated B cells. These cells are infrequent in the periphery and instead hone to the bone marrow and lymphoid organs. Plasma cells do not require additional licensing from T cells or cytokine stimulation to secrete antibodies.

Human memory B cells are readily identifiable as CD19+CD27+ [116, 117]. Memory B cells secrete more pro-inflammatory cytokines [118, 119] and are more capable of supporting antigen-independent autologous T cell proliferation than naïve B cells [120].

Supernatants from memory B cells, but not naïve B cells, stimulated through CD40 enhanced T cell IFN-γ secretion to polyclonal stimuli [121]. Studies have found both elevated [122] and diminished frequencies of memory B cells during relapse in different patient cohorts [123]. Despite these discrepancies in the periphery, activated and memory B cells are overrepresented in the CSF of MS patients and have greater capacity to migrate towards elevated chemoattractant factor CXCL13 in CSF from MS patients [123, 124]. These findings suggest that in the context of autoimmunity, antigen-specific memory B cells that have refined BCR affinity to antigen and secrete inflammatory/activating cytokines are poised migrate to the CNS to stimulate T cell responses.

2.4.4. Activation of B cells

B cells can respond to a variety of stimuli that allow them to participate in an immune response. Recognition of cognate antigen though the BCR leads to activation. BCR stimulation is important for the survival and activation of B cells. BCR crosslinking induces signaling through NFκB and PI3K, in addition to other pathways that allow for activation of B cells.

Interactions with T cells are critically important for B-cell activation. CD40 is a membrane bound receptor expressed on B cells, monocytes, macrophages and dendritic cells. The ligand for CD40 is CD40L (CD154), and is expressed upon activation in T cells. Ligation of CD40 on the B cell surface is important for B cell activation, particularly in the germinal center [125]. In fact, X-linked hyper-IgM syndrome, whose hallmark feature is the lack of B cell activation, is caused by defects in the CD40 and CD40L genes. CD40

stimulation leads to activation of canonical and non-canonical nuclear factor kappa B (NFκB) and phosphoinositide 3-kinase (PI3K) activation to induce a variety of genes that support survival, proliferation, differentiation and expression of cytokines including IL-6. Studies in mice found that prophylactic treatment of mice with a neutralizing antibody to CD40L prevented EAE [126]. In MS, CD40 expression is present in post-mortem CNS tissue on macrophages, microglia and B cells that are in close proximity to CD40L-expressing cells [126]. It likely also plays a critical role in setting up ectopic follicles and germinal center structures observed in SPMS [127].

Human B cells express a variety of Toll-like receptors (TLR) that allow B cells to respond to conserved pathogenic elements. TLR signals drive maturation of human B cells and induce cytokine production. Unlike murine B cells, human B cells respond poorly to lipopolysaccharide (LPS) due to low TLR4 expression; however, human B cells express TLR1, 6, 7, 8, 9 and 10 [128]. B cells respond robustly to the TLR9 agonist class B unmethylated CpG oligodeoxynucleotides (ODN) [129]. TLR9 stimulation leads to NFκB, interferon regulatory factor 7 (IRF7), and activator protein 1 (AP-1) all of which drive B cell activation.

2.4.5. The Impact of Cytokines Produced by B cells

Human B cells produce wide variety of pro-inflammatory and regulatory cytokines and growth factors [130-132]. Many of these cytokines produced by B cells have the potential to influence T-cell functions. For example, human B cells produce IL-6, IL-23, IL1-β, and TGFβ that can support Th17 development [121, 131-134]. In mice, B cells acquire an

effector cytokine phenotype that directs T cell responses against [135]. Whether or not human B cells have this capacity is not known.

Human B cells also produce tumor necrosis factor alpha (TNF-α) and Lymphotoxin alpha (LT-α). These cytokines can induce apoptosis or proliferation and survival, depending on the cell type. TNF-α mediates the acute phase reaction, increases macrophage phagocytosis, acts as a chemoattractant for neutrophils, and both as an inducer of proliferation or apoptosis. LT-α is well documented to have profound affects on the TNF-α deficient mice, which lack germinal centers and B cell follicles [136] and LT-α is critical for the germinal centers but not SHM [137]. Both cytokines can also contribute to T cell responses. For example, production of TNF-α by murine B cells is important for generating T cell responses to the fungal pathogen *Pneumocystis murina.* B cells from MS patients produced significantly more LT-α in the presence of CD40L, BCR stimulation and CpG or IFN-γ [138]. In the same study CD40L, BCR stimulation and IFN-γ resulted in higher levels of TNF-α by B cells from MS patients. While it is true that HD B cells generally make less of these cytokines than their myeloid-derived APC, it should not be discounted that B-cell derived cytokines can influence local environments. Yet, how and the extent to which human B cell-derived cytokines impact immune responses and autoimmunity is not well defined.

2.4.6. IL-10 Production by B cells

Recent observations in mice have identified CD5+ CD1d^{hi} IL-10-producing B cells [139] that are capable of suppressing autoimmunity through mechanisms involving IL-10, IL-35 [140]

or CD40 and IL-21 [141]. Interleukin 10 is pleiotropic cytokine capable of acting on both B and T cells, which is of great interest to regulatory B cell effector functions [142].

IL-10 is considered an anti-inflammatory/regulatory cytokine. It is secreted by regulatory T cells, macrophages [143], and by some subsets of dendritic cells [144, 145]. IL-10 can dampen immune responses in several ways. First, IL-10 limits antigen presentation through down-modulation of HLA-DR and co-receptor (CD80 and CD86) expression on APC [142, 146-148]. IL-10 can also directly suppress CD4+ T cell proliferation and cytokine secretion induced by CD28 stimulation in healthy control peripheral blood cells and T cell lines [147] and increases T cell sensitivity to apoptosis [149]. However, IL-10 is not solely regulatory in nature and acts as a potent B cell survival and differentiation factor [142, 150].

Healthy donor B cells readily secrete IL-10 in to the culture supernatant upon *in vitro* stimulation; however, B cells from MS patients exhibit a defect in the ability to secrete IL-10 [151] or to do so in a normal ratio to pro-inflammatory cytokines [138]. This may be partially explained by the reduced level of TLR9 expression in memory B cells from MS patients [151], yet this deficit in IL-10 production was also observed independently of TLR9 stimulation. These results suggest that the regulatory capacity of B cells in MS patients is diminished.

The subset of B cells IL-10 producing cells can also be identified by IL-10 by intracellular staining after stimulation PMA and ionomycin in the presence of a protein transport inhibitor (i.e. brefeldin A). This methodology allows for a delineation of the subset of B cells that produce IL-10 however there are divergent reports on the phenotype of IL-10

producing B cells. In separate reports, IL-10+ B cells were enriched in populations that expressed CD24hiCD27+[152], CD19+CD25+Cd71+CD73- [153], or CD19+CD24hiCD38hi [154, 155]. However, IL-10+ B cells are not exclusively confined within these phenotypes.

Human B cells that are IL-10+ directly *ex vivo* (B10 cells) by intracellular staining only account for approximately one percent of the total B cell pool [152] and are found in a similar frequency in HD and MS patients [152, 154]. Human B cells secrete IL-10 to a variety of stimuli including CD40L, BCR crosslinking [156], and CpG [157] or a combination thereof [158]. *In vitro* activation induces IL-10 production in approximately five percent of B cells (B10pro). Several studies have quantified B10pro cells in the peripheral blood of MS patients. One of these studies found that B10pro cells are less frequent in MS patients when stimulated with CD40L and LPS, but not CpG [111]. In contrast, Kippenburg *et al* found that when B cells from were stimulated with CpG MS patients harbored fewer B10pro cells compared to HD during relapse and remission [116]. Due to the different stimulation conditions and conflicting results using IL-10 quantification by intracellular staining alone, particularly given the contrasting results from quantifying IL-10 levels in culture supernatants of activated B cells.

Whether these B10 or B10pro cells represent a unique B cell subset is an area of active investigation. A comparison of IL-10+ and IL-10- B cells after 48 hour stimulation showed over 99 percent similarity in gene expression [159], although another study identified changes in gene expression [153]. Despite these conflicting results, it is clear that human IL-

10 producing B cells can modulate T cell responses *in vitro* [155, 160], which suggests that B10pro cells have unique regulatory capacities.

More recently the fate of IL-10 producing B cells has been investigated. In mice, utilizing a IL-10 promoter driven GFP, IL-10+ B cells were found to differentiate into antibody secreting plasmablasts/plasma cells [161]. Due to the phenotypic heterogeneity of human IL-10+ B cells are more difficult to study. Using IL-10 secretion capture assays, two studies have approached the question of B10 cell fate in humans. One study found that IL-10+ B cells secreted less IgG in culture compared to IL-10-negative cells [159]. However, another study found that IL-10+ naïve human B cells are primary producers of IgG4 [153]. Further studies are warranted to dissect the capabilities and fate of IL-10 and other cytokine producing B cells in humans.

2.4.7. Modulation of B cells by immunomodulatory therapies

There are few reports on the impact of MS therapeutics on B cells other than antibody production. Activated B cells exposed to IFNβ-1b *in vitro* had decreased CD40 and CD80 co-stimulatory molecule expression in MS patients but on CD40 in HD [162, 163]. *In vitro* IFNβ exposure decreased IL-23 and IL-1β secretion but increased IL-10, IL-12 and IL-27 production in HD and MS patients [162]. Supernatants from IFN-β-1b exposed B cell cultures inhibited IL-17 secretion by PBMC [162]. IFN-β-1b also reduced the ability of B cells from MS patients to induce proliferation and IFN-γ secretion by T cells [162, 164]. The frequency of CD27+ peripheral memory B cells is diminished with IFNβ therapy, as well as expression of CD86 [122, 123]. In the same study, CCR5 expression was also decreased on

total CD19+ and naïve B cells, but not memory B cells [122]. Patients on IFN therapy showed an increase in naïve B cells and decrease in class switched and non class-switched memory B cells (CD20+CD27+IgD+).

Other therapies and environmental factors have an impact on B cells in MS. For example, Fingolimod, a sphingosine-1 receptor targeting drug, decreases the frequency of total CD19+ B cells, increases naïve and transitional B cells, and decreases CD80 expression in MS patients [165]. In the same study, B cells from Fingolimod treated patients produced less TNF-α and more IL-10 from transitional B cells. Another study found a similar reduction in memory B cells, and increased B cells that produce IL-10 and less LT-α [165]. After Fingolimod treatment proliferating memory B cells, marked by Ki-67 are reduced within weeks after initiation of therapy, but plasmablasts were elevated [166]. It is also known that helminth infections that induce TH2 responses can overcome the deficit in peripheral B cell production of IL-10 in MS patients and restore it to that of HD [167, 168]. Collectively, these observations demonstrate that the existing therapies can impact effector functions of B cells that include co-stimulation, cytokine production and APC activity.

2.4.8. B cell Antigen Presentation

Studies with murine B cells demonstrated that antigen specific B cells are potent APC [169-172]. In fact, B cells are critical APCs in some mouse models of autoimmune diseases such as diabetes and SLE [173, 174]. Human B cells are also capable of APC function, as they uptake large soluble and particulate antigens [175-177] similar to the murine B-cell preference for protein antigen [178]. B cells primarily capture cognate antigen through their

BCR, which induces BCR mediated crosslinking and internalization [179, 180]. Of note, under certain circumstances, B cells can uptake antigen through pinocytosis [181]. Antigens taken up by B cells are targeted to the endocytic compartment where they are processed and subsequently presented to CD4+ T cells in the context MHC Class II [182].

Initial investigations of human B cells as APC were carried out utilizing Epstein-Bar virus (EBV) transformed B cells since they can process and present particulate exogenous antigen to T cell clones [176]. This methodology proved that B cells can support antigen-specific T cell proliferation to tetanus toxoid (TT) [175]. Others showed that EBV-transformed B cells had the most effective APC function when previously activated T cells were used as responders [183]. In addition, EBV B cells from melanoma patients expanded in the same fashion were effectively used as APC to generate melanoma-specific, MHC II restricted CD4+ T cell clones that proliferated and secreted IFN-γ in response to melanoma antigen [184]. A potential confounding factor in these early human EBV B cell APC assays is that EBV transformation can induce IL-10 production in B cells [156], which may have inhibited T cell proliferation and pro-inflammatory cytokine production.

The next approach used to study human B cell APC function was to activate total human B cells *in vitro* with CD40L and IL-4 (CD40-B) as a means to increase expression of co-stimulatory molecules and factors known to be important in the generation of T cell responses [82, 133, 185-188] and chemotactic molecules on B cells important for secondary lymphoid organ homing [189]. This phenotype is similar to antigen-experienced human memory B cells that express increased levels of co-stimulatory molecules (CD80, CD86 and

CD40)[117, 190]. Murine antigen-specific B cells activated in a similar manner rival the APC capacity of dendritic cells (DC) [191].

In addition, CD40-activated B cells from HDs or cancer patients expanded viral-specific memory, auto-antigen/tumor and neo-antigen specific CD8+ T cell responses [181]. Human PBMC stimulated with CD40L and IL-4 to activate B cells were highly effective in generating neuro-antigen specific autologous CD4+ and CD8+ T cell clonal proliferation and IFN-γ secretion [82]. Initial studies demonstrated that peripheral HD B cells expanded using the CD40-B system were effective APC in the generation of antigen-specific CD4+ T cell lines [169, 184].

Our laboratory previously demonstrated that peripheral CD40-B cells from MS patients or HDs stimulated autologous CD4+ T cell proliferation in the presence of foreign or neuro-antigens [192]. CD40 activation of B cells with CpG can induce Th1 responses by provision of IL-12 [193]. Taken together, these studies indicate that *in vitro* activated CD40-B cells act as APC for CD4+ and CD8+ T cells in antigen recall responses and in primary responses to foreign or auto-antigen, including neuro-antigens.

Peripheral memory B cells from MS patients are also able to induce autologous CD4+ T cell IFN-γ production or proliferation in response to neuro-antigens without prior activation [194]. Others have shown that T cells from MS patients uniquely require B cells to achieve maximal proliferative and IFN-γ responses [138]. While evidence from mice suggests that B cells are also important for Th17 responses in EAE [195], whether human B cells can directly influence Th17 responses as APCs has not been evaluated.

In addition to providing antigen in the context of HLA-DR, B cells express other receptors that reciprocally influence B-T cell stimulation. For example CD80 and CD86 interact with CD28 or CTLA-4 to stimulate and dampen T cell responses, respectively. CD27 is expressed on memory B cells and activated T cells. CD27 interacts with CD70 to mediate reciprocal activation of B and T cells [196, 197]. Stimulation of T cells by OX40 enhances T cell activation [198, 199]. OX40 ligand, on B cells, plays an especially important role in modulating T cells that express OX40 when B cells serve as an APC [200].

B cells possess all the required elements for proficient antigen presentation. Of particular interest to MS are antigen-specific memory B cells. Memory B cells express elevated co-stimulatory molecules and because they primarily take up their cognate antigen in a BCR-dependent manner, auto-reactive B cells may bolster self-reactive T cell responses. In addition, B cells are capable of secreting cytokines that can greatly influence T-cell function, including IL-6 and IL-10. Demonstrations of altered cytokine production by B cells from MS patients provoke questions regarding the contribution of B cell cytokines to autoimmune disease and immune responses to pathogens.

2.2.9. Evidence for Active B cell Trafficking to the CNS

Importantly, new findings comparing post-mortem tissues from the CNS and draining cervical lymph nodes identified clonally related B cells in patients with RRMS, SPMS and PPMS [201]. This is highly suggestive that antigen-experienced B cells traffic to and from the CNS since related clones with an increasing number of mutations were identified on both sides of the BBB. In another study, CSF and peripheral blood was sampled from six RRMS

and two PPMS patients and the study found that the majority of clones identified both in the CSF and PB were derived from class-switched memory B cells [202].

The most important findings regarding ongoing migration of immune cells to the CNS were made in the context of Natalizumab. This monoclonal antibody blocks α4 integrin interactions that mediate extravasation of lymphocytes into the CNS. To date, Natalizumab is the most effective MS therapeutic [203]; however, patients on Natalizumab are at risk for developing progressive multifocal leukoencephalopathy (PML) due to reactivation of the John Cunningham (JC) virus in the absence of CNS immune surveillance. Natalizumab decreased the frequency of B cells and T cells in the CSF [204] and diminished OCB in a subset of patients [205]. These results suggest that peripheral B cells are capable of trafficking across the BBB during disease and that dysregulated B cell responses in the periphery could contribute to immune responses within the CNS.

2.4.10. B cell Depletion Therapy in Multiple Sclerosis

Despite the presence of OCB, the majority of research MS research has focused on T cells. The turning point for B-cell oriented research in MS came from a clinical trial using B cell depletion therapy (BCDT) in patients with RRMS. BCDT improved in the clinical symptoms of MS, with a reduction in total and new gadolinium enhancing lesions, and relapse incidence [206-209]. BCDT reduced B and T cell infiltration into the CSF of RRMS patients [210]. Initially it was thought that depleting B cells would reduce the OCB and potentially pathogenic antibodies. While antibodies are thought to play a role in MS disease pathology, current BCDT do not target immunoglobulin (Ig) secreting plasma cells and total Ig levels

remain unchanged during the time when clinical symptoms were initially diminished [206-209]. One study showed a diminishment of serum anti-MOG and anti–MBP in the serum of some patients after 24 weeks of BCDT [210].

However, not all therapeutic agents that target B cells demonstrate similar efficacy. For example, Atacicept, an agent that neutralizes Tumor necrosis factor receptor superfamily member 13B (TACI), a receptor for BAFF and APRIL (A PRoliferation-Inducing Ligand), that blocks plasma cell development worsened MS symptoms in a number of patients [211]. It is thought that this was due to the inability of Atacicept to target memory B cells and an unpredicted down-modulation of regulatory B cells upon treatment. How B cells potentiate immune responses and why BCDT is effective in a T cell-mediated disease remains unclear. B cells likely influence MS disease pathology through antibody-independent effector functions.

2.5 Glatiramer acetate

Glatiramer acetate is an FDA approved therapy for MS. GA is a random synthetic copolymer consisting of amino acids (L-alanine, L-lysine, L-glutamic acid and L-tyrosine) in a similar ratio to myelin basic protein 0.14:0.34:0.43:0.09) with an average molecular weight of 5-9kDa. GA was designed to induce EAE but was found to be protective. GA has many reported mechanisms of action [212]. Modulation of the immune response occurs as early as 4-12 hours in MS patients by enhancing IL-10 and TNF-α production [213]. GA can bind directly to HLA molecules [214, 215] interfere with the activation of antigen-specific T cells

[214, 216-218] and skew T cell polarization toward Th2 [219-221] and CD4+ [222][213] and CD8+[105, 223] regulatory cells.

Interestingly, GA has been reported to directly influence the cytokine secretion of myeloid APC to favor Treg and Th2 skewing cytokines [221, 224, 225]. For example, GA reduced IL-6 secretion by monocytes and inhibited p-STAT3 and Th17 differentiation in Th17-polarizing conditions [226]. Monocytes treated *in vitro* with GA had increased IL-10 expression and decreased IL-12p70 [213, 224] and TNF-α [225]. Monocytes from GA treated patients exhibited enhanced phagocytosis *in vitro* [227]. DC differentiation carried out in the presence of GA resulted in less IL-12 secretion in a dose dependent manner and modestly suppressed IL-8 and TNF-α, but had no impact on IL-6 [210]. These DC induced Th2 cells *in vitro*.

There is evidence that B cells from peripheral blood can bind MBP or GA (>7.5kDA) directly [192, 194]; however, this occurs at a few low frequency in HD and treatment naïve MS patients. However, GA-specific antibody responses occur after GA therapy [228]. In patients on therapy for less than two years levels of total α-GA IgG and IgG2 were elevated; however, the highest levels of GA reactivity were seen in the IgG4 subclass in both short and long term treated patients [229]. Little or no α-GA IgE was detected [230]. In the same study they found that serum with α-GA antibodies did not interfere with T cell proliferation to GA. This is in line with observations showing that α-GA antibodies are not neutralizing [231]. Interestingly, at 18 and 24 months after initiation of GA therapy, patients with higher levels α-GA IgG experienced fewer relapses [232]. It is clear that GA can drive antigen-specific B

cell antibody production; however, the impact of GA on other aspects of B cell function has not been studied in humans.

2.6 Conclusions and Hypothesis

B cells have the capacity to act as regulatory and inflammatory effector cells in the context of relapsing remitting multiple sclerosis. Memory B cells in particular are fully equipped to participate in immune responses in an antibody secretion independent manner through provision of cytokines and as APC. We hypothesized that B cells from MS would exhibit dysregulated cytokine and proliferative responses that would be modulated by GA therapy. Furthermore, we hypothesized that B cells from MS patients would support neuro-antigen specific Th1 and Th17 responses.

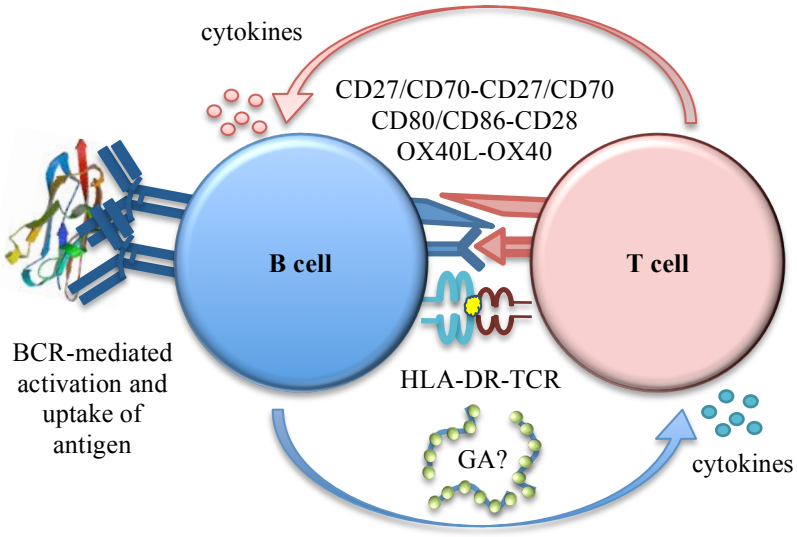


Figure 2-02. A model for B cell-T cell interactions in MS. B cells sense cognate antigen via BCR and present antigen to T cells on HLA-DR in the context of co-stimulatory molecules. B and T cells secrete cytokines leading to reciprocal activation.

CHAPTER THREE
Methodology

3.1 Patient and Healthy Donor Procurement and Processing

3.1.1 MS Patient Recruitment and Characteristics

MS patients were recruited through the University of Texas Southwestern Medical Center MS clinic in accordance with Institutional Review Board (IRB) guidelines. Patients gave oral and written consent to participate in these studies. Patient samples used in all of chapter four (Table 3.1), part of chapter five, and all of chapter six (Table 3.2) were treatment-naïve clinically definite RRMS or monosymptomatic multiple sclerosis patients, that subsequently converted to RRMS. These patients were enrolled in the controlled high-risk subjects Avonex multiple sclerosis prevention study (CHAMPS) study.

In chapter five, treatment naïve (TN)-MS patients had clinically definite RRMS and had not been on immunomodulatory or other disease modifying therapies, including corticosteroids for sixty days prior to sampling. Ten additional TN-MS patients were recruited for comparison to patients on GA therapy (GA-MS). For the additional TN-MS patients MS101-120 and GA-MS patients (table 3.1), 6-8mL of peripheral blood was collected in 8-10 acid citrate dextrose (ACD) tubes containing trisodum citrate (22.0g/L) citric acid (8.0g/L), and dextrose (24.5g/L)(BD Biosciences; San Jose, CA) and were processed by traditional ficoll or Sepmate to obtain PBMC (3.1.4 and 3.1.5). Patients receiving glatiramer acetate therapy (GA-MS) had been on therapy for at least three months at the time of sampling (Table 3.4). One patient was sampled prior to initiation of GA

therapy and at three and six months after initiation (patients MS103, GA103-2, GA103-3) and one patient was sampled prior to initiation of therapy and three months after initiating GA therapy (patient ID MS116 and GA116-2).

3.1.2 Healthy Donor Sample Procurement

Healthy donor (HD) samples were obtained from white blood cell filter bags (Carter Blood Care, Bedford TX) containing 40-100mL and were processed the same day as blood donation. Blood donations were evaluated by Carter Blood Care and found to be negative for the presence of Hepatitis B surface antigen, Hepatitis C antibody, Hepatitis B core antibody, Human Immunodeficiency Virus (HIV)-1 antibody, HIV-2 antibody, HIV-O antibody, Human T-lymphotorophic Virus type I and II antibodies, West Nile Virus, and Syphilis. HD leukapheresis packs (100-400mL)(Hemacare, Van Nuys, CA or Lonza, Walkersville, MD, USA) were shipped overnight to the University of Southwestern Medical Center and processed immediately upon receipt, within 24 hours of leukapheresis procedure. Mononuclear cell enriched peripheral blood obtained was screened and found negative for HIV-1, hepatitis B core, and hepatitis C antibodies. Due to the low amount of contaminating erythrocytes in leukapheresis five of the HD leukapheresis samples were processed without ficoll gradient density centrifugation (3.1.6 and 3.1.7).

Table 3.1 Treatment-naïve MS patient characteristics

Patient ID	Diagnosis	Time since diagnosis (years)	Age	Sex	MRI findings	EDSS
MS1#*§$@	RR	<2	23	F	+	1.5
MS2#*§$@	RR	<2	25	F	+	1.5
MS4*$@	RR	<2	43	F	+	1.5
MS5#*§$@	RR	<2	47	F	+	2.5
MS10#*§@	RR	<2	47	F	+	2
MS11§@	RR	<2	40	M	+	0
MS13*§@	RR	<2	41	F	+	1
MS14§@	RR	<2	41	F	+	2
MS15#*§@	RR	<2	32	F	+	1.5
MS17#*§@	RR	<2	44	F	+	3.5
MS18#*§@	RR	<2	37	F	+	2.5
MS19§@	RR	<2	35	F	+	2
MS20#*§@	RR	<2	27	M	+	1.5
MS101*⌘	RR	21	64	F	ND	ND
MS102*⌘	RR	<1	30	F	ND	ND
MS103*⌘	RR	4	54	F	ND	ND
MS113*⌘	RR	<1	65	M	ND	ND
MS114*⌘	RR	3.5	56	M	ND	ND
MS115*⌘	RR	<1	36	F	ND	ND
MS116*⌘	RR	1	41	M	ND	ND
MS117*⌘	RR	<1	28	F	ND	ND
MS119*⌘	RR	<1	52	F	ND	ND
MS120*⌘	RR	1.5	29	F	ND	ND

The mean age of patients was 40.7, EDSS was 1.8 and the ratio or females to males was 4.6:1. # Samples used in chapter four and five, and § samples used in chapter six. ⌘ Samples isolated from peripheral blood, these patients were not evaluated for MRI findings or scored on the EDSS.. $ Patients used in Harp *et al* 2008. @ Patients used in Harp *et al* 2010.

Table 3.2 Characteristics of patients on GA therapy used in chapter five

Patient ID	Diagnosis	Time since diagnosis (years)	Age	Sex	MRI findings	EDSS	Time on GA therapy (months)
GA-103-2	RR	4	54	F	ND	ND	3
GA103-3	RR	5	54	F	ND	ND	6
GA104	RR	0.8	64	M	ND	ND	10
GA105	RR	4.3	31	F	ND	ND	26
GA106	RR	2.3	50	F	ND	ND	28
GA107	RR	8.6	40	F	ND	ND	44
GA108	RR	2.8	63	M	ND	ND	32
GA109	RR	10	55	F	ND	ND	48
GA110	RR	4.6	42	F	ND	ND	54
GA111	RR	3.9	45	F	ND	ND	44
GA112	RR	2.5	31	F	ND	ND	28
GA116-2	RR	1.3	41	M	ND	ND	3
Characteristics of patients used for phenotypic studies and intracellular cytokine staining							
GA121	RR	3	25	M	ND	ND	40
GA122	RR	3.3	32	F	ND	ND	12
GA123	RR	5	52	F	ND	ND	12
GA124	RR	21	53	F	ND	ND	60
GA125	RR	10	54	M	ND	ND	66
GA126	RR	3.1	37	F	ND	ND	20
GA127	RR	1.5	24	F	ND	ND	20
GA128	RR	3.5	34	F	ND	ND	32
GA129	RR	3.7	56	F	ND	ND	42
GA130	RR	7	39	M	ND	ND	57

The mean age of patients was 41.5 and the ratio or females to males was 4.0:1. All samples were obtained from peripheral blood Patients receiving GA therapy were not screened for MRI findings or scored on the EDSS.

3.1.3 *Enumeration of Cells*

Peripheral blood samples were diluted in a 0.4% solution trypan blue (Sigma Aldrich, St. Louis, MO, USA) and 10μL of the diluted buffy coat material was added to the chamber of a hemocytometer (Hausser Scientific, Horsham, PA, USA). Cell number was enumerated under a light microscope at 40X-100X by counting 2 or 4 of the large grids of the counting chamber. The cell number was calculated by ((number of cells counted)/(number of large squares counted)) x (dilution factor) x (volume of cell suspension in mL) x 10^4, yielding a cell count in cells/mL.

3.1.4 *Leukapheresis Processing with Ficoll*

Leukapheresis packs were sprayed with 70% ethanol and wiped with a kimwipe until dry. A cut was made in the tubing of the leukapheresis pack and 25mL of leukapheresis material was added to 50mL conical tubes (Corning, Corning, NY, USA). Leukapheresis material was diluted 1:1 with PBS without calcium and magnesium or Roswell Park Memorial Institute, (RPMI) 1640 (Mediatech, Manassas, VA, USA) and as much as 30mL of the diluted leukapheresis material was slowly overlaid on to 15mL of ficoll-paque PLUS (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using a 25mL serological pipete (Costar, Washington, D.C., USA) in a new 50mL conical tube. Samples were centrifuged (Eppendorf 5810 R, Hamburg, Germany) at 1500-1900 revolutions per minute (rpm) for 25 minutes at room temperature with the brake off. The PBMC layer was removed from the interface of the plasma and ficoll layer using a 5 or 10mL serological pipette and transferred to a new 50mL conical tube. PBS or RPMI was added to bring the PBMC fraction

to a total volume of 50mL. The tubes were centrifuged at 1800rpm for 10 minutes at 4°c with the brake on. The supernatant was removed by vacuum aspiration or dumping (briefly inverting over a waste container), and the cell pellets were gently suspended by vortexing at half speed or by serological pipette. PBS or RMPI was added to a total volume of 50mL. Ten μL of material was transferred to a microcentrifuge tube, diluted 1:50 or 1:50 and 1:10 in trypan blue and enumerated (section 3.1.3). While cell counts were being prepared, PBMC were centrifuged at 1500rpm for 10 minutes at 4°C with the brake on. The supernatant was discarded and the cell pellet was re-suspended at $4x10^8$ cells/mL in human media (Table 3.8-01) and diluted in 2X freezing media (Table 3.8.1) to a final concentration of $2x10^8$ cells/mL. Cells were transferred to cryovials, in aliquots of $2x10^7$ to $4x10^8$. Cryovials were transferred to a Mr. Frosty (Nalgene, Rochester, NY, USA) and stored at -80°c overnight. The next day cell aliquots were transferred to a liquid nitrogen freezer for long-term storage at-130°c.

3.1.5 *Leukapheresis Processing without Ficoll*

Leukapheresis bags were treated the same as in 3.1.4. A maximum of 15mL of the leukapheresis material from the leukapheresis bag was transferred to a new 50mL conical tube and brought to 50mL in RPMI prior to centrifugation at 1800rpm for 10m at 4°c. Supernatants were discarded by vacuum aspiration and cell pellets suspended by serological pipet or vortexing at half speed in the remaining volume (~0.3mL). PBS or RPMI was added to bring the cell pellet to a total volume of 50mL. The tubes were centrifuged at 1800rpm for 10 minutes at 4°c with the brake on. The supernatant was discarded and the cell pellets were gently suspended by vortexing at half speed or by serological pipette. PBS or RMPI was

added to a total volume of 50mL, at which point cells were enumerated on as in 3.1.3 by preparing a 1:50 or 1:50 and 1:10 dilution in trypan blue. Cells were centrifuged at 1500rpm for 10 minutes at 4°c with the brake on. Cells were prepared for permanent storage after the supernatant was discarded as in 3.1.4.

3.1.6 *Processing of peripheral blood by traditional ficoll*

Peripheral blood samples collected in ACD tubes were centrifuged at 1400rpm for 10 minutes at room temperature with the brake off. The rubber stoppers were removed and the serum layer was transferred to a sterile 50mL conical tube and placed on ice. 1mL of serum was transferred to microcentrifuge tube stored at -80°c. The fraction containing erythrocytes and lymphocytes was transferred to 50mL conical tube by using a 10mL serological pipette to transfer the sample. Up to 25mL of peripheral blood was added to each 50mL conical tube where it was diluted 1:1 with room temperature PBS. 15mL ficoll was added to a new 50mL conical tube and up to 30mL of the blood/PBS mixture was overlaid on to the ficoll. The ficoll tubes were centrifuged at 1500-1900Rrpm at room temperature for 25 minutes with the brake off. The PBMC layer was removed from the interface after centrifugation by a 5-10mL serological pipette and transferred to a new 50mL conical tube. Up to 25mL of PBMC was pooled in each new conical tube and was diluted 1:1 with PBS. Ten μL of the PBMC was transferred to a microcentrifuge tube, diluted 1:10-1:50 in trypan blue and enumerated as in 3.1.3.

PBMC were centrifuged at 1500Rrpm for 10 minutes at 4°c with the brake on, the supernatant was removed by dumping and the cell pellet was suspended by vortex in the

remaining volume. The suspended cell pellets were combined into one 50mL conical tube and 10mL of PBS was added before the cells were centrifuged at 1500rpm for 7 minutes at 4°c. The supernatant was removed by dumping. The remaining pellet was suspended by vortex in the small remaining volume; 1mL of human freezing media was added for every $1x10^8$ PBMC and mixed thoroughly before transfer to a cryovial in 1mL aliquots. Cryovials were transferred to a Mr. Frosty and stored at -80°C overnight. The next day cell aliquots were transferred to a liquid nitrogen freezer for long-term storage.

3.1.7 *Processing of peripheral blood by sepmate tubes*

Serum was collected as in 3.1.6 and stored at -80°C for batched analysis of serum cytokine levels. Up to 25mL of erythrocyte/PBMC fraction was added to each 50mL conical tube where it was diluted 1:1 with room temperature PBS. 15mL ficoll was added to the bottom chamber of a Sepmate tube (Stemcell Technologies, Vancouver, BC) and up to 30mL of diluted blood PBS mixture was added to the top chamber. Sepmate tubes were centrifuged at 1900rpm for 10 minutes at room temperature with the brake on. After, the Sepmate tubes were inverted over new 50mL conical tubes for no more than 3 seconds to retrieve the LEL. This fraction was diluted 1:1 in PBS and centrifuged at 1500rpm for 10 minutes at 4°c. The supernatant was discarded by dumping and the cells were suspended in 10mL PBS; 10μL of the PBMC-enriched fraction was transferred to a microcentrifuge tube, diluted 1:10-1:50 in trypan blue, and enumerated as in 3.1.3. The PBMC were centrifuged at 1500rpm for 7 minutes at 4°c and the supernatant was discarded by dumping. The remaining pellet was suspended by vortex in the small remaining volume; 1mL of 1X freezing media was added

for every $1x10^8$ PBMC and mixed thoroughly before transfer to a cryovial in 1mL aliquots. Cryovials were transferred to a Mr. Frosty and stored at -80°C overnight. The next day cell aliquots were transferred to a liquid nitrogen freezer for long-term storage.

3.1.8 *Thawing of human samples*

Samples were retrieved from liquid nitrogen storage and immediately thawed in a water bath at 37°C until only a small fraction of the sample remained frozen. The samples were sprayed with 70% ethanol and wiped dry with a kimwipe before introduction into the hood. Samples were added drop-wise to at least 10mL minimal or human media or BSA buffer (3.8.1) and centrifuged at 1500rpm at 4°c for 5-7 minutes of 1600rpm for 3 minutes. Supernatant was discarded and the cell pellet was suspended by serological pipette or vortex. Ten mL of human media, BSA or FACS buffer was added. If the cells began to clump, a 70μm filter (Thermo Fisher Scientific, Waltham, MS, USA) was fitted on to a new 50mL conical tube and the cells were passed through the filter by transferring with a serological pipette. If the cell viability was particularly low as judged by cell clumping or by trypan blue incorporation PBMC were filtered, incubated on ice for 10-20 minutes and re-filtered. The filter was rinsed with an additional 5-10mL of BSA or FACS buffer. 10μL of the PBMC was transferred to a microcentrifuge tube and diluted 1:10-1:50 in trypan blue. Ten μL of the trypan blue dilution was enumerated as in 3.1.3. Cells were centrifuged at 1500rpm for 5 minutes at 4°c and the supernatant was discarded by vacuum aspiration or dumping and cells were suspended by vortex.

3.1.9. *Labeling of PBMC, B and T cells with carboxyfluorescein succinimidyl ester (CFSE)*

Cells were centrifuged at 1500rpm for 5 minutes at 4°C and the supernatant was discarded. The cell pellet was suspended by vortex and 10mL of room temperature PBS added. Cells were centrifuged at 1500rpm for 5 minutes at 4°c and the supernatant was discarded. PBMC, T cells and total B cells were suspended at $5x10^7$ cells/mL in room temperature PBS. Memory and naïve B cells were suspended in the volume remaining after the supernatant was discarded.

Lyophilized CFSE (Life Technologies, Grand Island, NY) was thoroughly suspended in 18μL 100% dimethylsulfoxide (DMSO) provided by the manufacturer and stored at -20°c. The reconstituted CFSE was thawed at room temperature and diluted 1:100 for PBMC, T cells and total B cells or 1:200 for memory and naïve B cells in PBS in a microcentrifuge tube and mixed by vortex. For PBMC, T cells and total B cells 110μL of CFSE was added for every $5x10^7$ cells and 200μL CFSE was added to memory and naïve B cells for every $5x10^7$ cells. CFSE was mixed with the cells by swirling and cells were incubated at room temperature for 10 minutes. Ten mL of minimal or human media was added and cells were centrifuged at 1500rpm for 5 minutes at 4°C. The supernatant was discarded, cell pellet suspended by vortex. This wash step was repeated once more to remove excess CFSE.

3.2 Isolation of B and T cells

3.2.1 *B cell isolation by Magnetic Selection*

Thawed and enumerated PBMC were suspended at a concentration of $8x10^7$ cells/mL in room temperature BSA buffer (3.8.1). CD19 microbeads (Miltenyi, Bergisch Gladbach, Germany) were suspended by vortex; 20μL of microbeads per $1x10^7$ cells and PBMC were incubated at room temperature for 20 minutes. Ten mL of room temperature BSA buffer was added to the PBMC and cells were centrifuged at 1500rpm for 5 minutes at 4°C.

During the centrifugation step, an LS colum (Miltenyi, Bergisch Gladbach, Germany) was placed on the MidiMACS magnetic column (Miltenyi, Bergisch Gladbach, Germany), which had been thoroughly sprayed with 70% ethanol and allowed to dry. Three mL of room temperature BSA buffer was applied to the column and allowed to drip into a sterile 12x75mm round bottom polystyrene tube (FACS tube, Corning, Corning, NY, USA). Once the column stopped dripping the FACS tube was removed and discarded. A fresh 15mL conical tube was placed beneath the column to collect the B cell depleted fraction.

The supernatant from the PBMC was discarded by vacuum aspiration or dumping and the PBMC were suspended by vortex. 500μL of room temperature BSA buffer was added to the PBMC and mixed thoroughly by pipetting. The 500uL of PMBC was applied to the top of the column and allowed to drip until it stopped. 3mL of room temperature BSA buffer was applied to the top of the column until it stopped dripping in to the 15mL conical tube, this was repeated two addition times. The LS column was removed from the magnet and inserted into a FACS tube, 2mL of room temperature BSA buffer was added to the top of the column and the B cells were extruded with the plunger. Ten μL of the B cell enriched fraction was transferred to a microcentrifuge tube and diluted 1:1 with trypan blue. The enriched fraction was enumerated as in section 3.1.3. The B cell enriched fraction was centrifuged at 1500rpm

for 3-5 minutes at 4°c and the supernatant was discarded and suspended by vortex for use in B cell culture or memory and naïve B cell solation by fluoresce activated cell sorting (FACS).

3.2.2 *T cell isolation by negative selection*

The B cell depleted fraction (BCDF) from section 3.2.1 was centrifuged at 1500rpm for 3-5 minutes at 4°C. BCDF was suspended by vortex and ice-cold BSA buffer was added to yield a final concentration of $8x10^7$ cells/mL. Pan T cell kit II (Miltenyi, Bergisch Gladbach, Germany) primary antibody was suspended by vortex and 10μL for every $1x10^7$ cells was added to the BCDF and was incubated on ice for 10 minutes. Then, 20μL of the microbeads from the Pan T cell kit II was added for every $1x10^7$ cells and the BCDF was incubated on ice for an additional 20-40 minutes. Ten mL of ice-cold BSA buffer was added and the BCDF was centrifuged for 5 minutes at 1500rpm at 4°C.

During the centrifugation step, an LS colum (Miltenyi, Bergisch Gladbach, Germany) was placed on the MidiMACS magnetic column, which had been thoroughly sprayed with 70% ethanol and allowed to dry. 3mL of room temperature BSA buffer was applied to the column and allowed to drip into a sterile FACS tube (Fisher Scientific, Pittsburgh, PA, USA). Once the column stopped dripping the FACS tube was removed and discarded. A fresh 15mL conical tube was placed beneath the column.

The supernatant from the BCDF was removed by dumping and the pellet was suspended by vortex. Five-hundred μL of ice-cold BSA buffer was added and cells were mixed thoroughly before transfer to the column. Once the column stopped dripping 3mL of

ice-cold BSA buffer was added until the column stopped dripping; this was repeated an additional two times. The B and T cell depleted fraction that remained bound to the column was discarded and the T cell enriched fraction that dripped through the column was centrifuged at 1500rpm for 3-5 minutes. The supernatant was discarded by dumping and the T cell fraction was suspended in 10mL human media or BSA buffer and placed on ice at 4°C until cultures were set up no more than 2 hours later. Ten microliters was transferred to a microcentrifuge tube and diluted 1:10 in trypan blue. The diluted T cell fraction was enumerated as in section 3.1.3.

Twenty to fifty microliters were transferred to a new FACS tube containing 300μL FACS buffer. The cells were stained with antibodies to 10μL CD3 (APC or V500, BD biosciences), 5μL CD4 (PE-Cy7, BD Biosciences) and 5μL CD8 (PE, APC or APC-Cy7, BD Biosciences) for 20 minutes on ice. After incubation, 3mL of FACS buffer was added and cells were centrifuged for 5 minutes at 1500rpm at 4°c. Supernatant was discarded, the cell pellet was suspended in 300μL of FACS buffer and placed on ice until analysis on a FACSAria (BD Biosciences). Only T cells above 96% purity with no evidence of myeloid cell contamination by forward/side scatter profile were used in B-T co-cultures.

3.2.3. *FACS of memory and naïve B cell populations*

Purity was typically above 95% for memory and naïve B cell sorts in chapter four and increased with time to >98% for studies on total B cells in chapter five. Total CD19+ B cells were stained with 5μL (PECy5, PE or Brilliant violet421), 10μL of CD27 (PE or FITC) per million cells and sorted into >98% pure naïve (CD19+CD27-) and memory (CD19+ CD27+)

populations on a FACSAria (BD Biosciences, custom order system) in FACS tubes or 15mL conical tubes containing 500µL or 1mL of FACS buffer, respectively, in a chamber cooled to 4°C (Figure 3.1).

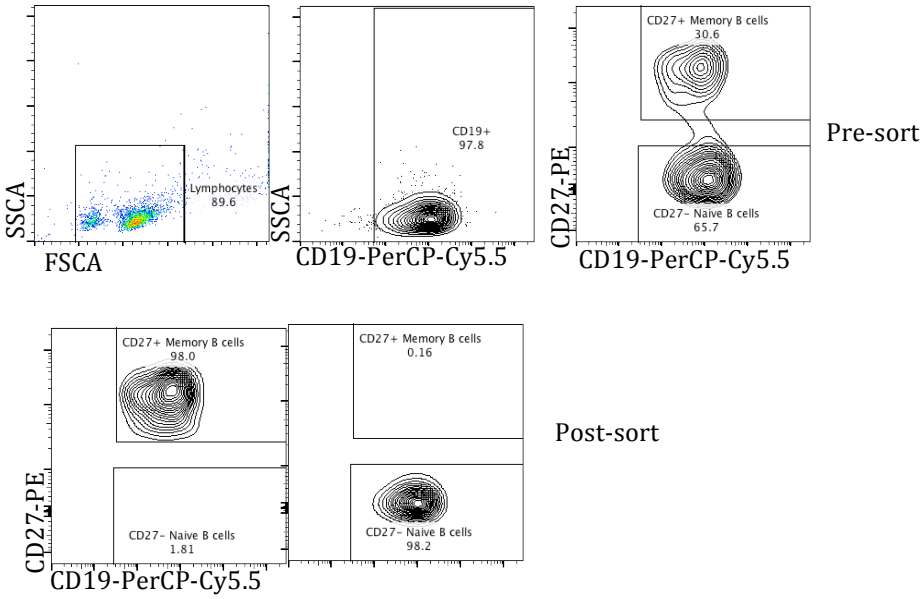


Figure 3.1. Representative gating strategy for memory and naïve B cell sorts. **A.** Cells were selected on forward-side scatter parameters. **B.** Cells were gated on CD19 positivity and sorted on expression of CD27 (**C**). Representative post-sorted memory (**D**) and naïve (**E**) B cells that were at least 98% pure.

3.3 B cell *in vitro* Stimulation

3.3.1 Maintenance and Preparation of NIH-3T3 Cells

NIH-3T3 is an immortalized cell line derived from Swiss mouse fibroblasts. NIH-3T3 were transfected by electroporation with a linearized plasmid containing the coding region of hCD40L derived from cDNA from activated human T cells and a neomycin phosphortransferase cassette that confers resistance to G418 to yield $CD40^{+}$ NIH3T3 cells (a

kind gift from Gordon Freeman).

$CD40^+$ NIH3T3 cells were stored in minimal freezing media (3.8.1) in a liquid nitrogen freezer. Cells were thawed in a 37°C water bath, sprayed with 70% ethanol and dried with a kimwipe. Thawed cells were added drop-wise to 10mL minimal media in a 15 or 50mL conical tube and centrifuged at 1500rpm for 5 minutes at 4°C. The supernatant was discarded by dumping or vacuum aspiration, the cell pellet was suspended by vortex, and 10mL of minimal media was added. Ten microliters of the cell suspension was transferred to a microcentrifuge tube and diluted 1:1 in trypan blue. 10μL of the trypan blue-diluted mixture was enumerated as in 3.1.3. The cell suspension was centrifuged at 1500rpm for 5 minutes at 4°C. The supernatant was discarded by dumping or vacuum aspiration; the cell pellet was suspended by vortex, and suspended at a final concentration of $5x10^4$ to $1x10^5$ cells/mL in G418 media. 20mL was transferred to a T75 flask with a gas-permeable cap (Thermo Fisher Scientific, Waltham, MA) in a horizontal orientation to facilitate adherence. $CD40^+$ NIH3T3 cells were cultured in a humidified NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA) at 37°C, 5% CO_2.

Cells were passaged every two-five days when cells were 80-95% confluent. The media was discarded by dumping, 10mL of room temperature PBS was added to the flask and swirled gently before being discarded, 3mL of 0.25% trypsin with 0.1% EDTA ethylenediaminetetraacetic acid (Hyclone Laboratories, Logan, UT, USA) was added for 1-3 minutes at room temperature or 37°C until cells were visualized to detach by eye. Seventeen mL of complete media was added and cells were transferred to a 50mL conical tube. Cells

were centrifuged at 1500rpm for 5 minutes at 4°C and the media was discarded. The cell pellet was suspended and 10mL complete media was added. Ten microliters of the cell suspension was transferred to a microcentrifuge tube and diluted 1:1 in trypan blue. Ten μL of the trypan blue-diluted mixture was enumerated on a hemocytometer as in 3.1.3. Cells were centrifuged at 1500rpm for 7 minutes at 4°c and the supernatant was discarded. For further expansion cells were suspended in G418 media and moved to new T75 flasks.

3.3.2. Preparation of $CD40^+$ NIH3T3 cells for Culture with Human B cells

For culture with B cells, $CD40^+$ NIH3T3 cells were suspended in 10mL minimal after the final centrifugation step in the passaging, section 3.3.1 and were irradiated with 96 Gy using a ^{137}Cs-source to inhibit proliferation. $CD40^+$ NIH3T3 cells were centrifuged at 1500rpm for 5 minutes at 4°c and the supernatant was removed. The cell pellet was suspended by vortex, 10mL of G418 media was added the cells were centrifuged 1500rpm for 5 minutes at 4°C. This wash step was repeated again. The cell pellet was suspended from in G418 media at the desired concentration of cell/mL. Cells were placed in sterile reagent reservoirs (Fisher Scientific, Pittsburgh, PA, USA) and 200μL of the cell suspension was added to wells of a round-bottom 96 well plate (Costar, Washington D.C., USA) with a growth area of $0.32cm^2$. Cells were incubated overnight to allow to adherence to the wells. Just prior to the addition of B cells, the supernatant was removed by vacuum aspiration and the wells were washed with 200μL minimal media to removed residual G418.

3.3.3 Optimization of $CD40^+$ NIH3T3 Stimulation of Human B cells

In order to test increases and decreases in B cell responses we optimized the concentration of $CD40^+$ NIH3T3 cells that stimulated approximately 25 and 50% to sensitize the assay. Irradiated $CD40^+$ NIH3T3 cells were suspended at a concentration of 0 to $4x10^4$ cells/mL in a 1:1 dilution series. The cells were incubated overnight and the following day the media was removed by vacuum aspiration. The wells were washed twice with 200µL minimal media to remove traces of G418.

CFSE-labeled (PBMC from one healthy donor were suspended at $50x10^6$ cells/mL and 200µL was added to each well containing the $CD40^+$ NIH3T3 cells. Cells were incubated for five days. PBMC were removed from the wells by pipetting and transferred to FACS tubes. Cells were washed with 3mL FACS buffer and centrifuged at 1500rpm for 5 minutes at 4°C. The cell pellets were suspended in 300µL FACS buffer by vortex. Cells were stained with 10µL CD19-PerCP-Cy5.5 (BD Biosciences, San Jose, CA, USA) for 20 minutes on ice. Cells were washed with 3mL of FACS buffer and the pellets were suspended in 300µL FACS buffer. Cells were kept on ice until analysis on an BD Accrui flow cytometer (BD Biosciences, San Jose, CA, USA) The concentrations chosen were 500 and 2,000 cells per well to stimulate approximately 25 and 50% B cell proliferation, respectively (Figure 3-

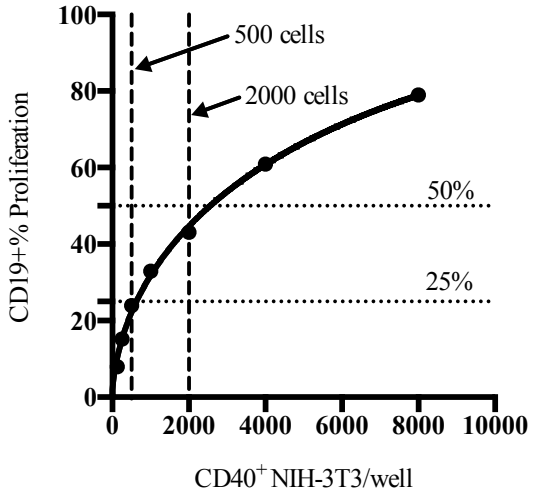


Figure 3-02. B cell proliferative response from one HD to $CD40^+$ NIH-3T3 cells. We fit the nonlinear data by power series, R^2=0.998. Dotted horizontal lines and dashed vertical lines indicate the concentration of $CD40^+$NIH-3T3 cells that induce approximately 25 and 50% proliferation.

02 intersection of dashed and dotted lines).

3.3.4 Human B cell Culture

Recombinant human IL-4 (R&D Systems, Minneapolis, MN, USA) and IL-2 (Invitrogen, Carlsbad, CA, USA) were diluted to 10ng/mL in PBS and stored in 10-15μL aliquots at -80°C and were not subjected to freeze thaw cycles. TLR9 agonist class B CpG oligonucleotide ODN 2006 (Invivogen, San Diego, CA, USA) was suspended at 500μg/mL in sterile water provided by the manufacturer and stored at -20°C. Polyclonal goat anti-human IgM/IgG H+L (Jackson ImmunoResearch, West Grove, PA, USA) was stored at 4°C in 50μL aliquots at 2.4mg/mL. All soluble B cell stimulation factors were prepared at a 2X concentration. IL-2 and IL-4 were diluted to 20ng/mL in minimal media. α-IgM/IgG was diluted 1:50 in minimal media before diluting to 1.0μg/mL in 20ng/mL IL-4 media. CpG was diluted to 10μg/mL in 20ng/mL IL-2 media.

Sorted memory and naïve B cells or total MACS purified B cells were stained with CFSE (section 3.1.8). Cells were suspended at $1x10^6$ cells/mL in minimal media. 100μL of B cells were added to wells containing irradiated $CD40^+$ NIH3T3 cells (3.3.2) seeded a low- or high-dose for a ratio of $CD40^+$ NIH3T3 to B cells 1:200 and 1:50, respectively. For cultures that did contain additional stimuli, 100μL minimal media was added. 100μL of 2X IL-4 media (final concentration 10ng/mL), 2X IL-4/α-IgM/IgG (final concentration10ng/mL IL-4, 0.5μf/mL α-IgM/IgG), or 2X IL-2/CpG (final concentration 10ng/mL IL-2, 5μg/mL CpG)

were added to the appropriate wells. One well containing B cells and 10ng/mL IL-4 was prepared as a negative control for proliferation.

3.3.5 *Collection and Storage of Supernatants from B cell Cultures*

On day three of culture, 100μL of media was gently removed from B cell cultures using a multichannel pipette being careful not to disturb cells at the bottom of the well. Supernatants were transferred to microcentrifuge tubes and stored at -80°C for batched analysis. One hundred μL of fresh minimal media was added to each well before cultures were returned to incubators. On day five, 150μL of supernatant was removed in the same manner.

3.3.6 *Preparation of B cell Cultures for Quantification of Proliferation by Flow Cytometry*

On day five, once supernatants were removed for further analysis, 150μL of BSA or FACS buffer was added to each well. B cells were suspended by pipetting with a multichannel pipette. Some samples were transferred to FACS tubes for staining whereas others were stained in the wells in which they were cultured. Ten microliters from one culture was removed and transferred to a separate tube to serve as a compensation control for CFSE.

Cells that were stained in wells were centrifuged for 5 minutes at 1500rpm at 4°C. After centrifugation the supernatants were removed using a multichannel pipette and the cells were suspended in 100μL BSA or FACS buffer before placing the cells on ice for the remainder of the procedure. 100μL of BSA or FACS buffer containing 10μL of CD-19-PE or CD19-PerCP-Cy5.5 was added and cells were incubated for 15-30 minutes. Cells were centrifuged at 1500rpm for 5 minutes at 4°C, supernatants were removed with a multichannel

pipette and samples were suspended in 200μL FACS buffer. Samples transferred to 1.2mL polypropylene micro dilution tubes (micoFACS tubes) (USA Scientific, Orlando, FL, USA) or FACS tubes prior to data collection.

For cells that were stained in FACS tubes, an additional 3mL of BSA or FACS buffer was added to the cells upon transfer to FACS tubes. Cells were centrifuged for 5 minutes at 1500rpm at 4°C. After centrifugation the supernatants were removed and the cells were suspended in 100μL BSA or FACS buffer before placing the cells on ice for the remainder of the procedure. 100μL of BSA or FACS buffer containing 10μL of CD-19-PE or CD19-PerCP-Cy5.5 was added and cells were incubated for 15-30 minutes. 3mL of FACS buffer was added and cells were centrifuged at 1500rpm for 5 minutes at 4°C, supernatants were removed before samples were suspended in 200μL FACS buffer. Compensation tubes were prepared by transferring approximately $5x10^5$ PBMC to three tubes. One was left unstained for a negative control. Another was stained with 5μL CD19-PE or -PerCP-Cy.5. Finally, 10μL of CFSE-stained B cells were mixed with unlabeled PBMC. Data was acquired on a BD FACSAria or BD ACCURI C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

3.3.7 Quantification of IL-6 and IL-10 mRNA by qPCR

MACS-purified B cells were suspended at $2x10^7$ cells/mL in minimal media. 100μL of B cells was transferred to a 96 well round bottom 96-well plate. Lyophilized recombinant human CD40L (rHCD40L)(R&D Systems, Minneapolis, MN, USA) was diluted in PBS and stored at -80°c in 10-50μL aliquots. rHCD40L was used instead of $CD40^+$ NIH3T3 cells to eliminate mRNA transcript contamination. A concentration that induced approximately 25%

B cell proliferation when incubated for 5 days with 10ng/mL IL-4 was selected for use in this assay (personal communication with Ding Chen, Ph.D.). rHCD40L was diluted to 1.0μg/mL in minimal media with α-IgM/IgG to 1μg/mL or CpG 10μg/mL for a 2X final concentration. 100μL of rHCD40La+α-IgM/IgG or CpG was added to B cells in the 96 well plate. For a negative control, 100μL minimal media was added to yield unstimulated B cells.

24 and 72 hours after the B cell culture was started, cells were suspended in the culture media and transferred to a microcentrifuge tube. 1mL of ice-cold PBS was added to the cells and tubes were centrifuged for 3 minutes at 1600rpm for 3 minutes at 4°C. The supernatant was removed by vacuum aspiration. 400μL of ice-cold PBS was added and the pellet was suspended by pipetting up and down. The tubes were centrifuged at 1600rpm for 3 minutes at 4°c. The supernatant was removed by vacuum aspiration, leaving as little liquid remaining as possible without disturbing the cell pellet. Cell pellets were placed on ice and snap-frozen by placing in liquid nitrogen. Frozen pellets were moved, without thawing, to -80°C for storage.

All steps following were carried out with general protocols for assuring RNA quality and integrity by working on clean surfaces, using barrier pipette tips, and wiping instruments and gloves with RNAse-Away. All reagents used for RNA extraction except 70% ethanol, 100% chloroform (Sigma Aldrich, St. Louis, MO, USA) and DNAse/RNAse free water (Biotecx Laboratories, Huston, TX, USA) were provided in the Qiagen RNeasy Plus Universal RNA isolation kit (Qiagen, Valencia, CA, USA). Cells were transferred to a chemical hood on dry ice. 900μL QIAzol lysis reagent was added to each tube and mixed by pipetting up and down. Tubes were left at room temperature for 5 minutes before 100μL

gDNA eliminator was added and tubes were shaken vigorously for 15 seconds. One hundred and eighty µL chloroform (Sigma Aldrich, St. Louis, MO, USA) was added to each tube, tubes were shaken vigorously for 15 seconds, and then allowed to rest for 5 minutes at room temperature. Tubes were centrifuged at 12,000g for 15 minutes at 4°C. The clear, upper, aqueous layer, with care to avoid contaminants from the interface layer, was transferred to a new microcentrifuge tube, and an equivalent volume of 70% ethanol was added.

Up to 700µL of the mixture was added to an RNeasy spin column attached to a collection tube, included in the RNA isolation kit. The tube was centrifuged for 15 seconds at 8,000-10,000g at room temperature. The flow through was discarded rom the collection tube by dumping and re-attached to the spin column. The remainder of the aqueous layer, ethanol mix was added to the spin column and centrifuged at 8,000-10,000g for 15 seconds at room temperature. The flow though was discarded and the spin column was placed back into the same collection tube. Buffer RWT and RPE (from the RNA isolation kit) were diluted to 1X with 90-100% ethanol (Sigma Aldrich, St. Louis, MO, USA). 700µL buffer RWT was added to the spin column and the tube was centrifuged for 15 seconds at 8,000-10,000g at room temperature. The flow through was dumped from the collection tube and re-attached to the same spin colum. 500µL buffer RPE was added to the spin column and the tube was centrifuged for 15 seconds at 8,000-10,000g at room temperature. The flow through was discarded from the collection tube then re-attached to the spin column. The tube was centrifuged for I minute at 8,000-10,000g at room temperature. The spin column was placed on a fresh collection tube and 25µL of DNase/RNase free water was added to the spin column. The tube was centrifuged for I minute at 8,000-10,000g at room temperature. The

eluted RNA was transferred to a microcentrifuge tube and immediately placed on ice. 2μL was removed from each tube and read at 260 and 280nm with a 320nm correction wavelength on an Epoch Microplate Spectophotometer (BioTek, Winooski, VT, USA) with Gen5 Data analysis software (BioTek, Winooski, VT, USA) equipped with the Take3 plate after blanking with DNase/RNase free water. RNA samples with an 260:280nm ratio of 1.75 or greater were stored at -80°c. Typically ~200-800ng of RNA was recovered from B cell stimulations.

3.3.8. cDNA synthesis from RNA

Reverse transcription (RT) of RNA to cDNA was achieved using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). All kit contents were thawed on ice, vortexed to mix and quick spun. A master mix mix of 4μL 5X buffer and 1μL iScript reverse transcriptase was prepared in a microcentrifuge tube. Two hundred ng RNA (variable volume), that was thawed briefly on ice, and and DNase/RNase free water sample to bring the RNA to 15μL volume were added to 0.2mL 8-strip PCR tubes with attached flat lids (USA Scientific, Ocala, FL, USA). 5μL of the master mix was added to each PCR tube containing RNA for a total volume of 20μL. Controls containing no template were included. PCR strips were quick spun to before they were placed in a PTC-100 programmable thermocycler (MJ Research, St. Bruno, Quebec, Canada) or Mastercycler EPgradient S (Eppendorf, Hamburg, Germany) set to 42°C for 30 minutes, 85°C for 5 minutes, with a heated lid, and 4°C indefinitely. Within 5 minutes of the start of the 4°C step the tubes were transferred to -80°C for storage or used directly for quantitative polymerase chain reaction (qPCR).

3.3.9. Detection of IL-6 and IL-10 by Quantitative Polymerase Chain Reaction (qPCR)

The contents of the iTaq Universal Green Supermix (Biorad, Hurcules, CA, USA) were brought to room temperature, protected from light. 10μL of Supermix, 8μL DNase/RNase free water, and 1μL of RT2 primer for human IL-6, IL-10 or the control gene Peptidylprolyl isomerase A (PPIA)(Qiagen, Venolo, Netherlands) were used to generate a master mix. 19μL master mix was added to a hard-shell semi-skirted low profile PCR plate (Biorad, Hercules, CA, USA). 1μL cDNA was spotted on to the side of the appropriate wells. All samples were run in duplicate or triplicate and controls included a no RT and no template wells. The plate was quick spun and placed in an Applied Biosystems 7500 Real-Time PCR System equipped with software (Applied Biosystems, Foster City, CA, USA). The plate was run on the ddct plate setting with custom amplification steps (15s at 95°C, 1m 60°C, plate read with SYBR Green detector) x 40 cycles and a dissociation curve 65°C to 95°C increasing at a rate of 2°C/minute was run to ensure the uniformity of amplicons. Wells that did not match the dissociation point were excluded from analysis.

The plate data was saved in plate format and exported into a ddct study using the Applied Biosystems software. The cycle threshold (CT) value was determined automatically and was within the exponential phase of amplification. Wells in which did not display the exponential/plateau phases or that began above the base fluorescence threshold were excluded. CT values were exported and analyzed by the Livak method. ΔCT were determined by subtracting the CT of the gene of interest from the CT of the control gene, PPIA. ΔΔCT values were calculated by subtracting the unstimulated (calibrator) ΔCT from

experimental ΔCT. The normalized gene expression was calculated by $2^{-\Delta\Delta CT}$. All calculations were made in Microsoft Excel.

3.4 Quantification of Cytokines and Soluble Factors

3.4.1 *Cytokine Enzyme Linked immunosorbent Assay (ELISA)*

Capture antibodies (3.1) were diluted to 1μg/mL in coating solution (0.1 M $NaHCO_3$ in deionized water) and 100uL was added to each well. Plates were incubated overnight at 4°C or at room temperature for four hours. Plates were washed three times with PBST (0.5% Tween20, 0.05% sodium azide in 1X phosphate buffered saline). Non-specific binding was inhibited by the addition of 200μL blocking buffer (1% bovine serum albumin Sigma Aldrich in 1X phosphate buffered saline) for 30 minutes at room temperature. Plates were washed twice with PBST before 100uL of samples and standards were added to the plates and incubated overnight at 4°C or room temperature for four hours. Supernatants and serum samples were thawed on ice prior to the addition to ELISA plates. The standards for TNF-α, LT-α, IL-6, -10 and -12 were eleven two-fold dilutions of 10,000pg/mL in blocking buffer. IFN-γ standards were eleven two-fold dilutions of 25,000-50,000pg/mL also in blocking buffer. All standards were run in duplicate or triplicate and included a blank/zero value. All samples were run neat. Samples and standards were incubated overnight or for four hours at room temperature before plates were washed 3 times with PBST and 100μL of biotinylated detection antibody (1μg/mL, table 3.1) diluted in blocking buffer was added to each well. Plates were incubated for 45 minutes at room temperature. Plates were washed three time with PBST and 100μL of a 1:2000 dilution of streptavidin-horseradish peroxidase (HRP)

(BD Biosciences, San Jose, CA, USA) in blocking buffer was added to each well. Plates were incubated at room temperature for 30 minutes and washed 5 times with PBST.

Table 3.4.1. Reagents used in ELISAs

Reagent	Company
α-IL-10 and α-IL-10-Biotin	BD Biosciences, San Jose, CA, USA
α-IL-6 and α-IL-6-Biotin	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
α-TNF-α and α-TNF-α-Biotin	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
α-LT-α and α-LT-α-Biotin	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
α-IFN-γ and α-IFN-γ-Biotin	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
α–IL-12 and α-IL-12-biotin	BD Biosciences, San Jose, CA, USA
rHu IL-10	Shenandoah Biotechnology Inc, Warwick, PA, USA
rHu IL-6	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
rHu TNF-α	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
rHu LT-α	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
rHu IFN-γ	BD Biosciences, San Jose, CA, USA or eBioscience San Diego CA, USA
rHu IL-12	BD Biosciences, San Jose, CA, USA

All antibodies were stored at 4°c. Recombinant human (rHu) proteins were received lyophilized and were reconstituted in PBS. Aliquots of protein 10-50µL were stored at -80°c.

Detection of HRP was carried out by the addition 100uL of 1mL 3,3',5,5'-Tetramethylbenzidine (TMB) at 1mg/mL in 100% dimethylsufoxide (DMSO) to 9mL phosphate-Citrate Buffer pH 5.0 (0.5M citrate in 1xPBS) and 2µL 30% hydrogen peroxide or 1X TMB substrate solution (eBioscience). Plates were developed in the dark for 2-15 minutes and the reaction was stopped with 100uL 1M hydrochloric acid (HCl)(Sigma Aldrich, St. Louis, MO, USA). Plates were read on an Epoch microplate spectrophotometer (Biotek, Winooski, VT, USA) at 450nm. Standard curves were generated in Microsoft excel

or Graphpad using linear or non-linear regression, only those plates with standard curves yielding an R-square value more than 0.94 were included in these studies. Unknown values were extrapolated using standard curves. The lowest detection level for IL6 and TNFα was 1pg/mL, 5pg/mL for LTα, 78.1-156.3pg/mL for IFN-γ, and 9.5-39.1pg/mL for IL12p70.

3.4.2 *IgM and IgG Enzyme Linked immunosorbent Assay*

Capture antibodies from Human IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) and Human IgM ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA) were diluted 1:100 and 1:250 in coating solution. 100μL of capture antibody was added to a flat-bottom ELISA plate and incubated overnight at 4°c. Plates were washed with 200μL PBST twice, 200μL blocking buffer was added and plates were incubated for 30 minutes at room temperature. Plates were washed twice with 200μL PBST before 100μL samples and standards were added. The standards for IgM and IgG were included in the kits. Lyophilized IgM was reconstituted with blocking buffer and incubated at room temperature for 10-15 minutes before 1:1 serial dilutions were prepared for a standard curve from 1.9-2000ng/mL with a blank. Five microliters of IgG standard was diluted in 22mL blocking buffer for a top standard of 1,000ng/mL, 1:1 serial dilutions were prepared yielding a standard curve from 0.9-1,000ng/mL with a blank. All standards were run in duplicate or triplicate. LLOD for IgM and IgG was 3.9ng/mL. Serum samples were diluted 1:20 and 1:100 for IgG, and 1:10 and 1:50 for IgM in blocking buffer. Serum Ig levels are reported in mg/dL and for culture supernatants in ng/mL. Samples and standards were incubated overnight at 4°c, plates were washed 3 times with PBST. HRP-conjugated detection

antibodies for IgM and IgG were diluted 1:250 and 1:100,000 in blocking buffer and 100µL was added to each well. Plates were incubated at room temperature for 60 minutes and washed 5 times with PBST. 100µL TMB solution was added to each well and plates were incubated in the dark for 5-10 minutes. The reaction was stopped with the addition of 100µL 1M HCl.

3.4.3 Reading ELISA Results

The optical density (O.D.) was read at 450nm on an E-max® plate reader (Molecular Devices, Sunnyvale, CA, USA) with Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA) or an Epoch Microplate Spectophotometer (BioTek, Winooski, VT, USA) with Gen5 Data analysis software (BioTek, Winooski, VT, USA). Sample concentrations were calculated based on the standard curve (linear or power series) in Graphpad Prism, La Jolla, CA, USA) or Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

3.4.4 *Bioplex*

Bio-Plex Pro Human Th17 Cytokine Panel 15-plex kit (Bio-Rad Laboratories, Hercules, CA, USA) allows for the simultaneous measurement of fifteen factors (IFN-γ, TNF-α, soluble CD40L IL-4, -6, -10, -17A, -17F, -21, -22, -23, -25, -31, and -33) from one 50µL sample. was brought to room temperature. High and low controls were reconstituted with 250µL diluent HB and incubated on ice for 30 minutes. The standard vial was reconstituted with 781µL of HB diluent and incubated on ice for 30 minutes. The standard was serially diluted 1:4 in diluent HB seven times to yield a total of 8 standards; blanks were included as a zero

value. All standards were run in duplicate. Samples were thawed on ice. The 20X capture beads were diluted to 1X in diluent HB or culture media vortexed, and 50μL was added to each well of the plate provided in the kit. The plate was attached to the Bio-Plex handheld washer (Bio-Rad Laboratories, Hercules, CA, USA) and washed twice by adding 200μL Bio-Plex wash buffer, inverting the magnet and gently placing on a paper towel while attached to the magnet. The plate was removed from the magnet and 50μL samples and standards were added. The plate was sealed with the included plate sealing tape and placed on a MS3 digital microplate shaker (Ika, Wilmington, NC, USA) that was slowly increased to 850±50rpm for 1 hour, protected from light. With 10 minutes of shaking left, the 20X detection antibodies were diluted to 1X in detection diluent and protected from light. The plate seal was removed and the plate was attached to the handheld magnet. The plate was washed 3 times and removed from the magnet. The detection antibodies were vortexed and 25μL was added to each well. A new plate seal was used to cover the plate and was transferred to the microplate shaker that was slowly increased to 850±50rpm for 30 minutes. With 10 minutes of shaking left the 100X streptavidin-PE (SA-PE) was brought to 1X in diluent HB and protected from light. The plate sealer was removed and the plate was placed in the magnet for washes. The plate was removed

Factor	ULOD	LLOD
IFN-γ	13492.3	7.5
IL-10	50018.0	3.9
IL-17A	24435.8	2.8
IL17F	14128.6	10.9
IL-1β	4506.8	0.3
IL-12	121812.8	16.4
IL-22	27352.7	6.6
IL-23	75981.6	17.6
IL-25	18312.9	1.4
IL-31	69145.0	6.5
IL-33	118672.6	8.5
IL-4	18503.9	7.0
IL-6	35022.4	2.7
TNF-α	9174.8	2.4
sCD40L	25990.8	2.5

Table 3.4.2. The mean upper limit of detection (ULOD) and lowest limit of detection (LLOD) for Bio-Plex reported in pg/mL.

from the magnet and 50µL SA-PE was added to each well after it was vortexed. The plate covered with a new plate sealer and was transferred to the microplate shaker that was slowly increased to 850±50rpm for 10 minutes. The plate sealer was removed and placed in the magnet for 3 washes. Finally, the plate was removed from the magnet and 150µL Bio-Plex assay buffer was added to each well.

The plate was stored on ice or at 4°C until it was read. Plates were read within one week of the assay, but typically on the same day. Bio-Plex data was read on a Magpix system (Luminex, Austin, TX, USA) with Xponent software (Luminex, Austin, TX, USA) and Bioplex manager (Bio-Rad Laboratories, Hercules, CA, USA) by the University of Texas Southwestern Medical Center Microarray Core facility. The LLOD and upper limit of detection (ULOD) are outlined in table 3.4.2.

3.5 Phenotypic Characterization of Immune Cells by Flow Cytometry

3.5.1 T lymphocyte Panel

Thawed PBMC (3.1.8) were suspended at $1x10^7$ cells/mL in FACS buffer. One hundred microliters was transferred to a FACS tube containing 95µL FACS buffer and 5µL mouse serum (Sigma Aldrich, St. Louis, MO, USA) to inhibit non-specific binding. Cells were incubated on ice for 15-25 minutes. A master mix of 5µL CCR7-FITC, CXCR3-Brilliant Violet 421, CD45RO-PerCP-Cy5.5, CD45RA-APC, CD3-V500, 10µL CD8-PE and 3µL CD4-PE-Cy7 (BD Biosciences, San Jose, CA, USA) were added to 60µL FACS buffer. For some samples, 5µL CD25-APC-Cy7 was included and antibodies were mixed with 55µL FACS buffer. 100µL of master mix was added to the tube containing the cells and was mixed

by vortexing. Unstained PBMC and PBMC stained with one antibody were used for compensation. Fluorescence minus one (FMO) controls were prepared by adding all antibodies but one, for each parameter to discriminate positive staining from negative. All tubes were incubated on ice for 30-45 minutes. Three mL FACS buffer was added to each tube and cells were centrifuged at 1500rpm for 5 minutes at 4°C. Supernatants were discarded by dumping, cell pellets were suspended by vortex, and tubes were brought to a volume of ~200µL with FACS buffer. Cells were stored on ice until data was acquired on the same day on a BD FACSAria.

3.5.2 B lymphocyte Panel

Thawed PBMC (3.1.8) were suspended at $1x10^7$ cells/mL in FACS buffer. One hundred microliters was transferred to a FACS tube containing 95µL FACS buffer and 5µL mouse serum (Sigma Aldrich, St. Louis, MO, USA) to inhibit non-specific binding. Cells were incubated on ice for 15-25 minutes. A master mix of 5µL CD27-Brilliant violet 421, 10µL HLA-DR-APC-Cy7, (BD Biosciences, San Jose, CA, USA) and 5µL of CD10-PE-Cy7, IgD-APC, and CD19-PE (eBioscience, San Diego, CA, USA) were added to 75µL FACS buffer. 100µL of master mix was added to the tube containing the cells and was mixed by vortexing. Unstained PBMC and PBMC stained with one antibody were used for compensation. Fluorescence minus one (FMO) controls were prepared by adding all antibodies but one, for each parameter to discriminate positive staining from negative. All tubes were incubated on ice for 30-45 minutes. 3mL FACS buffer was added to each tube and cells were centrifuged at 1500rpm for 5 minutes at 4°C. Supernatants were discarded by dumping, cell pellets were

suspended by vortex, and tubes were brought to a volume of ~200μL with FACS buffer. Unstained PBMC and PBMC stained with one antibody were used for compensation. Cells were stored on ice until data was acquired on the same day on a BD FACSAria.

3.5.3 Quantification of IL-10 and IL-6 receptor on Lymphocytes

Thawed PBMC (3.1.8) were suspended at $1x10^7$ cells/mL in FACS buffer. One hundred microliters was transferred to a FACS tube containing 95μL FACS buffer and 5μL mouse serum and incubated on ice for 15 minutes. One hundred μL of FACS buffer containing 5μL of IL-10 receptor-PE (IL-10r, CD210), IL-6 receptor-PE-Cy7 (IL-6r, CD126)(Biolegend, San Diego, CA, USA), gp130-APC (CD130)(R&D Systems, Minneapolis, MN, USA), CD3-V500, CD8-APC-Cy7, CD19-Brilliant Violet 421, CD27-PE-CF594, and 3μL CD4-PE CF594 (BD Bioscience, San Jose, CA, USA). Unstained PBMC and PBMC stained with one antibody were used for compensation. Fluorescence minus one (FMO) controls were prepared by adding all antibodies but one, for each parameter to discriminate positive staining from negative. Cells were incubated on ice for 30 minutes and 3mL FACS buffer was added. Cells were centrifuged at 1500rpm for 5 minutes at 4°C. Supernatant was discarded, cells were suspended by vortexing, and brought to ~200μL in FACS buffer. Cells were kept on ice until data was acquired on a BD FACSAria.

3.5.4 Detection of Cytokine Production by Intracellular Staining

To detect spontaneous cytokine secretion thawed PBMC (3.1.8) were suspended at $2.5x10^6$ cells/mL in minimal media with 1000X brefeldin A (BFA) and 500X Phorbol 12-Myristate

13-Acetate (40.5UM), Ionomycin (670uM)(PMA/I, eBioscience, San Diego, CA, USA) diluted to 1X. Unstimulated cells and cells treated with BFA or PMA/I were included as controls. For detection of T cell cytokine production *ex vivo* or after *in vitro* culture (B-T co-culture, section 3.7.1), cells were incubated for 18h with PMA/I. For B cell cytokine production *ex vivo* cells were stimulated for 4 hours with PMA/I. Alternatively, PBMC were cultured for 48h with and after *in vitro* culture with 5μg/mL CpG and 1μg/mL α-IgM/IgG to induce IL-10 production by B cells.

After incubation 3mL FACS buffer was added and cells were centrifuged at 1500rpm for 5 minutes at 4°C. Cell pellets were suspended by vortexing, volume was brought to ~200μL and cells were transferred to a v-bottom 96 well plate (Corning, Corning, NY, USA). The plate was centrifuged at 1600rpm for 3 minutes at 4°C, the supernatant was removed by vacuum aspiration (cells pelleted) and the cells were suspended in 100μL FACS buffer. B cells were stained with 5μL each of CD19-APC-Cy7, CD27-PE-CD594, CD1d-PE-Cy7, CD38-APC, and CD24 Brilliant violet 421 (BD Bioscience, San Jose, CA, USA) suspended in a total volume of 100μL of FACS buffer that was transferred to the 96 well plate. T cells or B-T cell co-cultures were stained with 5μL CD3, 10μL CD8-APC and 4μL CD4-PE-Cy7 (BD Bioscience, San Jose, CA, USA) in 100μL of FACS buffer. In some cases, the B-T co-culture staining panel was expanded to include 5μL CD45RO- or CD19-PerCP-Cy5.5. Cells were incubated on ice for 25-30 minutes. In addition, for B-T co-cultures CFSE labeled cells were used as a compensation control for the FITC channel in a similar manner to B cell cultures (3.3.6).

The cells were pelleted the cells were suspended in BD permeabilization /wash buffer diluted to 1X in deionized, sterile-filtered water (BD Biosciences, San Jose, CA, USA). The cells were pelleted and suspended in BD fixation and permeabilization buffer (BD Bioscience, San Jose, CA, USA). Cells were incubated at room temperature for 35-40 minutes and then pelleted. Cells washed by suspending in 200μL BD permeabilization /wash buffer and pelleting, twice. Cells suspended in 200μL BD permeabilization /wash buffer and incubated for 10 minutes. Cells were washed twice with BD permeabilization /wash buffer. B cells were suspended and incubated in a total volume of 50μL of BD permeabilization /wash buffer that contained 1μL IL-6-PE and 10μL IL-10-FITC (eBiosciences, San Diego, CA, USA) for 30 minutes. T cells and B-T co-cultures were suspended and incubated in a total volume of 50μL of BD permeabilization /wash buffer that contained 1μL IFN-γ-Pacific Blue (eBioscience, San Diego, CA, USA) and 10-15μL IL-17PE (BD Bioscience, San Jose, CA, USA) for 30 minutes. FMO controls were prepared for the antibodies used for intracellular detection of cytokines. 200μL BD permeabilization /wash buffer was added and cells were pelleted. 200μL of BD permeabilization /wash buffer was used to suspend the cells and they were transferred to microFACS tubes. Data was collected on a BD FACSAria on the same day the cells were stained. In some instances, one drop of OneComp eBeads (eBioscience, San Diego, CA, USA) were suspended in 200μL FACS buffer and stained in the same manner instead of PBMC for compensation controls in flow cytometry panels that had been validated using PBMC previously.

3.6 Enzyme-linked ImmunoSpot (ELISpot)

3.6.1. Enumeration of antigen-specific T cells

Capture and detection antibodies from Human IFN-γ and IL-17A ELISpot development Modules (R&D Systems, Minneapolis, MN, USA) were diluted in PBS with 1% BSA and stored at -80°C. A 1:60 dilution was made of the capture antibody was made in PBS and 100μL was added to Multiscreen-IP with under drain ELISpot plate with Immobilon-P polyvinylidene fluoride (PVDF), 45μm 96 well plate (EMD Milipore, Billericia, MA, USA). Plates were incubated overnight at 4°c. Well were washed three times by adding 200μL PBS , aspirating wells and inverting on a kim wipe. Wells were blocked with 200μL of PBS containing 1% BSA and 5% Sucrose (Sigma Aldrich, St. Louis, MO, USA) for 2 hours at room temperature.

Thawed PBMC from HD and MS patients were suspended at $2x10^7$ cell/mL in human media. MBP, MOG, MOG 35-55, and GA or a cocktail of TT, MV, RSV and Flu were diluted in human media to 100μg/mL. Wells were washed twice with 200μL human media and 50μL of PBMC and 50μL of antigen were added to the appropriate wells. All samples were run in quadruplicate and included no antigen control wells to assess background/spontaneous IL-17 and IFN-γ production. Plates were incubated at 37°C for 72h.

Wells were washed 3 times with 200μL PBST (Table 3.8.1). Detection antibodies were diluted 1:60 in PBS containing 1% BSA and 100μL was added to each well. Plates were incubated overnight at 4°c. Plates were washed three times with PBST. Detection of spots was carried out by the ELISpot Blue Color Module (R&D Systems, Minneapolis, MN, USA), all reagents were stored at 4°c. Streptavidin-AP was diluted 1:60 in PBS with 1%

BSA, and 100μL was added to each well. Plates were incubated for 2 hours and room temperature. Plates were washed three times with PBST and once with 200μL deionized water. One hundred μL BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) substrate was added to each well and plates were incubated in the dark for 20-30 minutes. Plates were washed 2-3 times with 200μL deionized water and allowed to dry for at least 24 hours. Plates were sent to ZellNet Consulting (Fort Lee, NJ, USA) were the number, area and intensity of spots and the total area covered by spots were quantified.

3.7. B-T Co-Culture Assay

3.7.1. B-T Co-Culture Assay Set-up

Tetanus toxoid (Umass Biologics, Worcester, MA, USA) was suspended in PBS at 5mg/mL and 10-30μL aliquots were stored at -80°C for long-term storage or -20°C for short term storage, up to two weeks. Native influenza (Flu, California/7/2009/NYMC X-179A, H1N1), mumps virus (MV, Ender's strain), respiratory syncytial virus (RSV, long strain) were inactivated by gamma-irradiation (Genway Biotech, San Diego, CA, USA). Recombinant human myelin oligodendrocyte glycoprotein (MOG) 1-125 comprising the extracellular domain was a kind gift of Jerri Ann Lyons or was generated in our laboratory as previously described [233], MOG 35-55 (CS Bio Co, Menlo Park, CA, USA), and bovine myelin basic protein (MBP, Genway Biotech, San Diego, CA, USA) was diluted to 5mg/mL in PBS and stored at -80°C in 10-15μL aliquots. A cocktail of control antigens (foreign antigens) was prepared by diluting MV, RSV, and TT to 25mg/mL and flu antigen to 15mg/mL in human media in the same tube. Neuroantigens MOG, MOG 35-55 and MBP were a diluted to

25mg/mL in separate tubes, and GA was diluted to 50mg/mL in human media in a separate tube.

Sorted memory and naïve B cells (3.2.3) were labeled with CFSE (3.1.9) and suspended at $1X10^6$ cells/mL in human media. MACS-enriched T cells (3.2.2) and PBMC were labeled with CFSE (3.1.9) and suspended at $1X10^7$ cells/mL in human media. $2x10^6$ PBMC or $1x10^6$ of T cells and $1x10^5$ memory or naïve B cells were added to polystyrene 12x75mm FACS tubes with a polyethylene cap. PBMC and B-T co-cultures were with foreign antigen cocktail, 10μg/mL MV, RSV, TT and 6μg/mL flu, or 10μg/mL MOG 35-55, MOG, MBP, or GA. In some cases, MBP and MOG or MBP, MOG and GA were combined. All cultures were brought to a total volume of 500μL in human media.

Cultures were incubated in a humidified incubator at 37°c. On day three of culture 250μL of the culture supernatant was removed, taking care to avoid disturbing cells, that settled to the bottom of the tube, and placed in a microcentrifuge tube. 250μL fresh, warmed human media was added to each culture to return the volume to 500μL. Cells were incubated until -18 hours before day 5 of culture when 450μL of and stimulated with PMA/I. After an 18-hour stimulation 3mL of ice-cold FACS buffer was added to each FACS tube used to culture the cells. Cells were stained with a panel of antibodies to track T cell populations and to detect intracellular IL-17A and IFN-γ (3.5.4).

3.7.2 Neutralization of IL-6 receptor

Cultures of PBMC or autologous B and T cells were set up as described in section 3.7.1. Ten micrograms of neutralizing mouse monoclonal anti-human IL-6 receptor clone B-R6, low

endotoxin, sodium azide free (Abcam, Cambridge, England, UK), was added to cultures at the time of set up. An additional 10μg was added to cultures on day 2.5 and no supernatants were collected on day three. Approximately 0.2μg of this antibody saturates IL-6r on one million PBMC.

3.8. Statistical Analysis

3.8.1 Statistical Analysis.

An unpaired student's T-test was performed to compare cytokine concentration and proliferative responses, unless otherwise stated. A p-value less than 0.05 was considered statistically significant. For ELIspots a T test with Holm-Sidak method, not assuming equivalent standard deviation. The number of responders in certain assays was defined as two standard deviations above the mean of the noted population. In some instances, chi-squared analysis was utilized to determine the statistical significance between numbers of responders. All statistical analyses were carried out in Microsoft Excel or GraphPad Prism.

3.9. Media and Buffers

Table 3.9.1. Media and buffers

Media	Contents	Preparation and Storage Conditions
Human Media	90% Roswell Park Memorial Institute meida without L-glutamine or sodium pyruvate (RPMI; Cellgro Mediatech, Manassas, VA, USA), 10% Human Serum AB (Corning, Corning, NY, USA), 2mM L-glutamine (Cellgro Mediatech, Manassas, VA, USA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Cellgro, Carlsbad, CA, USA) and 100 µg/mL Penicillin, 100 IU Streptomycin (Cellgro Mediatech, Manassas, VA, USA).	RPMI was stored at 4°c, all other reagents were stored at -20 to -80°c. After combining reagents, media was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at 4°C and used within six weeks.
G418 Media	90% RPMI, 10% Fetal Bovine Serum (FBS, Gibco, Carlsbad, CA, USA), 10 mM HEPES, 2 mM L-glutamine, 100 µg/mL Penicillin, 100 IU Streptomycin and 200µg/mL G418, Geneticin, (Invitrogen, Carlsbad, CA, USA).	RPMI was stored at 4°c, all other reagents were stored at -20 to -80°c. After combining reagents, media was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at 4°C and used within six weeks.
Minimal Media	90% RPMI, 10% Fetal Bovine Serum (FBS, Gibco), 10 mM HEPES, 2 mM L-glutamine, 100 µg/mL Penicillin, 100 IU Streptomycin.	RPMI was stored at 4°c, all other reagents were stored at -20 to -80°c. After combining reagents, media was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at 4°C and used within six weeks.
BSA Buffer	0.5% Bovine Serum Albumin (BSA; Sigma), 2 mM L-glutamine, 100 µg/mL Penicillin, 100 IU Streptomycin in Phosphate Buffered Saline (PBS; Cellgro Mediatech, Manassas, VA, USA).	PBS and BSA were stored at 4°c. After combining reagents, buffer was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at 4°C and used within six weeks.
Human Freezing Media	50 % Human Serum, 40% Human Media, 10% dimethylsulfoxide (DMSO; Sigma St. Louis, MO, USA)	RPMI was stored at 4°c, all other reagents were stored at -20 to -80°c. After combining reagents, media was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at -20°C and used within six weeks..
2X Human Freezing Media	50 % Human Serum, 30% Human Media, 20% dimethylsulfoxide (DMSO; Sigma St. Louis, MO, USA)	RPMI was stored at 4°c, all other reagents were stored at -20 to -80°c. After combining reagents, media was filter sterilized with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Media was stored at -20°C and used within six weeks.
PBST	PBS with 0.05% Tween20 (Sigma Aldrich, St Louis, MO, USA). For ELISpot PBST was supplemented with 1% BSA.	For ELISpot buffer was filter sterilized with a 0.22 cellulose acetate filter (Corning, Corning, NY, USA)
FACS Buffer	1-4% BSA in PBS	Store at 4°C. Expires 6 weeks after combining reagents.

CHAPTER FOUR
Dysregulated B cell Activation in Multiple Sclerosis

4.1 Introduction

The evidence for B cell involvement in the pathogenesis of MS has become increasingly compelling, yet the exact contributions of B cells to MS are not clear. Post-mortem tissues from MS patients show that B cells infiltrate perivascular regions and sites of demyelination [52]. In SPMS, B cell follicles are present in the CNS [127] and associated with earlier disease onset, increased disability and mortality [234]. B cells are present in the CSF of RRMS patients and their frequency correlates with CNS inflammation [235-237]. Antigen-experienced memory B cells that bear somatically hyper-mutated BCRs are highly enriched in the CSF of MS patients [238]. The presence of OCB and mounting evidence based on BCR clonal relationships show that CSF B cells are selected in germinal centers [50] and traffic from the periphery to the CNS [201, 202] suggesting ongoing antigen-driven B cell activation in MS. Finally, BCDT has shown efficacy in MS patients to reduce lesions and relapse rate [207, 209].

B cells could be activated due to extrinsic factors such as over-expression of CD40L on T cells in MS patients [239-241] or cytokine secretion by other immune components [242]. In addition, B cells respond to TLR and BCR stimulation that could lead to aberrant activation. There is some evidence that B cells from MS patients exhibit altered activation after stimulation with CD40, BCR [243, 244], and TLR [151].

We hypothesized that both naïve and antigen-experienced memory B cells from MS patients would exhibit altered proliferation and increased pro-inflammatory cytokine

production. To test this hypothesis, memory and naïve B cells were sorted to ≥98% purity (Figure 3-01) and exposed *in vitro* to CD40L as a means to simulate T cell stimulation. Two levels (high and low) of CD40L stimulation were utilized which induced proliferation by approximately 25 or 50 percent of B cells. The rationale for this approach was two-pronged. First, we wanted to sensitize the assay to observe positive and negative regulation of responses. Second, it is possible that over-expression of CD40L on T cells from MS patients may cause otherwise normal B cells to display altered activation.

In addition to CD40L, we stimulated with IL-4, a potent B cell activation and survival factor. To further interrogate B cell activation, we combined CD40L plus IL-4 stimulation with a polyclonal IgG/IgM crosslinking antibody (BCR stimulation) to simulate T cell help and antigenic stimulation. We also combined CD40L stimulation with IL-2 and CpG, a TLR9 agonist to test BCR-independent activation.

4.2. Results

4.2.1 Proliferation by memory B cells

Proliferation by memory and naïve B cells to CD40L and additional stimuli was quantified by CFSE dilution using IL-4 only as a negative control (Figure 4-01). For ease of interpretation, proliferation data has been divided into sections and is represented in multiple

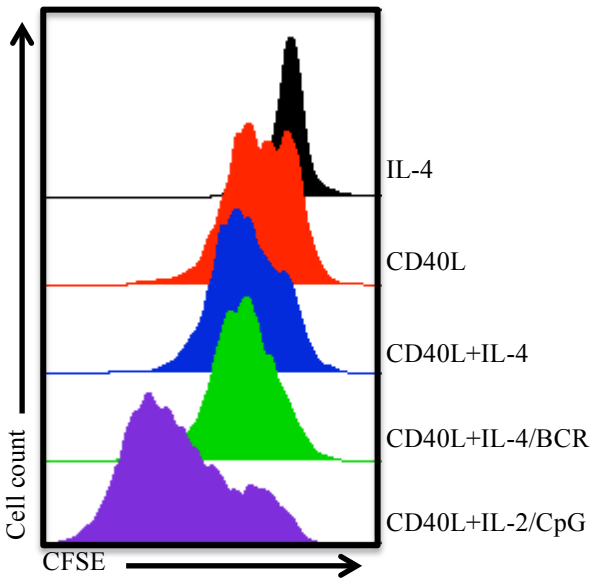


Figure 4-01. Representative proliferation by naïve HD B cells after a five-day culture. IL-4 was used as

figures, with only the noted comparisons represented in a given figure (Figures 4-02 and 4-03). All proliferation data is summarized in Table 4-01.

Proliferation by memory B cells from HD stimulated with low or high-dose CD40L and IL-2/CpG was significantly higher than CD40L alone (Figure 4-02A). The same was true for HD and MS memory B cells in low-dose CD40L conditions (Figure 4-03A and B). However, when MS memory B cells were stimulated in low-dose CD40L conditions additional stimuli failed to enhance proliferation (Figure 4-02B). Proliferation by memory B cells from MS patients was significantly lower than HD memory B cells in several culture conditions in high-dose CD40L cultures (Figure 4-02D). In contrast, low-dose CD40L elicited significantly more proliferation by memory B cells from MS patients compared to HD memory B cells (Figure 4-03D). This suggests that memory B cells from MS patients are more sensitive to CD40-engagement. We observed less proliferation by memory B cells from MS patients in response to IL-2/CpG (Figures 4-02D and 4-03D). As expected, we observed significantly more proliferation in high-dose CD40L conditions comared to low-dose CD40L for nearly all stimulatoin conditions (Table 4-01). However, memory B cells from HD achieved similar proliferation when exposed to low-dose CD40L plus IL-2/CpG compared to high-dose CD40L plus IL-2/CpG. Memory B cells from MS patients required high-dose CD40-activation achieve a maximal response to IL-2/CpG. This is not entirely surprising given previous reports that, at least in memory B cells, MS patients express less TLR9.

4.2.2 Proliferation by Naïve B cells

When stimulated with high-dose CD40L naïve HD B cell proliferation was enhanced by the addition of IL-4/BCR and IL-2/CpG (Figure 4-02A). In the same high-dose CD40L conditions proliferation by naïve B cells from MS patients was elevated by adding either IL-4 or IL-2/CpG, but not by IL-4/BCR (Figure 4-02B). There was a clear trend in HD naïve B cells for two or three signal stimulation favoring an increase in proliferation; this was not evident in naïve B cells from MS patients. In addition, naïve B cells from HD stimulated with high-dose CD40L proliferated more than naïve B cells from MS patients when stimulated with CD40L alone or with IL-2/CpG (Figure 4-02C).

Proliferation to low-dose CD40L stimulation by naïve B cells from HD was only enhanced by IL-2/CpG (Figure 4-03A). However, proliferation by naïve B cells from MS patients was significantly greater when any stimulation was combined with low-dose CD40L (Figure 4-03B). This suggests that when CD40-stimulation is limited naïve B cells from MS patients are more sensitive to further activation signals. However, when stimulated with high or low-dose CD40L and IL-2/CpG naïve B cells from MS patients proliferated less than HD naïve B cells (Figure 4-03 C).

4.2.2 Proliferative responses by memory B cells compared to naïve B cells

Memory B cells from healthy donors stimulated with high-dose CD40L proliferated significantly more than naïve B cells in response to IL-2/CpG (Figure 4-02-A). The same was true for HD B cells stimulated under low-dose CD40L conditions (Fiure 4-03A). In contrast, memory B cells from MS were more responsive to high-dose CD40L alone or CD40L plus IL-4 compared to naïve B cells from the same donors (Figure 4-02B). What may

exemplify the altered proliferation responses by MS B cells is the enhaced proliferative capacity by memory B cells to low-dose CD40L stimulatoin. Memory B cells from MS patients had a heightened ability to respond to low-dose CD40L, alone, or in combination with any other stimuli compared to their naïve counterparts (Figure 4-03B).

cell type	# points	Low-dose CD40L			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD naïve	4-7	24.4±9.1$	23.0±10.9$	28.7±10.5$	46.1±12.1*$
HD memory	4-6	25.8±16.2*$	33.6±24.8$	41.3±11.4$	74.8±6.9*$
MS naïve	12-13	15.1±8.2$	26.7±14.5$	24.5±11.3$	32.5±14.2*$
MS memory	10-12	51.7±8.5*#$	45.1±13.6#$	47.4±8.4#$	56.5±16.6*#
		High-dose CD40L			
HD naïve	10-14	53.8±22.7$	72.7±17.1*$	75.0±15.4*$	78.9±12.1#$
HD memory	9-13	65.9±20.7$	79.3±14.9*$	74.1±20.1$	92.4±5.3*#$
MS naïve	8-12	41.6±4.2#$	53.1±15.6*$	50.0±14.0*#$	66.7±18.7$
MS memory	9-11	61.6±4.3#$	63.3±7.7*$	63.0±9.2#$	70.5±19.6*

Table 4-01. B-cell proliferation after a five-day culture was quantified by CFSE dilution. Values are expressed as the mean percentage of cells that have undergone proliferation ± standard deviation. *P≤ 0.05 RRMS compared to HD, #P≤ 0.05 memory compared to naïve, and $P≤ 0.05 low versus high CD40L. P-values based on less than five data points were excluded from statistical analysis.

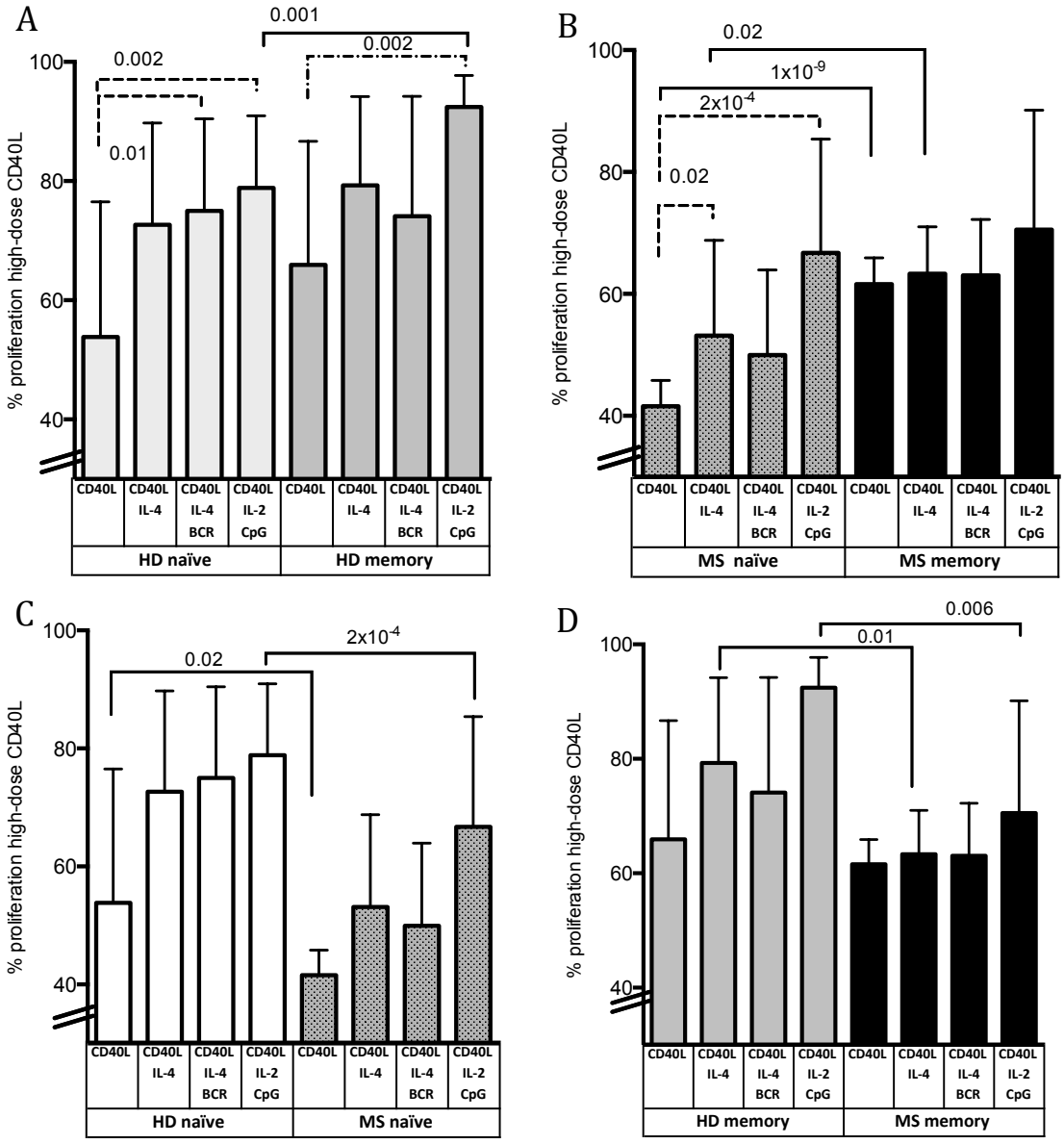


Figure 4-02. Proliferation by memory and naïve B cells *in vitro* in high-dose CD40L conditions. Comparisons between naïve and memory in HD **(A)** and MS patients **(B)** within the same donor group**.** Comparison of naïve B cells form MS patients and HD **(C)** and memory B cells from HD and MS patients **(D).** Dashed lines are comparisons made within a group; solid lines are comparison between two cell or donor types.

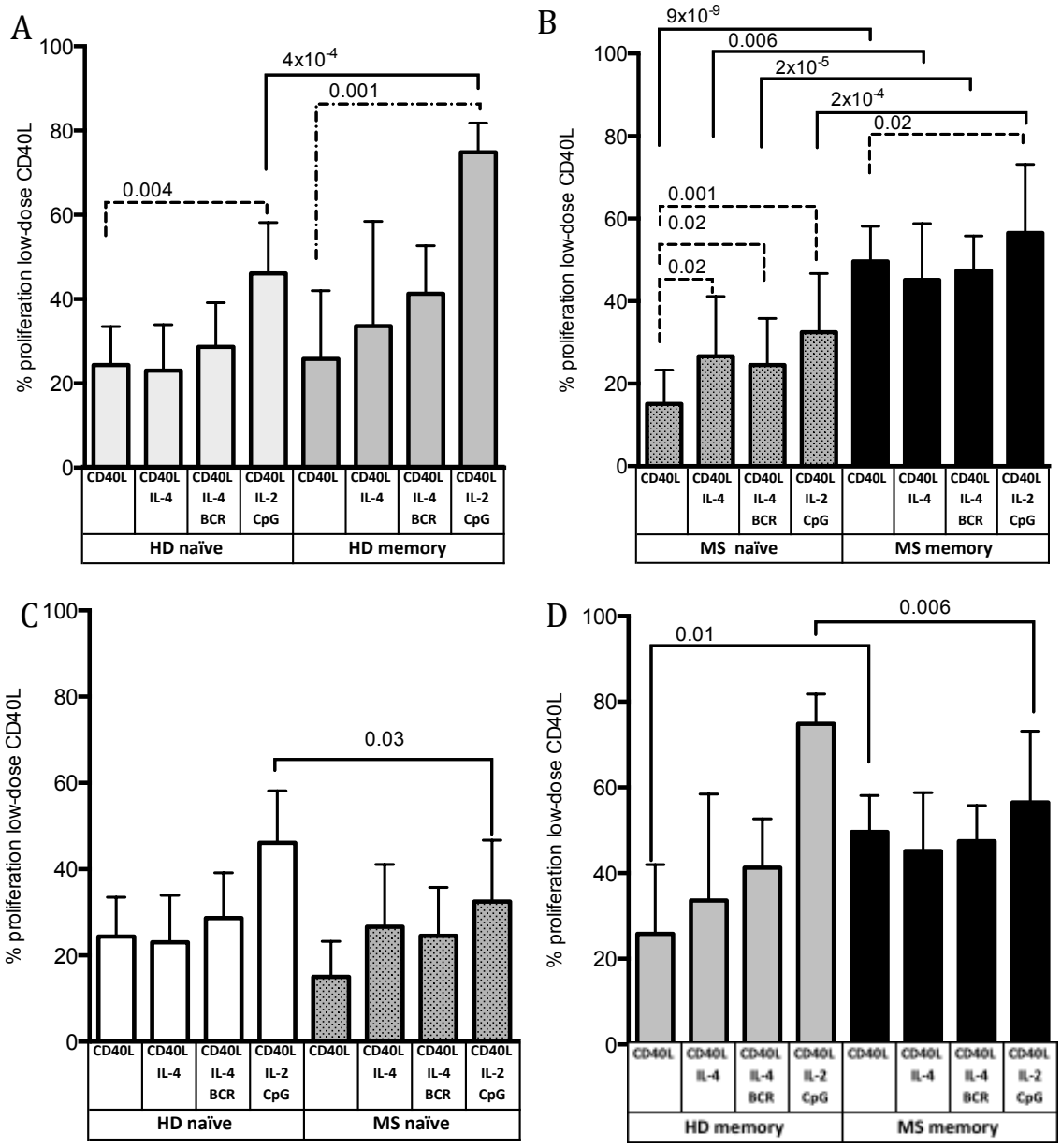


Figure 4-03. Proliferation by memory and naïve B cells *in vitro* in low-dose CD40L conditions. Comparisons between naïve and memory in HD **(A)** and MS patients **(B)** within the same donor group**.** Comparison of naïve B cells form MS patients and HD **(C)** and memory B cells from HD and MS patients **(D).** Dashed lines are comparisons made within a group, solid lines are comparison between two cell or donor types.

4.2.3. TNF-α Production by B cells

Table 4-02. TNF-α production is similar in HD and MS patients.

cell type	data points	TNF-α Low Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD naïve	3	22.0±36.4	1.0±0.1	33.7±44.9	1.0±0.1
HD memory	3	0.1±1.2	5.5±6.3	1.0±0.1	1.1±0.2
MS naïve	9-10	1.8±1.7	2.1±1.8	2.6±2.7	2.9±3.9
MS memory	7-9	11.9±29.3	1.8±1.7	2.9±3.4	22.6±59.1
		TNF-α High Day 3			
HD naïve	9-11	7.5±17.9	598.3±979.9	76.2±145.3	61.5±141.6
HD memory	8-9	357.3±1006.0	80.0±155.9	95.8±234.4	146.1±308.9
MS naïve	7	2.3±1.8	2.6±2.8	2.6±1.9	2.2±2.0
MS memory	9-11	1.6±1.0$	7.6±9.7	4.7±5.6	3.2±5.4
		TNF-α Low Day 5			
HD naïve	2	4.5±4.9	1.1±0.1	1.1±0.1	1.1±0.1
HD memory	2-3	1.7±0.9	2.2±1.7	172.0±125.0	6.5±0.7
MS naïve	8-9	1.9±1.3	3.6±3.6	2.7±3.4	2.4±2.0
MS memory	5-6	2.1±1.0	1.8±1.0	2.1±1.3	2.1±1.0
		TNF-α High Day 5			
HD naïve	8-15	603.3±1036.6	157.7±398.0	757.1±1448.8	299.1±363.2*
HD memory	7-11	141.1±368.6	77.6±168.5	255.0±559.5	393.8±691.7
MS naïve	5-7	1.5±1.3	2.6±1.8	2.2±1.7	1.4±0.9*
MS memory	9-11	3.6±2.7$	2.6±1.0	1.8±1.0	1.9±1.1

Production of TNF-α on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in pg/mL ± standard deviation. *P≤ 0.05 MS compared to HD, #P≤ 0.05 memory compared to naïve, and $P≤ 0.05 day 3 compared to day 5. P-values based on less than five data points were excluded from statistical analysis.

In the context of most autoimmune diseases, TNF-α mediates inflammatory and humoral immune responses [245]. In fact, in rheumatoid arthritis (RA), Crohn's disease, and psoriasis, TNF-α inhibitors are used as therapeutics. However, MS patients exposed to a TNF-α inhibitor exhibited no change in EDSS scores or lesion load and surprisingly, the frequency of exacerbations was increased in patients receiving this agent [246]. We measured TNF-α production by B cells because of its important role in the formation of germinal centers and humoral immunity.

B cell pools produced little TNF-α in response to any low-dose CD40L condition (Table 4-02). No significant differences were observed between the cohorts in low-dose CD40L conditions. In response to high-dose CD40L stimulation, no significant differences were observed despite a trend toward higher TNF-α production by HD naïve B cells on day three. This is likely due to heterogeneous TNF-α production. On day five, naïve B cells from healthy donors stimulated with high-dose CD40L and IL-2/CpG produced significantly more TNF-α than naïve B cells from MS patients (P=0.02).

4.2.4. LT-α Production by B cells

LT-α production is not well studied in the context of RRMS. However, LT-α is required for germinal center formation and may impact the activation of B cells within the CNS. We hypothesized that B cells from MS patients would produce more LT-α given the presence of memory B cells in the CNS and evidence of ectopic follicles in SPMS patients. Levels of LT-α tended to be higher in cultures with high-dose CD40L, but did not reach statistical significance (Table 4-03). We found that LT-α production by naïve B cells from HD and MS patients was similar on day three and five in low-dose CD40L conditions, although naïve B cells from MS patients produced significantly more LT-α when stimulated on day three of culture in response to low-dose CD40L plus IL-2 and CpG (P=0.02). On day five, Naïve B cells from HD produced more LT-α in response to high-dose CD40L plus IL-4/BCR compared to HD memory B cells (P=0.04) and naïve B cells from MS patients (P=0.02).

Table 4-03. LT-a by memory and naïve B cells is similar in most conditions.

cell type	data points	LT-α Low Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD naïve	4	2.0±2.0	6.10±6.4	19.8±22.9	6.3±7.1*
HD memory	4	169.6±327.0	22.0±28.9	54.0±61.5	175.5±161.8
MS naïve	8-10	4.1±2.2	56.5±71.7	68.3±78.5	56.5±180.3*
MS memory	8	16.5±26.4	36.5±39.8	40.9±72.3	101.9±166.6
		LT-α High Day 3			
HD naïve	10	17.0±39.4	435.4±853.7	486.2±1046.0	1200.4±2390.3
HD memory	4	169.6±327.0	22.0±28.9	54.0±61.5	175.5±161.8
MS naïve	8-10	36.0±54.8	82.6±129.7	135.5±224.0	125.5±174.6
MS memory	9-10	211.9±493.2	27.8±33.1	105.7±145.1	136.5±223.8
		LT-α Low Day 5			
HD naïve	3	2.3±2.3	82.7±80.5	87.7±93.1	53.3±19.2
HD memory	2-3	6.0±5.6	30±35.4	251.0±178.2	181.3±157.8
MS naïve	8-9	26.2±63.4	96.0±44.7	53.6±47.9	78.1±59.1
MS memory	9-10	3.0±4.5	13.4±16.9	12.4±16.4	6.9±9.9
		LT-α High Day 5			
HD naïve	8-15	323.0±570.7	590.3±988.5	982.0±1259.4*#	1019.5±1919.5
HD memory	6-12	52.0±59.4	212.0±289.2	234.7±374.5#	87.7±112.5
MS naïve	5-7	9.9±12.9	85.4±64.5	115.7±101.6*	170.5±112.8
MS memory	8-10	37.2±67.1	82.5±127.8	88.32±75.6	149.2±181.0

Production of LT-a on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in pg/mL ± standard deviation. *P≤ 0.05 MS compared to HD, #P≤ 0.05 memory compared to naïve, and v#P≤ 0.05 day 3 compared to day 5. P-values based on less than five data points were excluded from statistical analysis.

4.2.5. IL-12 production by B cells

B cell are capable of secreting IL-12 [247], a cytokine that acts as a potent inducer of Th1 cells. We attempted to measure IL-12p70 in the supernatant of B cell cultures; however the lowest limit of detection of IL-12p70 by ELISA was quite variable among experiments, ranging from 9.5-38.0 pg/mL. Thus, compared to other cytokines, IL-12p70 ELISAs were much less sensitive. Despite levels of up to 405.0 pg/mL IL-12 from some HD B cell cultures, the majority of supernatants were below the LLOD. The results from this study are inconclusive with respect to IL-12.

4.2.6. IL-10 production by B cells

Recently the ability of B cells to produce IL-10 and act in a regulatory manner to control inflammation and autoimmunity has been appreciated. While it is not entirely clear in humans whether B cells that produce IL-10 are phenotypically distinct or a terminally differentiated cell type, it is clear that in MS, IL-10 is associated with protective responses. Despite some conflicting reports on B-cell production of IL-10 in MS [243], we hypothesized that memory and naïve B cells from MS patients would produce less IL-10 than healthy donors.

We found that on day three of culture in low-dose conditions there were no significant differences between any of the populations studied (Table 4-04). In contrast, naïve B cells from HD produced significantly more IL-10 compared to naïve B cells from MS patients in low-dose CD40L conditions on day five (Figure 4-04A). Memory B cells stimulated with low-dose CD40L secreted little IL-10 in most culture conditions and no differences were observed between HD and MS patients (Figure 4-04B). In high dose-CD40L conditions, naïve HD B cells produced significantly more IL-10 compared to MS patients in nearly every condition tested on day five (Figure 4-04C). The same was true for memory B cells (Figure 4-04D).

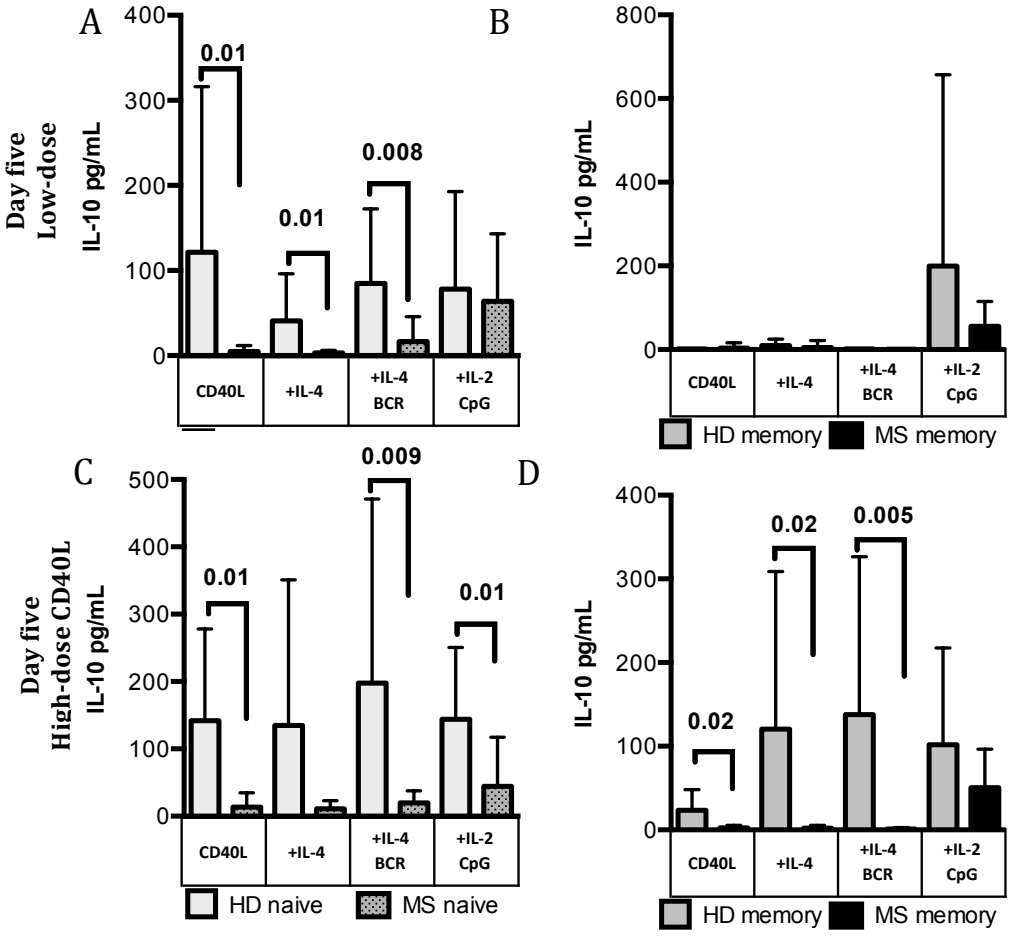
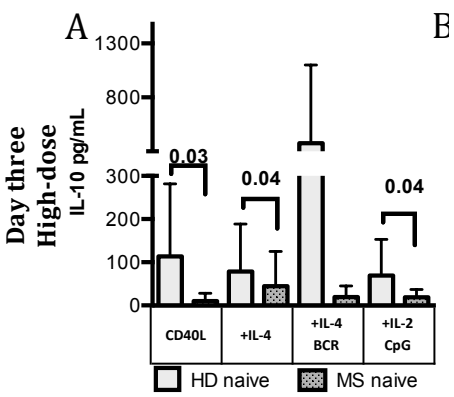


Figure 4-04. Production of IL-10 by B cells on day five of culture in low- and high-dose CD40L conditions. **A.** Production of IL-10 by naïve B cells to low-dose CD40L. **B.** Memory B cell secretion of IL-10 to low-dose CD40L. **C.** Naïve B cells from HD produce more IL-10 compared to MS patients. **D.** Memory B cells tended to produce less IL-10 than naïve B cells; however, memory B cells from MS patients failed to produce similar levels of IL-10 compared to healthy donors. Mean cytokine production (pg/mL) +/-standard deviation.

When B cells were stimulated with high-dose CD40L, naïve B cells from HD produced significantly more IL-10 on day three compared to naïve B cells from MS patients in response to CD40L plus IL-4/BCR and IL-2/CpG (Figure 4-05A). Memory B cells from HD tended to produce more IL-10 than MS patients on day three in high-dose CD40L conditions (Figure 4-05B). This was statistically significant when memory B cells were stimulated with CD40L and IL-4/BCR. In addition, we observed that naïve B cells from HD

and MS patients tended to produce higher levels of IL-10 compared to memory B cells (Table 4-04).



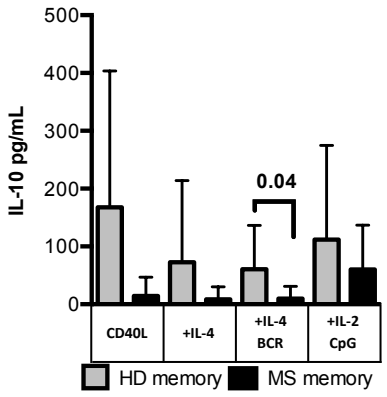


Figure 4-05. Production of IL-10 by B cells on day three of culture in low- and high-dose CD40L conditions. **A.** Production of IL-10 by naïve B cells to high-dose CD40L. **B.** Memory B cell secretion of IL-10 to high-dose CD40L. Mean cytokine production (pg/mL) +/-standard deviation.

Table 4-04. IL-10 production is deficient in MS patients.

cell type	data points	CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
		IL-10 Low-dose CD40L Day 3			
HD naïve	2-6	26.3±56.2	1.0±0.1	4.5±5.4$	12.8±12.6
HD memory	2-7	50.3±80.3	5.0±5.7	2.3±1.9	36.7±29.2
MS naïve	12-14	10.2±19.0	5.4±10.2	9.9±20.8	6.7±12.7
MS memory	8-12	10.4±26.2	44.5±65.6	3.0±6.4	62.9±111.5
		IL-10 High-dose CD40L Day 3			
HD naïve	9-11	113.7±168.0*	78.7±110.0	375.3±725.0*	69.2±84.0*
HD memory	9-10	167.1±236.2	72.7±141.3*	60.7±75.8	111.8±162.9
MS naïve	12-15	9.9±18.4*	44.5±80.7	19.6±25.6*	18.7±18.4*
MS memory	13-15	14.4±32.3	8.6±21.8*	9.8±21.0	56.6±75.4
		IL-10 Low-dose CD40L Day 5			
HD naïve	6-7	121.4±194.6	40.7±55.3	84.7±87.6$	78.3±114.6
HD memory	5-7	1.6±0.5	9.7±14.9	1.5±0.4	199.4±457.5
MS naïve	12-15*	4.7±7.1*	3.1±2.9*	16.6±29.3$	63.8±79.4
MS memory	13-17	3.5±12.9	4.8±17.2	1.1±0.7	55.5±59.7
		IL-10 High-dose CD40L Day 5			
HD naïve	9-14	131.6±140.3*#	134.9±216.1*	189.9±278.8*	153.3±104.6*
HD memory	10-15	23.4±24.8*#	120.7±188.2*	137.9±188.5*	101.9±115.8
MS naïve	12-15	13.6±21.3*	10.8±12.1*#	19.9±17.7*#	44.2±73.2*
MS memory	14-16	2.6±2.7*	2.2±3.4*#	1.3±1.6*#	50.4±46.1

Production of IL-10 on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in pg/mL ± standard deviation. *P≤ 0.05 MS compared to HD, #P≤ 0.05 memory compared to naïve, and $P≤ 0.05 day 3 compared to day 5. P-values based on less than five data points were excluded from statistical analysis.

4.2.7. IL-6 production by B cells

IL-6 plays a supporting role in many autoimmune diseases and is even a therapeutic target in rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), and other B cell malignancies [248]. However, B-cell derived IL-6 is not a well-appreciated feature of these disorders. At the time of this study, it was not known whether B cells from MS patients produced IL-6 or what possible role it could play in disease pathology. Since then, several studies in EAE have identified B cell-derived IL-6 as a pathogenic factor. In fact, IL-6 is obligatory for initiating EAE [249, 250] and treatment of EAE with an IL-6 neutralizing antibody diminishes disease severity [251].

We measured the level of IL-6 in the supernatants from memory and naïve B cell cultures and found, a consistent overproduction of IL-6 by B cells from MS patients when stimulated in culture with high-dose CD40L on days three and five (Figure 4-06, Table 4-05). This was consistent for both naïve (Figure 4-06A and C) and memory (Figure 4-06B and D) B cells. We observed less IL-6 in low-dose CD40L conditions and there were no significant differences between naïve B cell secretion of IL-6 between HD or MS patients on day three (Figure 4-06E) or five (Table 4-05). We found a significantly elevated IL-6 in memory B cell cultures from MS patients on day three in low-dose CD40L plus IL-4 (Figure 4-06F) but no differences on day five (Table 4-05).

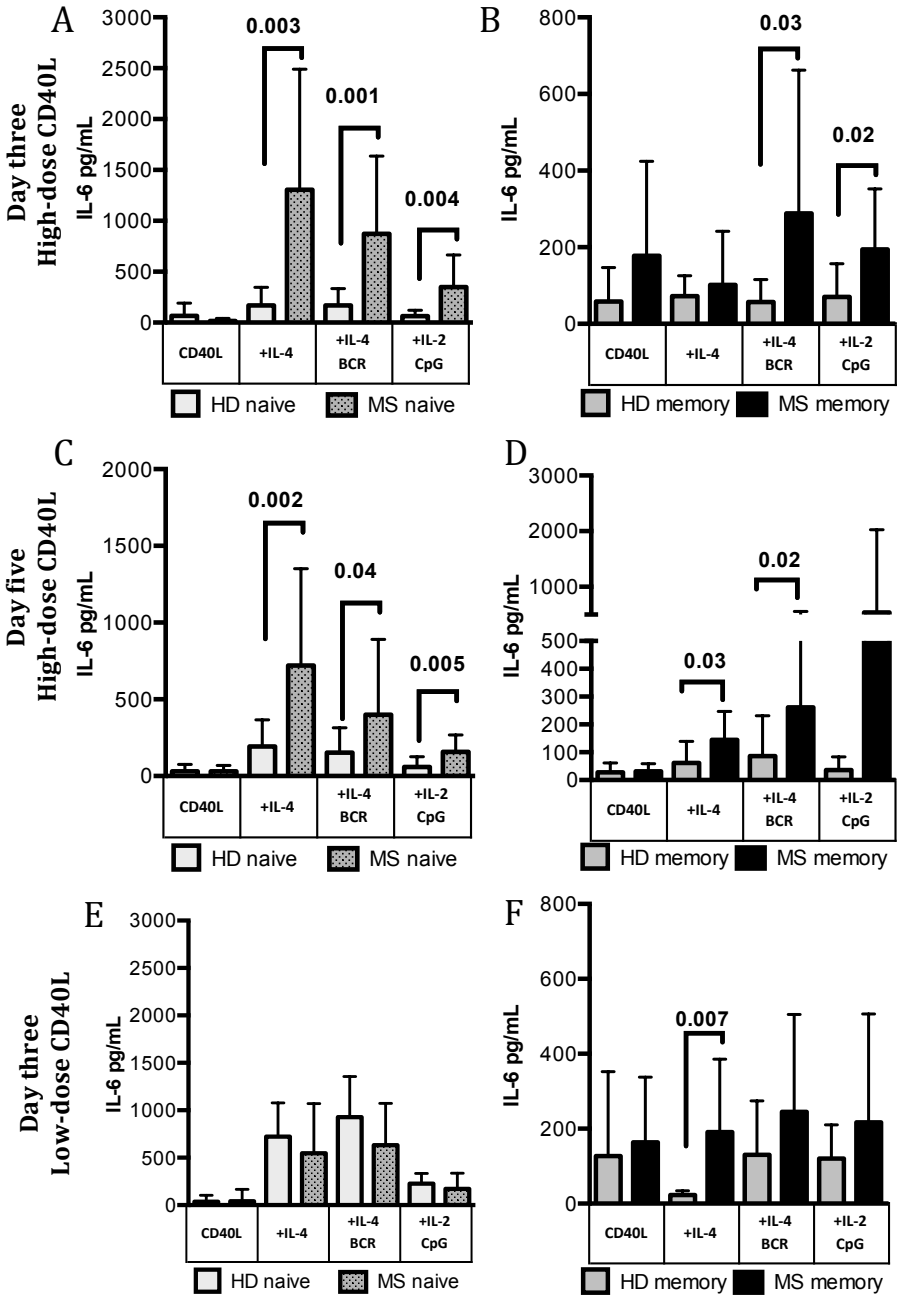


Figure 4-06. Production of IL-6 by B cells on day three and five of culture in high-dose CD40L conditions and day three in low-dose CD40L conditions. Production of IL-6 by naïve **(A)** and memory **(B)** B cells on day three of culture in high-dose CD40L conditions. IL-6 levels on day five in high-dose CD40L cultures from naïve **(C)** and memory **(D)** B cells. IL-6 levels in low-dose CD40L cultures on day three from naïve **(E)** and memory **(F)** B cells. Mean cytokine production ± standard deviation.

Table 4-05. IL-6 production is elevated in MS patients

cell type	data points	IL-6 Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD naïve	3-5	36.8±66.3	897.3±723.4#	928.2±462.9#$	226.4±108.2
HD memory	2-11	127.1±225.4	23.0±11.3*#	130.4±143.7#	120.2±89.8
MS naïve	14-17	42.2±125.0#	546.2±523.9	632.6±441.41#	171.6±165.4$
MS memory	14-17	163.9±173.6#$	194.3±200.9*	244.9±259.9#	216.4±287.8
		IL-6 High-dose CD40L Day 3			
HD naïve	12-14	66.2±126.3	168.9±177.2*#	168.9±165.9*	64.5±57.7*
HD memory	11-14	58.4±88.4	72.6±54.7#	57.0±58.2	70.5±85.2
MS naïve	13-18	16.2±26.0#	1303.0±1186.2*#	872.1±763.4*#	350.0±314.6*
MS memory	14-18	187.8±251.7#	98.1±144.4#	301.2±392.7#	196.1±163.0
		IL-6 Low-dose CD40L Day 5			
HD naïve	8-10	20.9±17.6	372.0±384.2	565.2±348.2#	92.6±92.5
HD memory	3-7	16.3±9.1	69.0±68.4	81.5±56.1#	103.3±102.9
MS naïve	13-15	79.4±169.0	581.6±625.6#	314.1±312.6	103.1±99.7
MS memory	9-14	28.1±30.0	147.4±115.1#	145.7±115.4	74.9±61.4
		IL-6 High-dose CD40L Day 5			
HD naïve	12-21	31.2±44.7	192.1±175.1*#	152.6±161.5*	59.5±67.3*
HD memory	11-17	31.0±36.2	63.0±84.9*#	78.4±149.0*	37.1±48.7
MS naïve	10-13	30.3±38.3	720.1±632.0*#	399.3±492.0*	157.7±109.6*
MS memory	7-10	31.0±27.4	145.0±102.0*	261.2±294.1*	539.4±1488.7

Production of IL-6 on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in pg/mL ± standard deviation. *P≤ 0.05 RRMS compared to HD, #P≤ 0.05 memory compared to naïve, and $P≤ 0.05 day 3 compared to day 5. P-values based on less than five data points were excluded from statistical analysis.

4.2.9. IL-10 and IL-10 Expression by qPCR

To determine whether the levels of IL-6 and IL-10 that were detected in culture supernatants were resultant of differential expression or consumption, we carried out qPCR for IL-6 and IL-10 transcripts from activated total CD19+ B cells in 3 MS patients and 3 HD. To avoid contaminating mRNA from CD40L feeder cells, recombinant human CD40L

(rHCD40L) was used to activate B cells in combination with IL-4/BCR or IL-2/CpG. Expression was normalized to a control housekeeping gene PPIA and to unstimulated B cells.

In agreement with the results from culture supernatants, B cells from MS patients expressed elevated levels of IL-6 transcripts after only 24 hours of stimulation. We did not observe a statistical difference with regard to IL-6 transcripts but there was a clear trend for higher expression in MS patients' B cells compared to HD (Figure 4-07A). While we did not observe differences in IL-10 expression from B cells stimulated with IL-4/BCR, we did find that B cells from HD stimulated with IL-2/CpG expressed significantly higher levels of IL-10 message (Figure 4-06B).

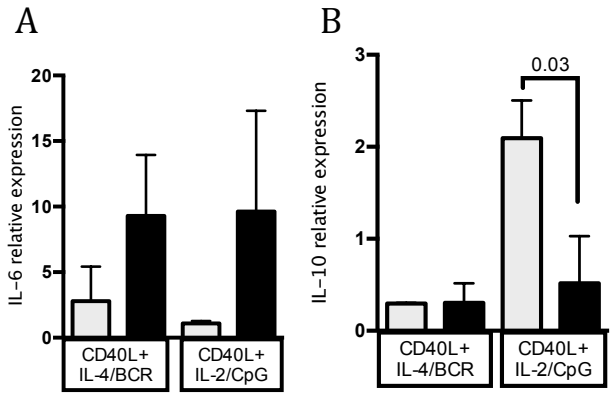


Figure 4-07. Transcripts of IL-6 and IL-10 from stimulated B cells. HD B cells (open bars) and MS patients (black bars). **A.** MS patients tended to have higher levels of IL-6 transcripts after 24-hours in culture. **B.** IL-10 transcripts were elevated in HD B cells' stimulated with rHCD40L plus IL-2/CpG

CHAPTER FIVE
The impact of Glatiramer Acetate on B cells

5.1 Introduction

Despite growing evidence for the involvement of B cells in the pathogenesis of MS, it is unclear whether the current immunomodulatory therapies can impact dysregulated B cell functions. The studies described here in chapter four, as well as several other studies, have identified a loss in the ability of B cells from MS patients to produce IL-10, but an enhanced ability to secrete potentially inflammatory cytokines, such as IL-6 and LT-α [138, 252, 253]. There are few studies focused on B cell responses to MS therapies, which are summarized in the literature review.

We became particularly interested in GA because it has been reported to directly impact the cytokine profiles of human myeloid APC. Observations made in mice that suggest that GA therapy influences B cell cytokine secretion [254-256]. We hypothesized that GA therapy would re-establish normal IL-10 production by B cells from MS and diminish IL-6 production. First, we sought to determine whether GA has a direct impact on B-cell proliferation or cytokine production by supplementing *in vitro* memory and naïve B cell cultures with GA. Second, we tested whether GA therapy modulated responses by B cells from MS patients.

5.2 Direct influence of Glatiramer Acetate on B cells *in vitro*

5.2.1. The Impact of Glatiramer Acetate on Proliferation

We quantified proliferation by naïve and memory B cells using CFSE dilution on day five of culture in the presence or absence of GA. Naïve B cells from HD cultured with low-dose CD40L (tended to proliferate less in the presence of GA but this was not statistically significant (P=0.08). In fact, the addition of GA to cultures had no impact on the proliferative capacity of memory or naïve B cells from MS patients or HD (Table 5-01).

Table 5-01. Exposure to GA *in vitro* does not alter B-cell proliferation

cell type	Low-dose CD40L			
	CD40L	CD40L+ IL-4	CD40L+ IL-4/BCR	CD40L+ IL-2/CpG
HD naïve	0.08	0.75	0.97	0.23
HD memory	0.46	0.29	0.52	0.55
MS naïve	0.25	0.72	0.96	0.79
MS memory	0.26	0.69	0.13	0.24
	High-dose CD40L			
HD naïve	0.57	0.8	0.75	0.83
HD memory	0.2	0.32	0.59	0.16
MS naïve	0.15	0.91	0.53	0.58
MS memory	0.78	0.5	0.12	0.53

Table 5-01. P-values comparing proliferation in cultures with and without GA from memory and naïve B cells from HD and MS patients. No significant differences were observed between cultures in the presence or absence of GA.

5.2.2 The Impact of Glatiramer Acetate on Cytokine Production by B cells

In some cases, cell numbers were limited and we were not able to test all conditions for cytokine production in the presence of GA. Those conditions where cells or supernatants were insufficient for measurement are labeled not determined (ND) in the tables that follow. To our surprise, memory and naïve B cells from MS patients and HD secreted similar levels of IL-10 in the presence or absence of GA in all of the conditions tested (Table 5-02). Similarly, levels of IL-6 were not different in the presence of GA *in vitro* (Table 5-02).

Table 4-02. B cell IL-6 and IL-10 production are not modulated by direct exposure to GA

cell type	IL-10 Low-dose CD40L Day 3				IL-6 Low-dose CD40L Day 3			
	CD40L	CD40L+ IL-4	CD40L+ IL-4 BCR	CD40L+ IL-2 CpG	CD40L	CD40L+ IL-4	CD40L+ IL-4 BCR	CD40L+ IL-2 CpG
HD naïve	0.33	0.42	0.26	0.60	0.50	ND	ND	0.65
HD memory	0.16	0.57	0.27	0.78	ND	ND	0.66	ND
MS naïve	0.88	0.39	0.45	0.17	0.15	0.23	0.48	0.41
MS memory	0.17	ND	0.55	0.69	ND	ND	ND	0.69
	IL-10 High-dose CD40 Day 3				IL-6 High-dose CD40 Day 3			
HD naïve	0.11	0.20	0.98	0.44	0.51	0.50	0.34	0.21
HD memory	0.11	0.20	0.98	0.44	0.57	0.27	0.54	0.54
MS naïve	0.92	0.64	0.24	0.99	0.21	0.77	0.74	0.54
MS memory	0.92	ND	0.24	0.99	0.86	ND	0.29	0.29
	IL-10 Low-dose CD40 Day 5				IL-6 Low-dose CD40 Day 5			
HD naïve	0.33	0.76	0.24	0.42	0.92	0.58	0.16	0.89
HD memory	0.44	ND	ND	0.46	0.93	ND	0.93	0.90
MS naïve	0.29	0.19	0.35	0.95	0.17	0.31	0.43	0.91
MS memory	0.46	ND	ND	0.31	0.37	ND	0.11	0.90
	IL-10 High-dose CD40 Day 5				IL-6 High-dose CD40 Day 5			
HD naïve	0.44	0.53	0.72	0.97	0.90	0.36	0.36	0.99
HD memory	0.07	0.92	0.05	0.85	0.59	0.14	0.92	0.85
MS naïve	0.50	0.78	0.85	0.60	0.46	0.36	0.94	0.09
MS memory	0.41	ND	0.47	0.38	0.41	ND	0.16	0.59

P-values for unpaired student's with a Welch's correction comparing the production of IL-10 or IL-6 in cultures with or without GA on days three and five of culture. Conditions with less than three donors were left out of statistical analyses and are listed as ND.

Despite previously observing few differences in LT-α and TNF-α between HD and MS patients, we examined whether GA altered production of these cytokines by B cells from MS patients. We found that the addition of GA to cultures of memory or naïve B cells did not significantly change the amount of LT-α or TNF-α secreted by B cells from HD or MS

patients (Table 5-03). From these results we can conclude that GA does not directly modulate the proliferation or cytokine secretion by B cells for any of the parameters measured here.

Table 4-03. B cell TNF-α and LT-α production are not modulated by direct exposure to GA

cell type	TNF-α Low Day 3				LT-α Low Day 3			
	CD40L	CD40L+ IL-4	CD40L+ IL-4 BCR	CD40L+ IL-2 CpG	CD40L	CD40L+ IL-4	CD40L+ IL-4 BCR	CD40L+ IL-2 CpG
HD naïve	0.42	0.97	0.28	0.93	0.41	0.82	0.93	0.68
HD memory	0.62	ND	0.49	0.27	0.39	0.40	0.81	0.63
MS naïve	0.11	0.16	0.23	0.26	0.29	0.86	0.81	0.86
MS memory	1.00	ND	ND	0.31	0.37	ND	ND	0.22
	TNF-α High Day 3				LT-α High Day 3			
HD naïve	0.42	0.28	0.77	0.19	0.17	0.68	0.62	0.20
HD memory	0.63	ND	0.39	0.38	ND	ND	ND	ND
MS naïve	0.81	0.96	0.65	0.28	0.71	0.43	0.46	0.28
MS memory	0.27	ND	0.53	0.46	0.65	ND	0.41	0.46
	TNF-α Low Day 5				LT-α Low Day 5			
HD naïve	ND	ND	ND	ND	0.46	0.90	0.57	0.40
HD memory	ND	ND	ND	ND	0.47	ND	ND	ND
MS naïve	0.28	0.44	0.45	0.57	0.51	0.80	0.52	0.63
MS memory	ND	ND	ND	ND	0.15	0.09	0.48	0.09
	TNF-α High Day 5				LT-α High Day 5			
HD naïve	0.24	0.38	0.94	0.86	0.16	0.33	0.19	0.47
HD memory	0.67	0.70	0.85	0.59	0.58	0.41	0.45	0.34
MS naïve	0.93	0.28	0.58	0.67	0.17	0.18	0.95	0.20
MS memory	0.25	ND	0.94	0.13	0.39	ND	0.15	0.95

P-values for comparison of TNF-α or LT-α production in memory and naïve B cell cultures with or without GA on days three and five of culture. Conditions with less than three donors were are left out of statistical analyses and are listed as ND.

5.3 Modulation of B cell Responses by Glatiramer Acetate therapy

5.3.1. Introduction

Collectively, our data suggested that B-cell proliferation and production of the cytokines quantified here are not directly impacted by exposure to GA *in vitro*. This is not entirely surprising as the mechanism(s) by which GA might directly influence cytokine secretion is unknown. Furthermore, the observations we made in this *in vitro* system do not preclude the hypothesis that GA modulates B-cell function in MS patients on GA therapy. To address this hypothesis, we next studied B cells from treatment-naïve and GA-treated RRMS patients (TN-MS and GA-MS, respectively) compared to healthy donors.

5.3.2 Glatiramer Acetate Therapy Modulates Proliferation by B cells

We compared low to high-dose CD40L conditions within each group. B cells from HD, GA-MS, and TN-MS patients proliferate significantly more to high-dose CD40L than low-dose CD40L (Figure 5-01D). B cells from HD and TN-MS patients proliferate significantly more to additional stimuli when cultured with high-dose CD40L compared to the same condition with low-dose CD40L (Figure 5-01F), except in the case of TN-MS patients with IL-2/CpG. Most notably, B cells from GA-MS patients do not display this CD40L-dose effect when combined with additional stimuli and instead, proliferated to a similar extent when exposed to either low- or high-dose CD40L (Figure 5-01E).

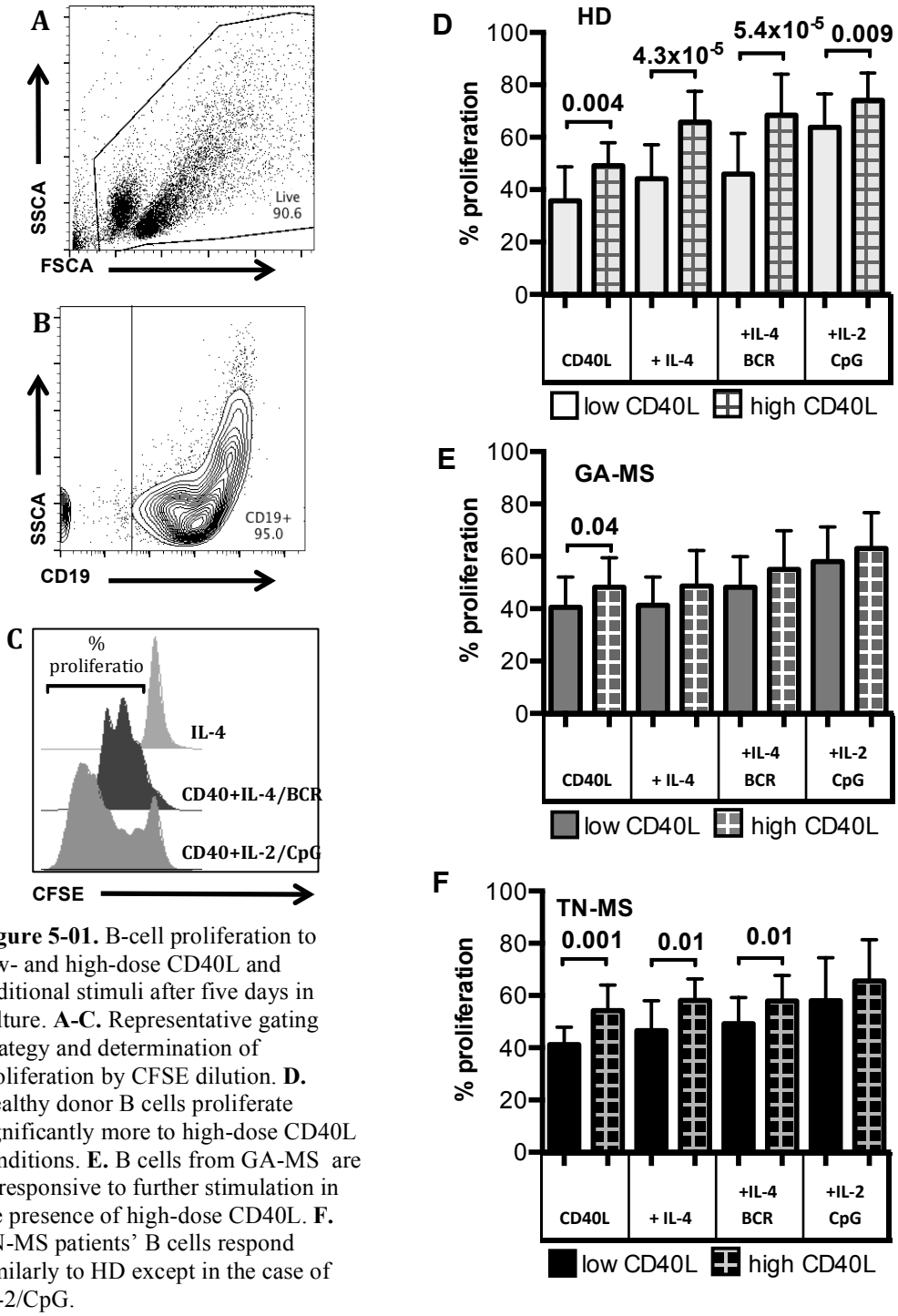


Figure 5-01. B-cell proliferation to low- and high-dose CD40L and additional stimuli after five days in culture. **A-C.** Representative gating strategy and determination of proliferation by CFSE dilution. **D.** Healthy donor B cells proliferate significantly more to high-dose CD40L conditions. **E.** B cells from GA-MS are unresponsive to further stimulation in the presence of high-dose CD40L. **F.** TN-MS patients' B cells respond similarly to HD except in the case of IL-2/CpG.

A comparison of different conditions within the same donor groups showed that in the presence of low-dose CD40L, the addition of IL-2/CpG enhanced proliferation compared to CD40L only for HD ($P=6.1x10^{-7}$), GA-MS ($P=4.2x10^{-4}$) and TN-MS ($P=8.8x10^{-4}$). B cells from TN-MS patients proliferated more to CD40L plus IL-4/BCR than to low- CD40L ($p=0.01$). When HD B cells were exposed to high-dose CD40L, the addition of IL-4 ($P=3.1x10^{-5}$), IL-4/BCR ($P=1.8x10^{-5}$) and IL-2/CpG ($P=1.3x10^{-11}$) enhanced proliferation. However, in GA-MS patients and TN-MS patients, proliferation was only enhanced when high-dose CD40L was combined with IL-2/CpG ($P=2.7x10^{-4}$ and 0.02, respectively).

Table 5-04. GA therapy alters B-cell proliferation

cell type	# points	Low			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	12-17	35.7±13.0§	44.2±12.9§	45.9±15.5§	63.8±12.8§
GA-MS	10-18	40.6±11.5§	41.3±10.8	48.2±11.8	57.9±13.2
TN-MS	14-19	41.2±6.71§	46.6±11.5§	49.3±9.9§	57.9±16.5
		High			
HD	19-25	49.2±8.71§	65.8±11.7*$§	68.4±15.7*$§	74.2±10.3*$§
GA-MS	16-25	48.2±11.3§	48.6±13.6*#	55.0±14.8*	63.0±13.7*
TN-MS	15-19	54.3±9.8§	58.2±8.3#$§	57.9±9.8$§	65.6±15.8$

Table 5-04. Proliferation by B cells after five days of culture. Values are expressed as the percentage of cells that had undergone proliferation ±standard deviation. P-values less than 0.05 were considered statistically significant. *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § low versus high

Further analysis revealed that B cell proliferation from HD, TN-MS and GA-MS patients were not significantly different in any of the culture conditions containing low dose CD40L (Table 5-04). When cultured on high-dose CD40L with IL-4, IL-4/BCR or IL-2/CpG. B cells from GA-MS and TN-MS patients proliferated significantly less compared to HD (Figure 5-02). B cells from GA-MS patients were less responsive to high-dose CD40L plus IL-4 than TN-MS patients (Figure 5-02). These results suggest a defect in the ability of

B cells from MS patients to respond to high-dose CD40L in combination with antigen that is more pronounced in patients on GA therapy.

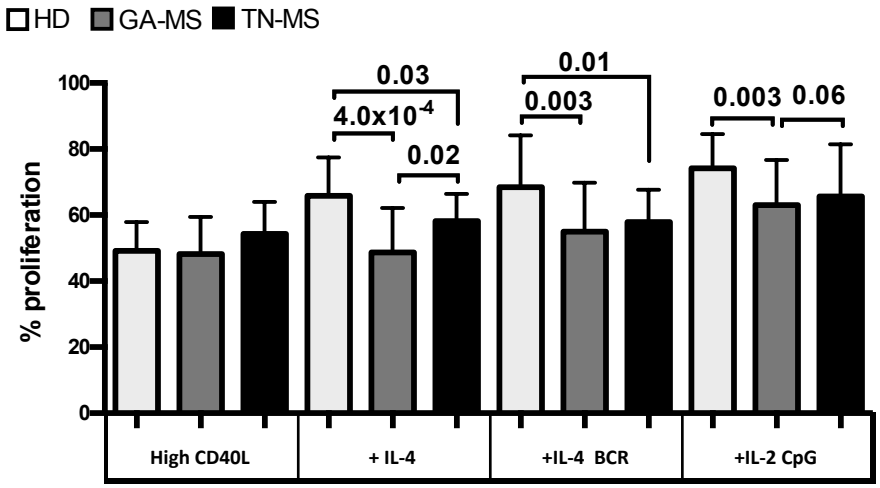


Figure 5-02. Comparison of B-cell proliferation to high-dose CD40L among donor populations. B cells from GA-MS patients are less responsive than HD in all conditions with additional stimuli. GA-MS to CD40L plus IL-4 and IL-2+CpG compared to HD and TN-MS patients. HD B cells proliferated more to CD40L plus IL-4 or IL-4

5.3.4. GA therapy Restores IL-10 Production by B cells

Using total B cells we observed that B cells from MS patients fail to induce expression of IL-10 compared to HD (Figure 5-03, Table 5-05). B cells from GA-MS patients produced significantly more IL-10 compared to TN-MS patients in nearly all high-dose conditions tested on day three (Figure 5-03A) and all conditions on day five (Figure 5-03B). The same observations were also made in low-dose CD40L conditions (Table 5-05). In fact GA therapy restored the ability of B cells to produce IL-10 at an equivalent level as B cells from HD.

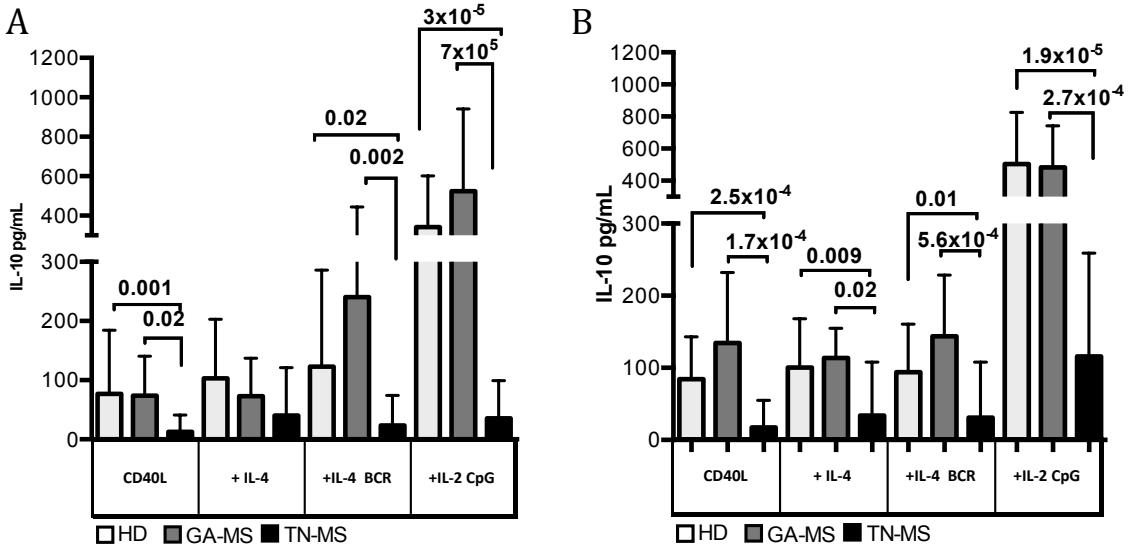


Figure 5-03. Production of IL-10 by B cells on three **(A)** and day five **(B)** of cultures in high-dose CD40L conditions.

Table 5-05. GA therapy restores IL-10 production by B cells

cell type	# points	IL-10 Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	15-18	44.4±96.1§	47.2±111.5	83.8±101.9*$	278.8±244.1§
GA-MS	10-16	61.354.6#	59.0±42.2#	219.0±182.1*#§	521.3±428.5#
TN-MS	12-17	4.4±10.4#	16.0±25.7#	18.7±30.8#$	62.9±73.4#$
		IL-10 High-dose CD40L Day 3			
HD	15-18	76.7±107.6$	103.7±99.8$	122.9±163.2$	338.9±256.1$
GA-MS	10-18	73.9±66.5#§	72.9±64.3	240.3±203.9#	523.4±417.8#
TN-MS	9-13	12.6±28.6#$	40.3±80.6$	23.4±50.8#$	35.6±63.5#$
		IL-10 Low-dose CD40L Day 5			
HD	20-24	111.8±95.4$§	118.3±119.9	113.8±119.9$	476.9±352.1$§
GA-MS	9-16	137.3±212.8#	99.1±52.9	109.9±69.38#§	446.7±264.0#
TN-MS	10-14	16.8±40.1#$	58.3±112.8	12.8±45.4#$	62.5±119.6#$
		IL-10 High-dose CD40L Day 5			
HD	12-25	84.2±58.9$	100.4±67.6$	93.9±66.6$	499.9±321.4$
GA-MS	10-17	134.2±97.6#§	113.7±41.2#	143.7±84.9#	483.3±258.2#
TN-MS	10-14	17.2±37.7#$	33.8±73.9#$	30.9±78.9#$	115.8±143.1#$

.Production of IL-10 by total CD19+ B cells from TN-MS, GA-MS and HD on day three and five of culture as measured by ELISA. Measurements are expressed as pg/mL±standard deviation in pg/mL. P-values less than 0.05 were considered statistically significant. *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day three versus day five.

5.3.5. Glatiramer Acetate Therapy Transiently Modulates IL-6 Production by B cells

Our next goal was to determine whether GA therapy diminished the elevated IL-6 levels secreted by memory and naïve B cells from TN-MS that we previously observed. We found that B cells produced minimal levels of IL-6 when stimulated with CD40L alone (Table 5-06). In response to low-dose CD40L conditions, B cells from TN-MS patients produced significantly more IL-6 compared to HD on day three when stimulated with CD40L plus IL-4 (P=0.02) or IL-4/BCR (P=0.02). Similarly, we found that TN-MS patients produced more IL-6 in high-dose CD40L conditions when stimulated with CD40L plus IL-4, IL-4/BCR, or IL-2 CpG (Figure 5-06). In contrast to our findings with memory and naïve B cells, we identified significantly more IL-6 production by HD B cells on day five to low-dose CD40L combined with IL-4 (p=0.01) or IL-4/BCR (p=0.004).

When analyzing IL-6 production by GA-MS patients, we found that GA-MS patients surprisingly produced more IL-6 on day three in low-dose conditions (p=0.01). Upon more careful examination, a clear trend emerged where a group of GA-MS patients produced high levels of IL-6, that were two standard deviations above the mean level of HD in the same condition, and a group that produced scant amounts of IL-6. In fact, we observed this two-tiered IL-6 grouping of GA-MS patients in all conditions and time points when CD40L was combined with other stimuli. We hypothesized that these two groups of GA-MS patients (IL-6^{high} and IL-6^{low}) were distinct. Despite the heterogeneous nature of human responses, we did not observe this clear separation on a per-donor basis for any of the other cytokines we measured.

Table 5-06. GA does not have a clear impact on IL-6 production in all GA treated patients

cell type	data points	IL-6 Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	16-18	39.7±24.7	253.2±211.9*$	218.6±221.3	256.7±233.6
GA-MS	8-15	69.4±108.47	589.9±758.2*	1032.3±1181.5	494.9±618.2
TN-MS	12-26	35.5±25.6	1528.9±1713.74$	846.9±1152.1	490.5±438.3
		IL-6 High-dose CD40L Day 3			
HD	14-17	40.3±30.9	979.7±859.6$	669.6±1006.9$	372.7±243.3
GA-MS	14-17	38.8±30.6	1353.2±2138.4	1602.2±2047.3	597.2±784.9
TN-MS	17-24	67.1±121.0	2311.8±2012.1$	1894.9±2085.7$	673.9±659.6
		IL-6 Low-dose CD40L Day 5			
HD	15-21	12.9±14.7	806.1±786.9	1133.5±1056.8	208.1±203.9
GA-MS	14-23	27.5±58.7	246.6±508.3	589.5±901.2	322.6±487.9
TN-MS	9-10	46.5±86.2	233.4±273.9	326.6±406.6	289.01±149.1
		IL-6 High-dose CD40L Day 5			
HD	24-30	43.2±54.3	1140.9±1491.5	949.3±1009.1	367.3±389.4
GA-MS	16-26	72.7±78.9	573.5±1226.1	1203.2±2126.1	511.9±600.5
TN-MS	14-18	89.5±97.9	1051.7±2356.4	873.5±2072.6	481.6±752.1

Production of IL-6 by B cells from TN-MS, GA-MS (all donors), and HD on day three and five of culture as measured by ELISA. Measurements are expressed as pg/mL±standard deviation in pg/mL. P-values less than 0.05 were considered statistically significant. *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day three versus day five.

To address this hypothesis, we tested whether clinical parameters between these two patient groups were different. Age (p=0.44) and time since diagnosis (p=0.75) or last relapse (P=0.1) were not significantly different between the IL-6^{high} and IL-6^{low} groups. Those patients that secreted high levels of IL-6 had been on GA therapy for more than 32 months (p=0.008)(Figure 5-04A). We then compared the levels of IL-6 produced by patients on GA therapy for less than or more than 32 months to HD and TN-MS patients.

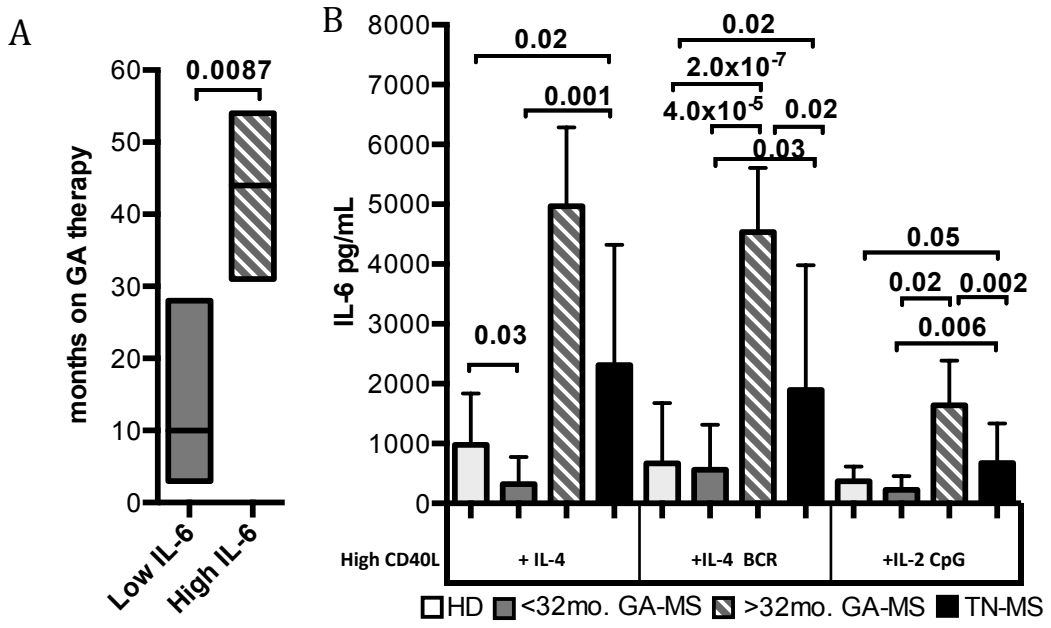


Figure 5-04. Dissection of IL-^{6low} and IL-^{6high} patients from GA-MS patients. **A.** Patients on GA for more than 32 produced high levels of IL-6. **B.** Comparison of patients on GA therapy for more or less than 32 months compared to HD and TN-MS patients revealed a transient diminishment of IL-6 production by B cells.

Separating the patients in this way allowed us to observe that GA therapy diminishes IL-6 production transiently (Figure 5-04B). IL-6 levels produced by B cells from patients that had been on GA therapy for more than (>32mo) or less than (<32mo) 32 months revealed that in response to CD40L plus IL-4, there is diminished IL-6 production by B cells from those GA-MS patients that had been on therapy for less than 32 months compared to TN-MS patients and HD (Figure 5-04B). Since only two of the three >32mo patients had data available in the CD40L plus IL-4 condition, we were not able to establish whether the level of IL-6 produced by B cells from these GA-MS patients was statistically different from other patient groups. In the presence of CD40L plus IL-4/BCR or IL-2/CpG, B cells from GA-MS patients had significantly reduced IL-6 production compared to TN-MS and GA>32mo. In contrast, B

cells from those patients on GA therapy for more than 32 months produced elevated levels of IL-6 compared to B cells from HD, GA<32mo and TN-MS patients.

5.3.6. TNF-α Secretion is Influenced by Glatiramer Acetate Therapy in Some Conditions

In contrast to memory and naïve B cells described in the previous chapter where we observed some donors that produced high levels of TNF-α, we did not detect any high levels of TNF-α in any culture supernatants of total CD19+ B cells from HD, GA-MS or TN-MS patients (Table 5-07). In fact, on day three, HD tended to produce more TNF-α compared to TN-MS patients with low-dose CD40 stimulation. GA-MS patients produced more TNF-α than TN-MS patients when stimulated with IL-4/BCR and low-dose CD40L. In high-dose CD40L cultures, only HD stimulated with IL-4/BCR produced more TNF-α than TN-MS patients. On day five, no significant differences were observed between HD and TN-MS patients. However, in response to low- or high-dose CD40L and IL-4 or high-dose CD40 and IL-2/CpG, GA-MS patients produced significantly more TNF-α compared to TN-MS patients.

We also examined whether additional stimulation modulates cytokine secretion. Additional stimuli did not enhance TNF-α production on day three or day five to low-dose CD40L. However, on day three in response to high-dose CD40L, we observed enhanced TNF-α secretion by HD B cells when combined with IL-4 ($p=0.01$) or IL-4/BCR($p=0.02$). B cells from HD, GA-MS or TN-MS patients did not display this response at day five. HD.

Table 5-07. TNF-α production by B cells is not affected by GA therapy

cell type	# points	TNF-α Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	16-20	12.6±10.4$§	15.1±12.5$§	13.7±8.7$§	17.2±16.4§
GA-MS	10-17	19.0±26.0	17.4±19.5	23.5±25.5#	22.8±26.9§
TN-MS	15-20	5.9±8.3$	7.2±7.5$§	7.1±7.6$#	9.5±7.7§
		TNF-α High-dose CD40L Day 3			
HD	15-19	13.6±10.6§	26.2±15.6$§	35.7±36.6§	19.2±18.1§
GA-MS	11-20	21.1±31.3	27.9±25.7	32.2±33.9§	25.9±27.2
TN-MS	14-19	8.6±10.8§	18.6±32.1$	14.9±18.8	16.7±19.7§
		TNF-α Low-dose CD40L Day 5			
HD	9-11	5.3±4.9§	6.5±6.2§	6.8±6.0§	5.7±6.5§
GA-MS	10-17	6.9±10.4	7.2±4.9#	9.1±12.2	6.1±4.8#§
TN-MS	9-12	4.5±4.1	2.9±1.2#§	8.2±9.0	2.5±1.6#§
		TNF-α High-dose CD40L Day 5			
HD	9-11	3.3±2.3§	6.1±6.99§	10.8±15.8§	6.6±7.0§
GA-MS	13-21	14.8±33.8	10.7±7.5#	11.8±24.1§	12.0±17.1
TN-MS	9-13	2.3±3.5§	4.5±2.5#	7.7±15.4	5.9±5.4§

Table 5-07. Production of TNF-α by B cells from TN-MS, GA-MS and HD on day three and five of culture as measured by ELISA. Measurements are expressed as pg/mL±standard deviation in pg/mL. P-values less than 0.05 were considered statistically significant. *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day three versus day five.

5.3.7. Secretion of LT-α in B cell Cultures

In the mouse model of MS, inhibition of LT-α/β reduced B and T cell CNS infiltration and EAE relapse [257]. In addition, SNPs in the promoter region of LT-α have been associated with MS [258]. Thus we also measured LT-α production in these cultures. On day three, B cells from TN-MS patients produced more LT-α compared to HD when low- or high-dose CD40L was combined with other stimuli (Table 5-08; high-dose CD40L, Figure 5-05). On day three, GA-MS patients produced more LT-α in response to low- and high-dose CD40L compared to HD but significantly less than TN-MS patients when stimulated with high-dose CD40L and IL-4/BCR or IL-2/CpG (Table 5-08; high-dose CD40L, Figure 5-05). In contrast, on day five in low-dose conditions, we observed no differences among the cohorts; however,

HD B cells produced more LT-α compared to GA-MS and TN-MS patients in response to high- dose CD40L and IL-2/CpG.

In low-dose CD40L conditions, LT-α production by TN-MS patients decreased significantly by day five when other stimuli were present. On day three, IL-4/BCR (p=0.002) and IL-2/CpG(p=0.001) enhanced LT-α secretion by HD B cells compared to low-dose CD40L only, but not by GA-MS or TN-MS patients. HD B cells also had increased LT-α production in response to CD40L IL-4 ($P=5.0x10^{-4}$), IL-4/BCR ($P=5.7x10^{-4}$), and IL-2/CpG ($P=1.0x10^{-4}$). Levels of LT-α declined in the cultures by day 5 to less than 150pg/mL in all GA-MS and TN-MS cultures. On day five, further stimulation did not significantly enhance LT-α production in any donor group compared to low-dose CD40L, alone. However, in response to high-dose CD40 stimulation, B cells from GA-MS patients produced more LT-α when stimulated with IL-4 (P=0.03) or IL-2/CpG (P=0.02) compared to CD40L alone. The same was true for B cells from TN-MS patients stimulated with IL-2/CpG (P=0.03).

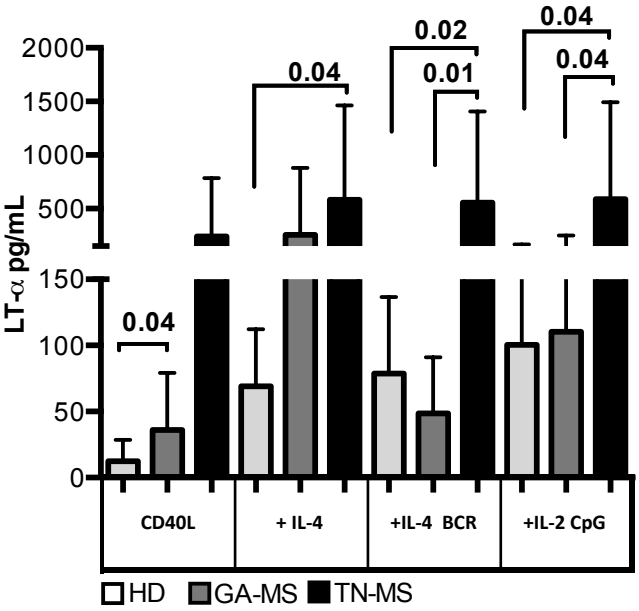


Figure 5-05. Secretion of LT-α by B cells on day three of culture in response to high-dose CD40L. TN-MS patients produced more LT-α than HD or GA-MS patients when additional stimuli were present in the culture.

Table 5-08. LT-α is production by B cells is modulated by GA therapy

cell type	data points				
		LT-α Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	14-18	11.3±11.6*	18.9±12.7$	27.2±16.3$	46.5±37.3$
GA-MS	10-16	123.35±190.4*	516.5±1534.1	419.9±823.3	365.6±873.8
TN-MS	15-20	351.7±976.5	699.1±988.9$§	745.9±1258.4$§	613.4±875.6$§
		LT-α High-dose CD40L Day 3			
HD	13-16	12.6±15.9*	68.8±43.2$	78.4±57.8$	100.3±67.5$
GA-MS	10-17	35.9±43.3	254.4±627.2	48.6±42.6#	110.4±140.4#
TN-MS	15-20	241.4±544.2*	579.8±880.9$	555.8±849.9$#	589.6±904.5$#
		LT-α Low-dose CD40L Day 5			
HD	12-16	144.9±278.7	153.5±226.1	299.8±511.7	194.9±341.7
GA-MS	8-15	280.3±741.5	197.3±359.6	217.0±447.5	128.7±249.9
TN-MS	9-13	39.9±108.9	44.3±75.9§	53.4±82.7§	88.9±101.8§
		LT-α High Day-dose CD40L 5			
HD	12-16	363.2±791.2	336.9±566.2	371.4±602.0	376.8±368.1
GA-MS	9-17	26.7±21.8	86.0±71.6	104.9±160.5	97.4±97.9
TN-MS	9-13	13.7±22.8	77.0±104.1	109.3±193.4	142.9±170.6

Production of LT-α by total CD19+ B cells from TN-MS, GA-MS and HD on day three and five of culture as measured by ELISA. Measurements are expressed as pg/mL±standard deviation in pg/mL. Student's unpaired T-test with Welch's correction was used to compare groups. P-values less than 0.05 were considered statistically significant. *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day 3 versus day 5.

5.3.8. Intracellular detection of IL-6 and IL-10 producing B cells does not explain in vitro alterations in IL-10 production

Recent observations in mice and humans have underscored the importance of IL-10 producing B cells in the regulation of autoimmune diseases. Given the modulation of IL-6 and IL-10 production by B cells from GA-MS patients *in vitro,* we hypothesized that the frequency of B cells that produce IL-10 spontaneously (B10 cells) or that have the capacity to secrete IL-10 upon short-term stimulation ($B10_{PRO}$ cells)[152] would be elevated while those B cells that produce IL-6 would be decreased in GA-MS patients. To test this hypothesis, we stimulated cells HD, GA-MS and TN-MS patients directly *ex vivo* and for 48 hours with BCR and CpG stimulation [160] and measured IL-10 and IL-6 production by flow cytometry using intracellular staining.

Negative controls (FMO, PMA/Iono only and BFA only) showed <1% cytokine positive cells. Both B and non-B cells producing IL-6 and IL-10 were detectable by flow cytometry (Figure 5-06A-D). Cells were gated on CD19 for further analysis. HD had significantly more IL-6+ B cells *ex vivo* (Figure 5-06E) compared to GA-MS and TN-MS patients. However, the intensity of IL-6 staining was similar among the groups (Figure 5-06F). After stimulation, B cells from TN-MS patients had significantly more IL-6+ B cells than GA-MS patients (Figure 5-06G) and the intensity of IL-6 staining was significantly higher in TN-MS than GA-MS patients and HD (Figure 5-06H). This suggests that B cells from TN-MS patients were producing a larger quantity of IL-6, in agreement with what is excreted into the supernatants during *in vitro* stimulation. Of note, the majority of IL-6+ B

cells in all patient groups were naïve B cells (CD27-) that expressed CD24 and high levels of CD1d.

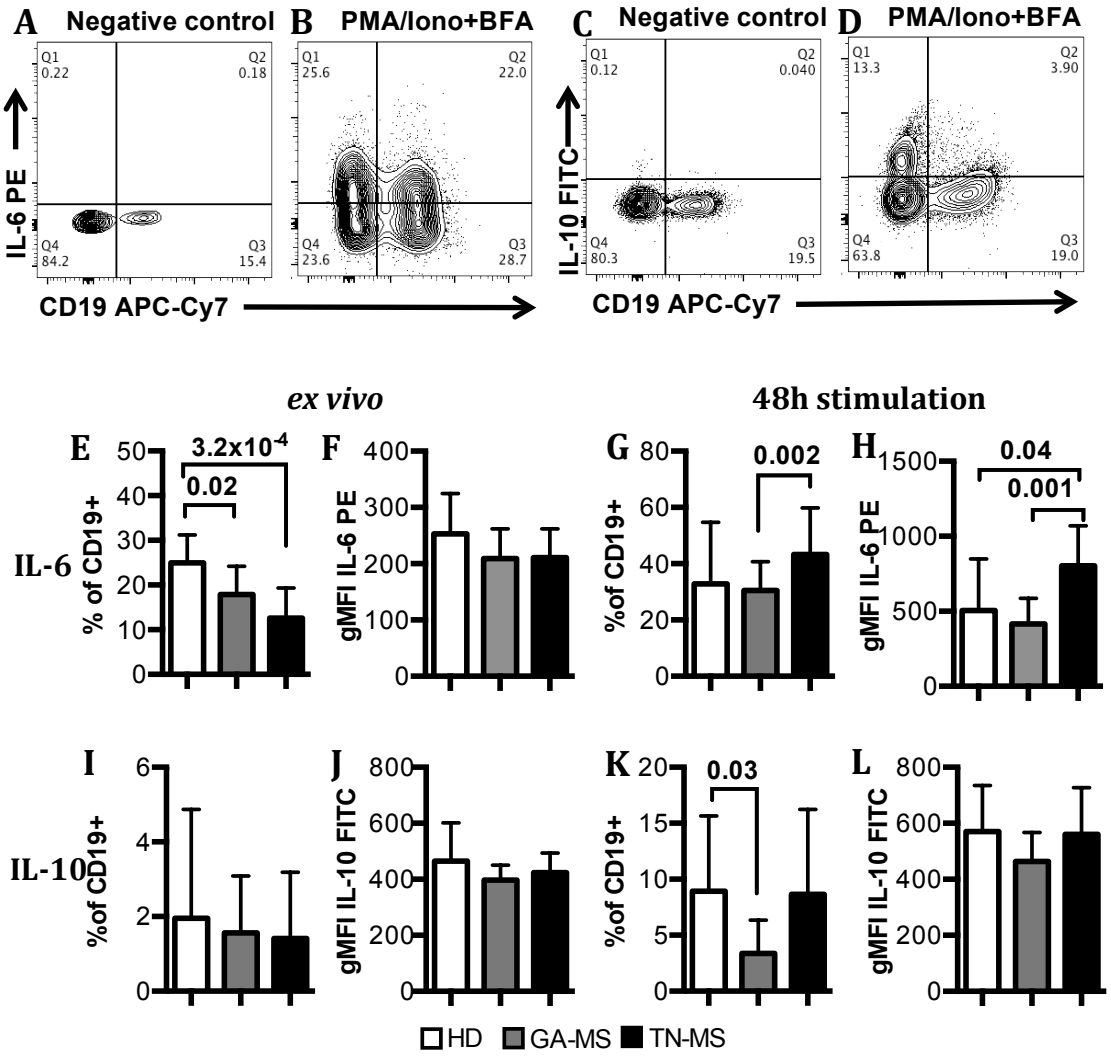


Figure 5-06. Intracellular detection of IL-6 and IL-10 production by B cells. **A-D**. Representative negative controls for IL-6 and IL-10 detection and representative expression, gated on lymphocytes. **E and I.** Percentage of CD19+ B cells positive for IL-6 or IL-10 *ex vivo*. **F and J.** Intensity of IL-6 and IL-10 staining *ex vivo*. **G and K.** Percentage of B cells positive for IL-6 and IL-10 after *in vitro* stimulation. **H and L.** Fluorescence intensity of IL-6 and IL-10 after *in vitro* stimulation. Negative controls are FMOs.

The frequency of IL-10+ B cells and the intensity of IL-10 staining were not significantly different *ex vivo* (Figure 5-06I and J). Surprisingly, after a 48-hour stimulation with BCR/CpG, GA-MS patients had fewer IL-10+ B cells than HD (Figure 5-06K). Despite a diminished frequency of IL-10+ B cells in the GA-MS patients, the intensity of IL-10 staining by these IL-10+ B cells from all 3 cohorts was similar (Figure 5-06L).

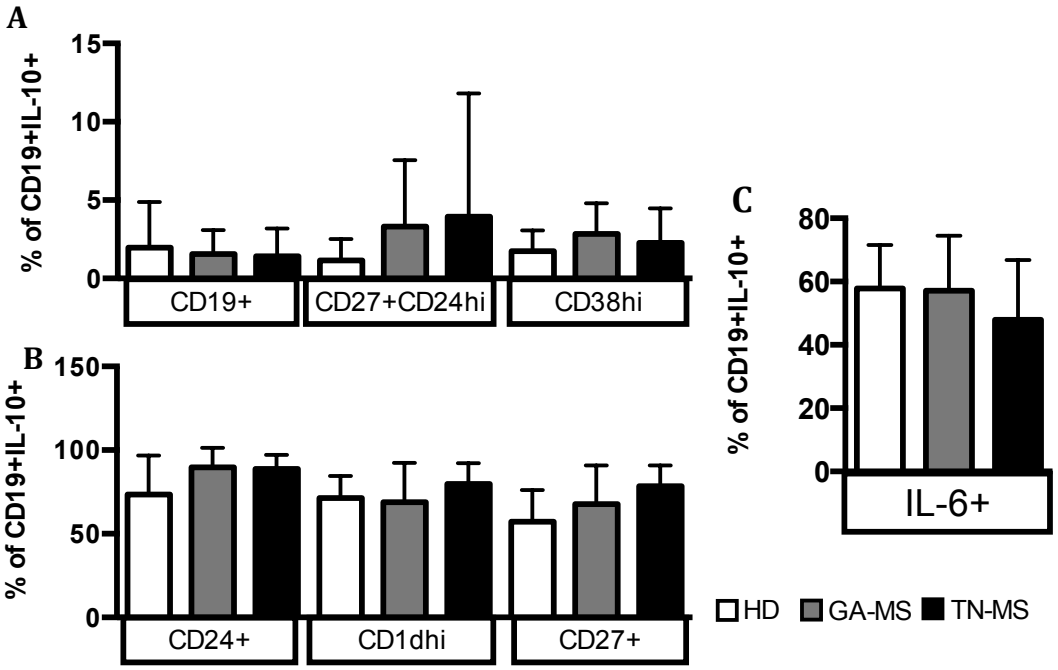


Figure 5-07. Distribution of IL-10+ B cells detected by flow cytometry *ex vivo* **A.** The frequency of IL-10+ B cells was not enriched in CD27+CD24hi or CD38+ populations. **B.** The majority of B cells that were IL-10+ expressed CD24, high levels of CD1d, and CD27. **C.** The majority of B cells that express IL-10 also express IL-6.

Previous reports suggested that IL-10 producing B cells are enriched in memory B cells that express high levels of $CD24^{hi}$ [152]. This subset is associated with less severe SLE [259]. Another study reported that IL-10 production is enriched in transitional $CD24^{hi}CD38^{hi}$ B cells [155, 260], which harbors B cells that have yet to pass peripheral tolerance

checkpoints. We found no such enrichment of IL-10+ B cells in CD27+CD24^{hi} cells (Figure 5-07A). In fact, the proportion of CD24^{hi}CD38^{hi} cells was so low we were unable to use this population to make conclusions. Also, within the total CD38^{hi} B cell pool, that could include a small number of circulating plasmablasts, we did not observe an enrichment of IL-10+ B cells (Figure 5-07A). Our results show that the overwhelming majority of B cells that were IL-10+ *ex vivo* expressed CD24, CD1d^{hi} and CD27 (Figure 5-07B). Surprisingly, B cells that expressed IL-10 co-expressed IL-6. Whether this co-expression of IL-6 and IL-10 can distinguish between B cells capable of acting in a suppressive manner is not known. Finally, it is not clear how the proportion of B cells that make IL-10 *ex vivo* or after short-term culture relates to production of IL-10 *in vitro* after longer culture. This is an important question in determining whether all B cells that are capable of producing IL-10 do so in a manner that is regulatory. We carried out intracellular staining in a separate cohort of GA-MS patients and were unable to make any conclusions about this potential relationship.

5.3.9. Immunoglobulin secretion is enhanced by glatiramer acetate therapy in vitro

In addition to measuring cytokines from B cell cultures, we also assayed for secretion of IgM and IgG (Tables 5-09 and 5-10). The majority of Ig secretion was observed in response to IL-2/CpG. This is not surprising because IL-2 selectively activates memory B cells that express the high-affinity IL-2 receptor and TLR9 stimulation is known to drive Ig production. No significant differences in IgM levels were observed among donor groups in response to low-dose CD40L on day three. However, on day five, B cells from GA-MS patients secreted significantly more IgM than HD and TN-MS patients in all conditions tested. In addition, B

cells from GA-MS patients had enhanced IgM secretion in response to IL-2/CpG compared to CD40L only (Table 5-09).

Table 5-09. GA therapy enhances IgM secretion by B cells *in vitro.*

cell type	data points	IgM Low-dose CD40L Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	8-11	12.2±14.1	7.8±11.9	16.9±28.11	225.4±286.7
GA-MS	10-16	6.5±3.5§	7.1±5.6§	6.3±5.4§	65.9±122.6§
TN-MS	15-20	11.7±12.9	9.3±10.7	7.7±6.2	33.8±74.3
		IgM High-dose CD40L Day 3			
HD	7-9	16.5±29.3	17.4±33.6	13.0±27.9	20.4±16.4
GA-MS	13-20	9.5±6.6	7.7±5.7§	8.5±9.1§	37.1±52.6§
TN-MS	14-19	7.1±5.3	7.9±7.9	12.0±19.4	12.3±30.6
		IgM Low-dose CD40L Day 5			
HD	7-9	5.8±6.0*	7.8±9.3*	5.9±6.1*	12.8±14.4*
GA-MS	9-13	16.8±14.9*#§	28.3±12/9*#§	16.3±15.4*#§	356.8±388.5#*§
TN-MS	9-12	5.5±6.8#	4.5±6.6#	5.1±6.3#	45.7±108.6#
		IgM High-dose CD40L Day 5			
HD	7-9	9.6±6.1	16.0±16.8	12.8±10.5	18.0±16.1*
GA-MS	9-16	17.5±15.2#	23.4±17.1#	20.0±15.8#	373.5±370.2*#§
TN-MS	9-13	5.2±5.9#	5.3±5.9#	6.8±6.7#	11.9±21.7#

Table 5-09. Production of IgM on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in ng/mL ± standard deviation. *P≤ 0.05 *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day 3 versus day 5.

In response to high-dose CD40L on day three, again, no differences were observed among the donor groups except that IL-2/CpG enhanced IgM secretion in the GA-MS group (p=0.04) compared to CD40L only. On day five, HD and GA-MS patients produced similar levels of IgM (Figure 5-08A), except when B cells from GA-MS patients were incubated with IL-2/CpG (p=0.004). In contrast, B cells from TN-MS patients secreted significantly less IgM than GA-MS patients in every condition tested. On day five we also that adding IL-2/CpG significantly increased IgM production by B cells from GA-MS patients (P=0.004).

The levels of IgM in supernatants from GA-MS patients increased significantly on day five in the presence of IL-4 (p=0.01), IL-4/BCR (p=0.01) and IL-2/CpG compared to day three (p=0.006)(Table 5-09).

Table 5-10. GA therapy enhances IgG production *in vitro*

cell type	donors	IgG Low Day 3			
		CD40L	CD40L IL-4	CD40L IL-4 BCR	CD40L IL-2 CpG
HD	14-18	2.6±2.5	2.9±2.7	2.2±1.5§	3.4±4.2*
GA-MS	8-11	6.7±5.5	7.9±8.0	6.7±7.8	28.8±27.2*#§
TN-MS	10-15	4.0±2.5	3.4±0.8	3.0±0.9§	8.4±10.5#
		IgG High Day 3			
HD	13-15	2.9±2.3	2.6±2.9	2.0±1.5§	5.8±9.9*
GA-MS	9-13	5.7±5.2	7.4±7.5	3.2±2.0§	23.5±22.2*#§
TN-MS	9-14	2.7±1.3	3.8±1.7§	5.1±6.0	4.0±2.9#
		IgG Low Day 5			
HD	11-16	32.9±84.6	9.3±11.2	9.9±11.1§	103.2±301.2*
GA-MS	6-10	11.0±8.7#	22.0±22.0	53.3±136.4#	209.6±135.3*#§
TN-MS	9-12	2.3±2.8#	2.5±2.2	1.8±1.5#§	2.0±1.7#
		IgG High Day 5			
HD	14-17	7.3±8.4	8.4±10.9	6.7±7.4§	14.4±16.1
GA-MS	7-11	12.8±16.6	23.4±21.6	8.7±6.7§	242.6±162.2§
TN-MS	9-13	2.0±16.6	1.4±1.0§	5.8±15.7	3.8±4.4

Table 5-10. Production of IgG on days 3 and 5 of culture. Levels of cytokines in cultures were quantified by ELISA and are described as the mean in pg/mL ± standard deviation. *P≤ 0.05 *HD versus GA-MS, #GA-MS versus TN-MS, $HD versus TN-MS, § day 3 versus day 5.

B cells from GA-MS patients produced significantly more IgG in response to low-dose CD40L alone on day three than HD (p=0.04). The addition of IL-2/CpG enhanced IgG GA-MS patients to a greater level compared to HD (p=0.01) and TN-MS patients (p=0.04) when stimulated with IL-2/CpG (Table 5-10). On day five, in low-dose CD40L conditions, GA-MS patients' B cells produced more IgG in response to CD40L compared to TN-MS patients (p=0.002), HD when stimulated with IL-4/BCR (p=0.002), and TN-MS patients

when incubated with IL-2/CpG (p=0.002). On both days, IL-2/CpG enhanced IgG secretion by GA-MS patients compared to CD40L only (day three p=0.03; day five p=0.004). HD accumulated more IgG in cultures by day five in low conditions in culture with IL-4/BCR (p=0.01) whereas TN-MS patients had decreased IgG (p=0.01) compared to day three. IgG production by GA-MS patients was greatly enhanced by IL-2/CpG on day five compared to day three (p=0.006)(Table 5-10).

In high-dose CD40L conditions on day three, B cells from GA-MS patients produced significantly more IgG than HD (p=0.02) and TN-MS patients (p=0.01) in response to high-dose CD40L plus IL-2/CpG (Table 5-10). On day five, GA-MS patients produced more IgG than HD in every condition except IL-4/BCR and more than TN-MS patients in response to IL-4 and IL-2/CpG (Figure 5-08B). Similar to low-dose conditions, the addition of IL-2/CpG enhanced IgG secretion by B cells from GA-MS patients on day three (p=0.02) and day five (p=0.002). Interestingly, HD produced more IgG in response to CD40L plus IL-2/CpG compared to TN-MS patients (p=0.01). In fact, we observed very little IgG production by TN-MS patients in any condition tested with a maximum of 57.8 ng/mL from one patient on day five when stimulated with high-dose CD40L plus IL-4/BCR. This is notably not the condition that produced maximal IgG from HD or GA-MS patients

IgG levels from HD and GA-MS patients were higher in cultures on day five than day three when B cells were stimulated with high-dose CD40L plus IL-4/BCR. Levels of IgG were increased on day five in response to IL-2/CpG in cultures with GA-MS B cells. In contrast, IgG levels were lower on day five than on day three when TN-MS patients' B cells were stimulated with high-dose CD40L plus IL-4 (P=0.002). It is not clear whether the

decrease in IgG levels in cultures with TN-MS patients' are a result of enhanced degradation, or perhaps express more of the inhibitory FCγRIIb [259] that binds free IgG. We did sample approximately half of the culture volume on day three and replace it with fresh media. This is likely why we observed less Ig on day five. We did not have sufficient culture supernatant to determine whether the isotopes from IgG subclasses were altered or if the antibodies produced were GA-specific.

5.3.10. Serum levels of Immunoglobulin M and G are unchanged by glatiramer acetate therapy

Given the enhanced *in vitro* production of IgG and IgM in patients on GA therapy, we tested serum levels of IgG and IgM by ELISA. We found that HD, GA-MS and TN-MS patients had similar levels of IgM and IgG (Figure 5-08C and D). This suggests an alteration in activation status of GA-MS patients rather than a systemic increase in Ig *in vivo.*

5.3.11. Glatiramer acetate does not impact serum Th1 or Th17 cytokine levels

We assessed whether GA modulated the cytokine levels in the serum of patients on therapy compared to TN-MS patients and HD. A total of fifteen factors and cytokines were quantified (Figure 5-09A-O). We found no significant difference in any of the parameters measured among the donor groups. Others have reported that levels of Th2 cytokines, such as IL-5 and IL-13, are elevated in the serum of GA-MS patients and indicate a clinical response [261]. We did not extensively evaluate the patients in this study for expression of Th2 cytokines.

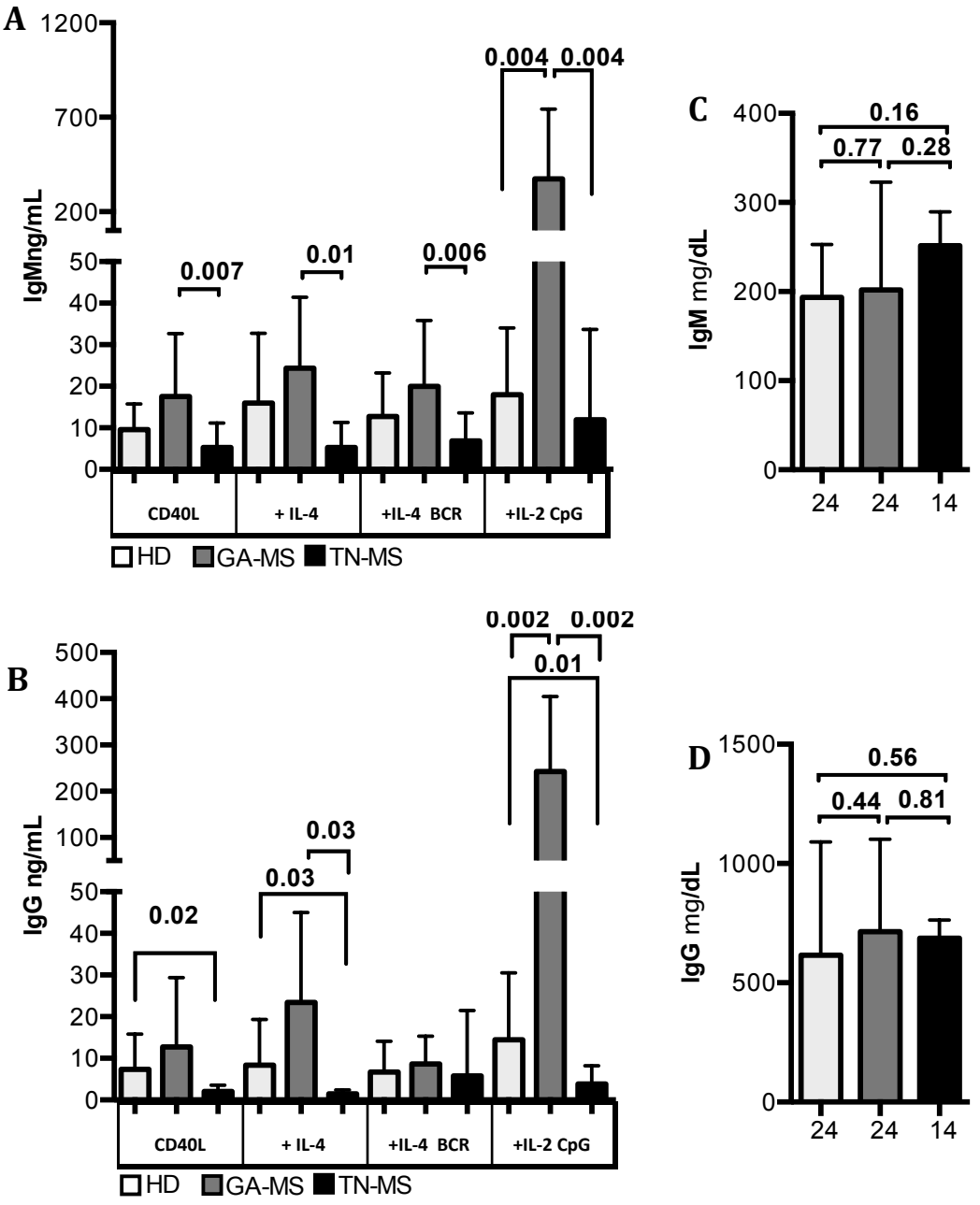


Figure 5-08. IgM **(A)** and IgG **(B)** levels from B cell cultures on day five in response to high-dose CD40L. Serum levels of IgM **(C)** and IgG **(D)** from TN- and GA-MS patients compared to HD; number of donors tested are indicated.

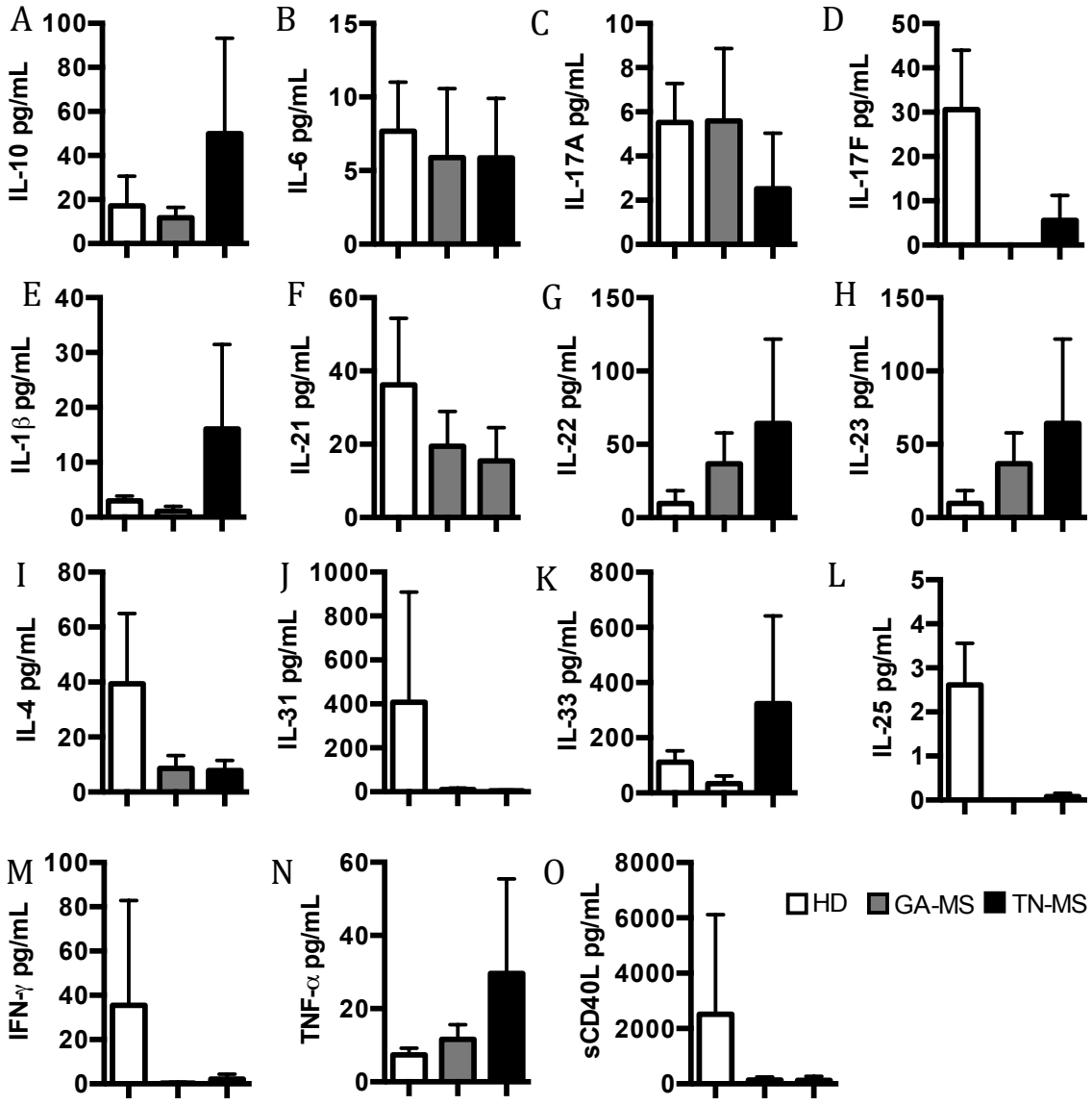


Figure 5-09 . Concentration of serum cytokines and soluble CD40L (sCD40L) from HD, GA-MS and TN-MS patients measured by Bioplex. No significant differences were observed among donor groups for any of the parameters measured.

5.3.12. *Glatiramer acetate alters the phenotypic composition of B cells*

B cells can be subdivided into subsets, each of which plays a unique role in the immune system based on the expression of specific surface molecules [262]. To determine whether GA therapy induces a remodeling of the B cell compartment that may contribute to differential cytokine secretion, we carried out immunophenotyping of B cells by flow cytometry (Figure 5-10).

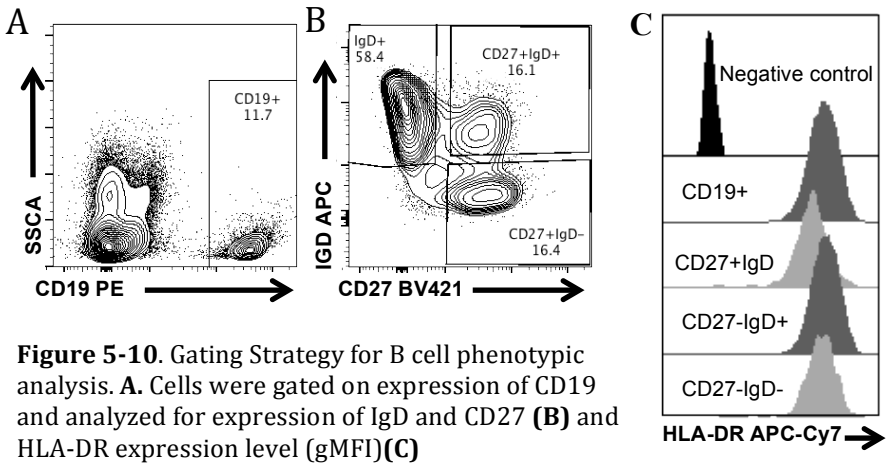


Figure 5-10. Gating Strategy for B cell phenotypic analysis. **A.** Cells were gated on expression of CD19 and analyzed for expression of IgD and CD27 **(B)** and HLA-DR expression level (gMFI)**(C)**

The percentage of total CD19+ B cells was decreased in GA-MS compared to HD and TN-MS patients (Figure 5-11A). While the proportions of total naïve and memory B cells was similar (Figure 5-11B and C) among the groups, the frequency of IgD+CD19+ B cells was elevated in GA-MS patients (Figure 5-11D). In addition, the percentage of $CD27^{bright}$ [259] plasamablasts was decreased in GA-MS patients (Figure 5-11E). Class-switched (CD27+IgD-) and non-class switched (CD27+IgD+) memory B cells were diminished in GA-MS patients compared to HD and TN-MS patients and a concomitant increase in CD27-IgD+ naïve B cells was observed (Figures 5-11F-H). We also measured

levels of HLA-DR expression and found that the expression on total CD19+, CD27- naïve B cells and CD27+ memory B cells was similar among the groups (data not shown). However, we found that class-switched memory B cells from GA-MS patients had higher levels of HLA-DR compared to TN-MS patients (Figure 5-11I). Unswitched memory B cells from HD had higher levels of HLA-DR compared to TN-MS patients but expression on CD27-IgD+ naïve B cells was similar (Figure 5-11J and K). This data suggests that patients on GA therapy have a shift in the B cell pool toward a naïve phenotype and diminished expression of HLA-DR that might limit APC capacity.

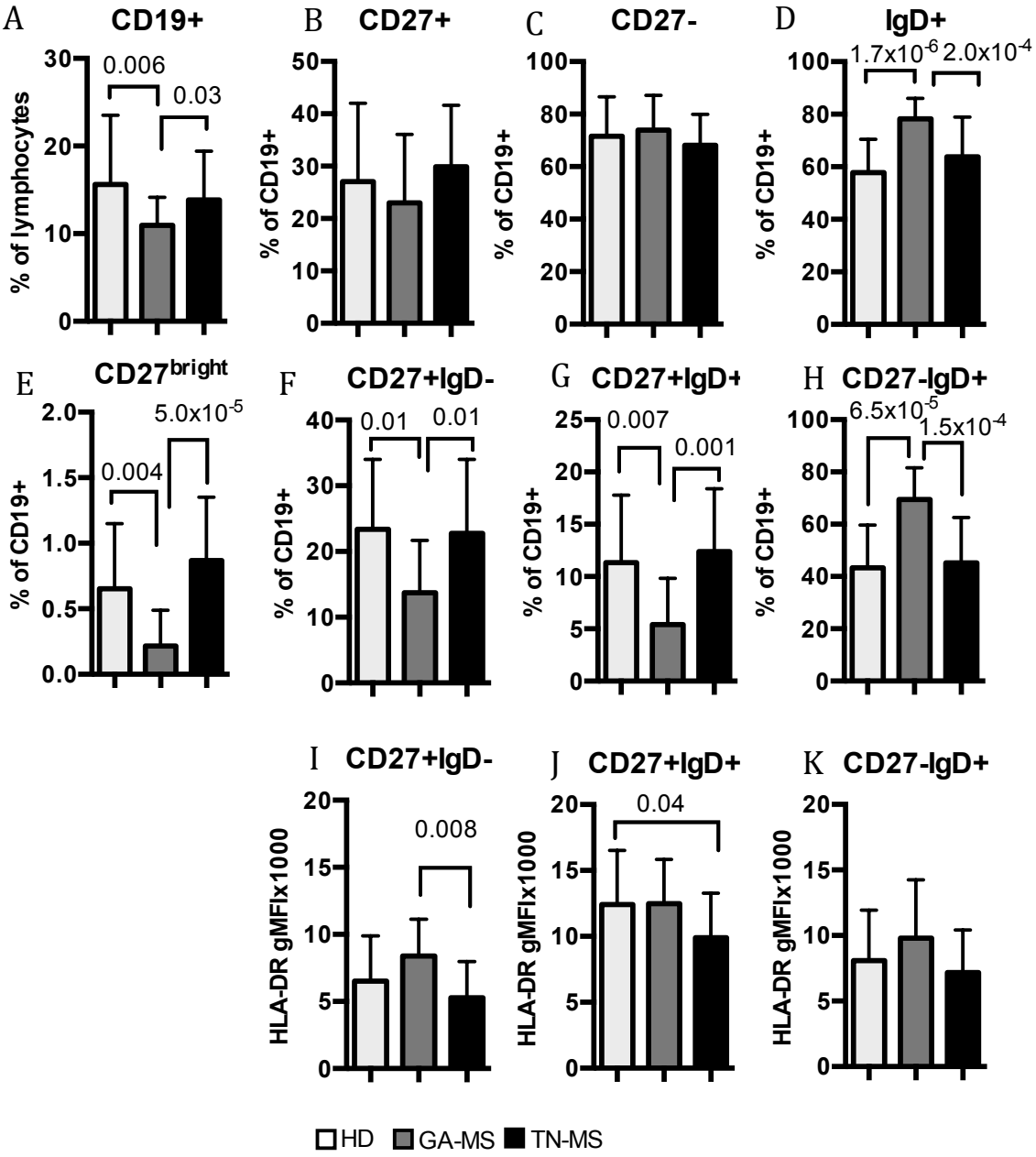


Figure 5-11. Phenotypic analysis of B cells from HD, GA-MS and TN-MS patients. **A.** CD19+ B cells. **B-E.** Memory, naïve, IgD+ and plasmablasts, gated on B cells**. F-H**. Distribution of CD27 and IgD, gated on B cells. **I-K.** HLA-DR expression level on groups in F-H.

CHAPTER SIX
B-T cell interactions in Multiple Sclerosis

6.1 Introduction

Previously, our laboratory demonstrated that memory B cells from a subset of MS patients supported proliferation and/or IFN-γ production by autologous T cells in response to MOG or MBP (neuro-antigens). However IFN-γ production and proliferation were not correlated. Many observations have been made in murine models suggesting a role for B cells in supporting or directing T cell responses to infection and in autoimmune responses [263]. These observations have not been thoroughly investigated in humans and the majority of research has supported a role for B cells in restraining T cell responses. In the context of MS and EAE, studies have pointed towards a role of B cells in Th17 responses. Indeed, multiple reports in both mice [195] and humans [243] have found a attenuation of IL-17-producing T cells when B cells are depleted. Glatiramer acetate has been reported to disrupt interactions between T cells and APC by promiscuously binding to HLA-DR. Given these observations, we hypothesized that B cells from MS patients could support neuroantigen specific Th1 and Th17 responses to neuroantigens and that GA would diminish these T cell responses.

To address this hypothesis, we characterized the phenotypic distribution and frequency of neuro-antigen specific T cells to determine whether MS patients harbored altered T cell subsets that might impact their proliferative capacity. Next, we compared antigen-specific T cell responses in autologous B-T co-cultures to determine if the frequency of T cells capable of responding in an antigen-specific manner was similar. Given our more

recent observations regarding IL-6, which can have a dramatic impact on T cell polarization and activation status, we also undertook studies to determine whether blocking IL-6 would inhibit the B cell driven neuro-antigen response by T cells. To this end, we evaluated the expression of membrane bound IL-6Rα (CD126), it's co-receptor gp130 (CD130) and IL-10R1 (CD210) to determine if T cells from MS patients had different expression levels compared to controls. Furthermore, it is not known whether the aberrant B cell responses were due to a heightened ability of T cells from MS patients to respond in these culture conditions. To address this, we conducted experiments using allogeneic B-T cell mixtures from MS patients and HD.

6.2 Characterization of T cells from MS patients

6.2.1. Phenotypic analysis of T cell subsets showed little difference between HD and MS patients

T cells can be subdivided into central memory (TCM) and effector memory (TEM) subsets discriminated by the expression of CCR7 and CD45RO [264](Figure 6-01E). We stained PBMC from HD, TN-MS and GA-MS patients for T cell markers and analyzed the CD3+ T cell populations for TEM, TCM based on the established strategy described by Mahnke *et al.* [265]. We also tested whether GA therapy impacted the phenotypic distribution of T cells in ten GA-MS patients. In addition to identifying TEM and TCM by this approach, we interrogated the expression of CXCR3 on CD45RO+ cells. CXCR3+CD45RO+ TEM cells are of particular interest in MS as they can migrate across an

in vitro blood brain barrier model [266] and IFN-γ expressing Th1 TEM are highly enriched in the CSF of MS patients (unpublished observations and [67]).

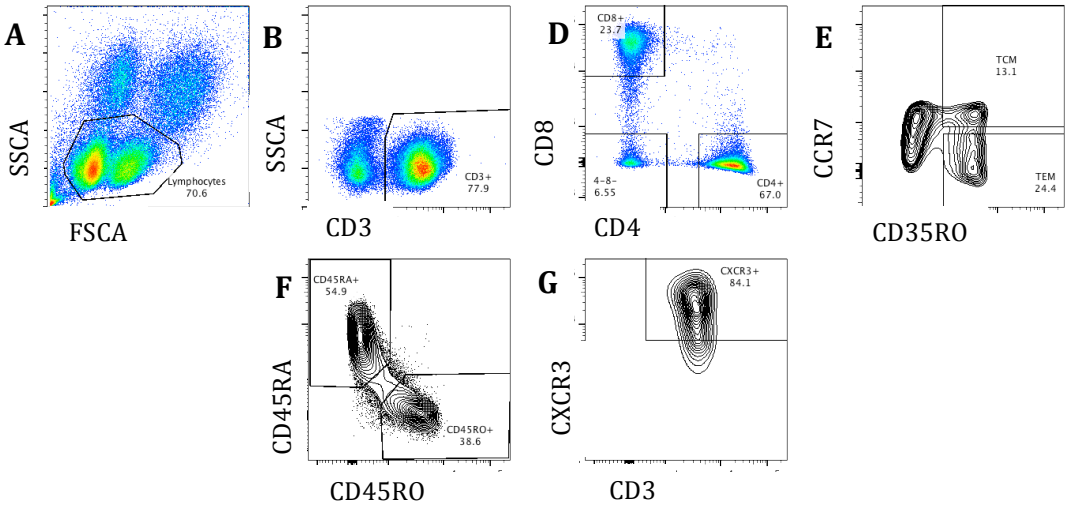


Figure 6-01. Gating Strategy for identifying differences in T cell populations. A. Lymphocytes were gated on CD3 positivity (**B**), and divided into CD4+ and CD8+ populations for further analysis. CD4+ and CD8+ populations were interrogated for expression of CD45RO and CCR7 to delineate TEM and TCM populations (**D**), CD45RA versus CD45RO expression (**E**). CD45RO+ T cells were characterized for the expression of CXCR3 (**F**).

We sampled 16-37 HD, 18-33 TN-MS patients and 10 GA-MS patients for the expression of T cell markers. We detected a significantly elevated frequency of CD3+ T cells in TN-MS and GA-MS patients compared to HD (Figure 6-02A). CD4+ T cells were also elevated in TN-MS patients compared to HD; however the frequency of CD8+ T cells was similar between all donor groups (Figure 6-02.B and C). The frequency of CD8+ T cell TEM was significantly higher in HD and TN-MS patients compared to GA-MS patients (Figure 6-02D). CD8+ T cells expressing CD45RA were significantly elevated in GA-MS patients compared to HD and TN-MS patients (Figure 6-02E). Correspondingly, the percentage of

CD8+ CD45RO+ was decreased in GA-MS patients compared to the other donor groups (Figure 6-02E).

Within the CD4+ T cell population, there was an increased fraction of TCM in TN-MS patients compared to HD (Figure 6-02G). Four of the 10 GA-MS patients had lower frequencies of TCM compared to the other GA-MS patients, but we could identify no corollary for this in patient demographics for age, time since diagnosis, sex, time since last relapse or time on GA therapy (data not shown). The frequency of TEM was elevated in HD and TN-MS patients compared to GA-MS patients (Figure 6-02H). We examined the expression of CXCR3 on CD45RO+ (memory) CD4+ and CD8+ T cells. We identified an elevated percentage of both CD4+ and CD8+ memory T cells in GA-MS and TN-MS patients compared to HD (Figure 6-02 I and J).

6.2.2. *Detection of cytokine secretion ex vivo*

We wanted to measure IFN-γ and IL-17 as readouts for pro-inflammatory responses and IL-10 as a regulatory response to neuro-antigens in B-T cultures. However, differences in the frequency of cells capable of spontaneously secreting these cytokines in different donor populations might impact interpretation of the data. To address this issue, we examined the spontaneous cytokine secretion of IFN-γ and IL-17A by T cells after a four-hour incubation with PMA/ionomycin in the presence of Brefeldin A. HD had a higher frequency of CD4+IFNγ+ and IL-10+ T cells compared to TN-MS patients (Figure 6-03A and B). We found a similar frequency of IL-17A+ and IFN-γ+IL17A+ CD4+ T cells in HD and MS

patients (Figure 6-02C and D). Similarly, HD and TN-MS patients harbored similar numbers of CD8+IFN-γ+ T cells (Figure 6-03E).

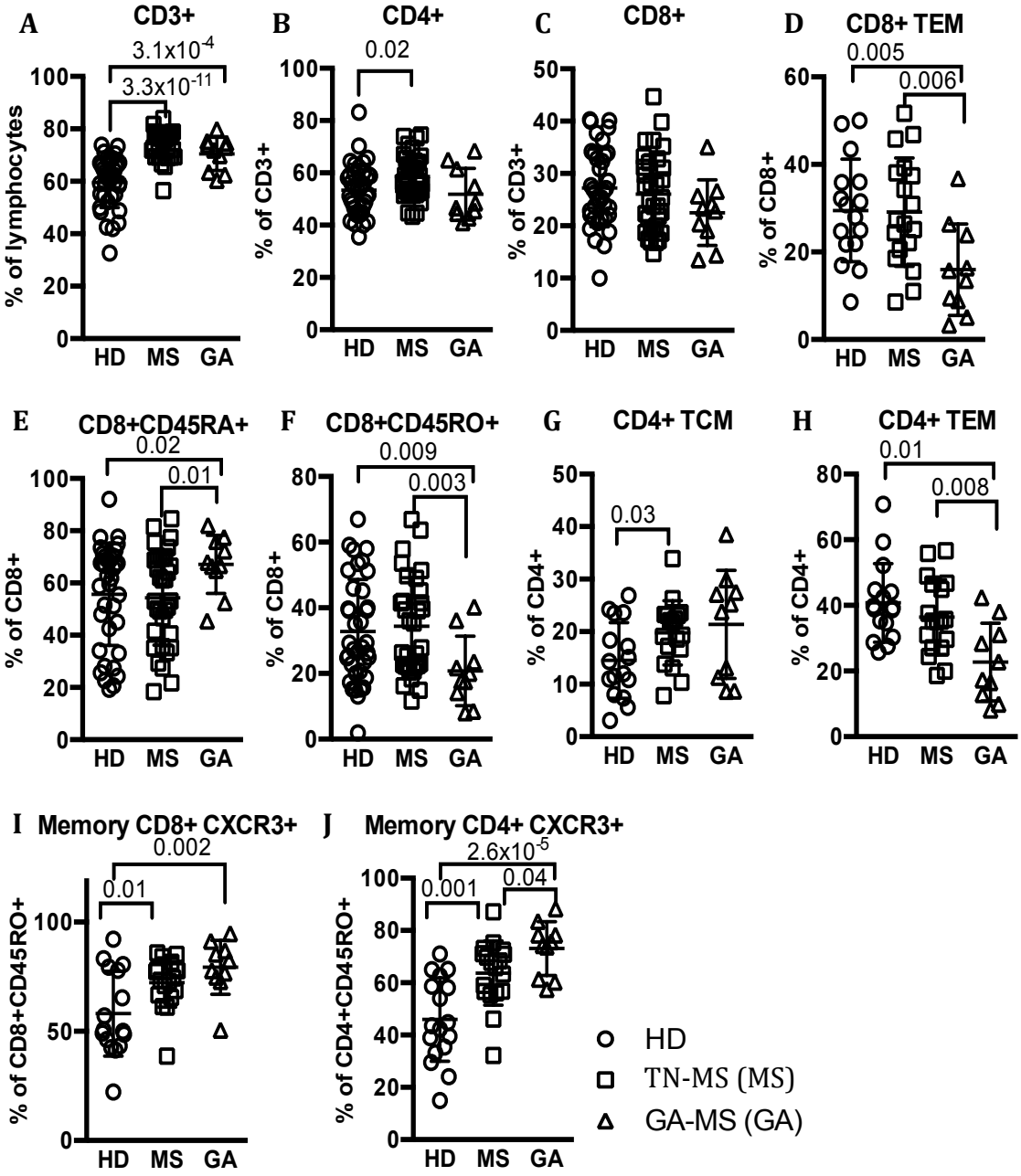


Figure 6-02. The phenotypic distribution of T cells in HD (open circles), MS (open squares) and GA-MS patients (open triangles) was assessed by flow cytometry. The frequency of CD3+ (**A**), CD4+ (**B**), and CD8+ (**C**) T cells. Sub-populations of T cells were identified based on the gating strategy in Figure 6-01. **D-H.** Subpopulations of CD45RO, TCM or TEM within CD4 or CD8+ T cells. **I-J.** Expression of CXCR3 on CD45RO+ T cells. All populations described were compared, but only those with significant differences for T cell subpopulations are represented here, for all other comparisons donor groups were similar.

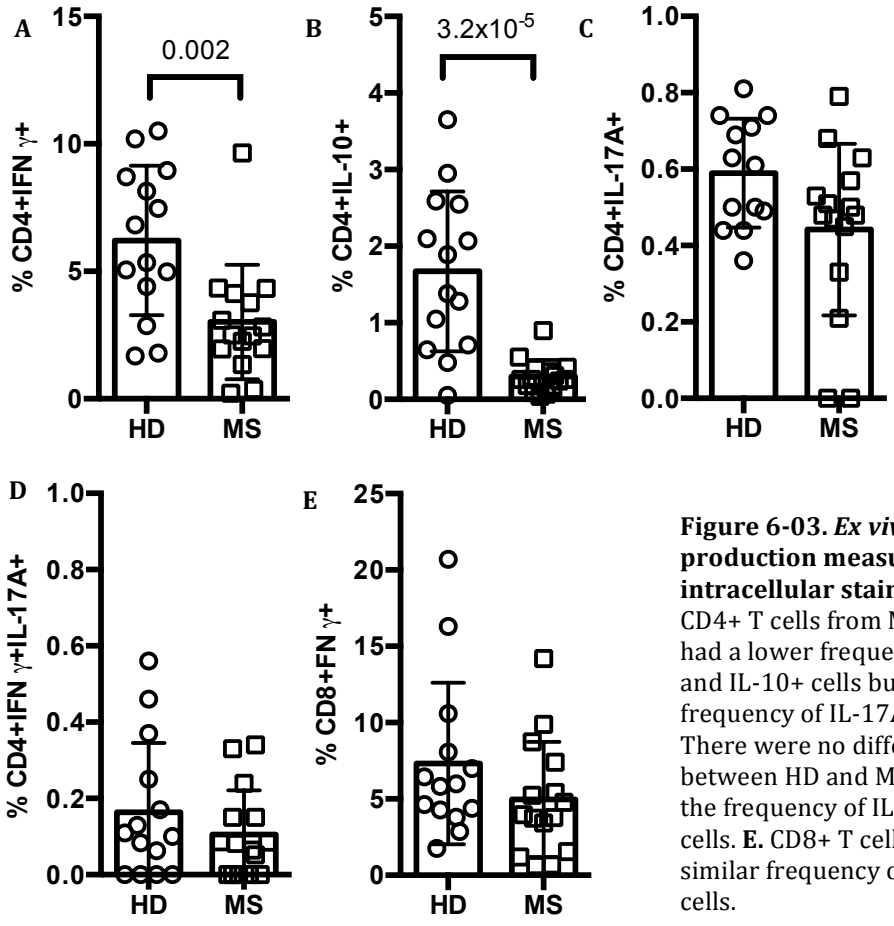


Figure 6-03. *Ex vivo* cytokine production measured by intracellular staining. A-C. CD4+ T cells from MS patients had a lower frequency of IFN-g+ and IL-10+ cells but a similar frequency of IL-17A+ cells. **D**. There were no differences between HD and MS patients in the frequency of IL-17A+IFNg+ cells. **E.** CD8+ T cells had a similar frequency of IFN-g+ cells.

6.2.3. The frequency of antigen-specific T cells is largely similar between HD and MS patients

In order to understand responses in B-T co-cultures, we carried out ELISpot for IFN-γ and IL-17A from 10 TN-MS patients and 10 HD. Representative results for one TN-MS patient and one HD in response to no antigen (negative control), foreign antigens (tetanus toxoid, mumps virus, respiratory syncytial virus and influenza A), MOG 35-55 peptide, MBP, MOG, and GA is shown in Figure 6-04A. We observed a small frequency of cells in both HD and

MS patients that spontaneously secreted IFN-γ or IL-17A in the absence of antigenic stimulation (Figure 6-04A and D). We observed no significant differences between the number or total area covered by IL-17A spots between HD and TN-MS patients to any antigen (Figure 6-02B and C). We observed an elevated number of IFN-γ spots in TN-MS patients in response to MOG but the area covered by spots was similar (Figure 6-04D and E). This suggests that during a quiescent disease state, the frequency of peripheral blood T cells that are specific to neuro-antigens are relatively similar in HD and TN-MS patients. Of note, we only measured IFN-γ and IL17-secreting T cells in this assay. MOG-specific T cells in HD and MS patients also secrete IL-10, IL-4, and TGFβ [267]. It is likely that the modest, but significant elevation in MOG-specific T cells we observed here is a result of an inflammatory-biased approach.

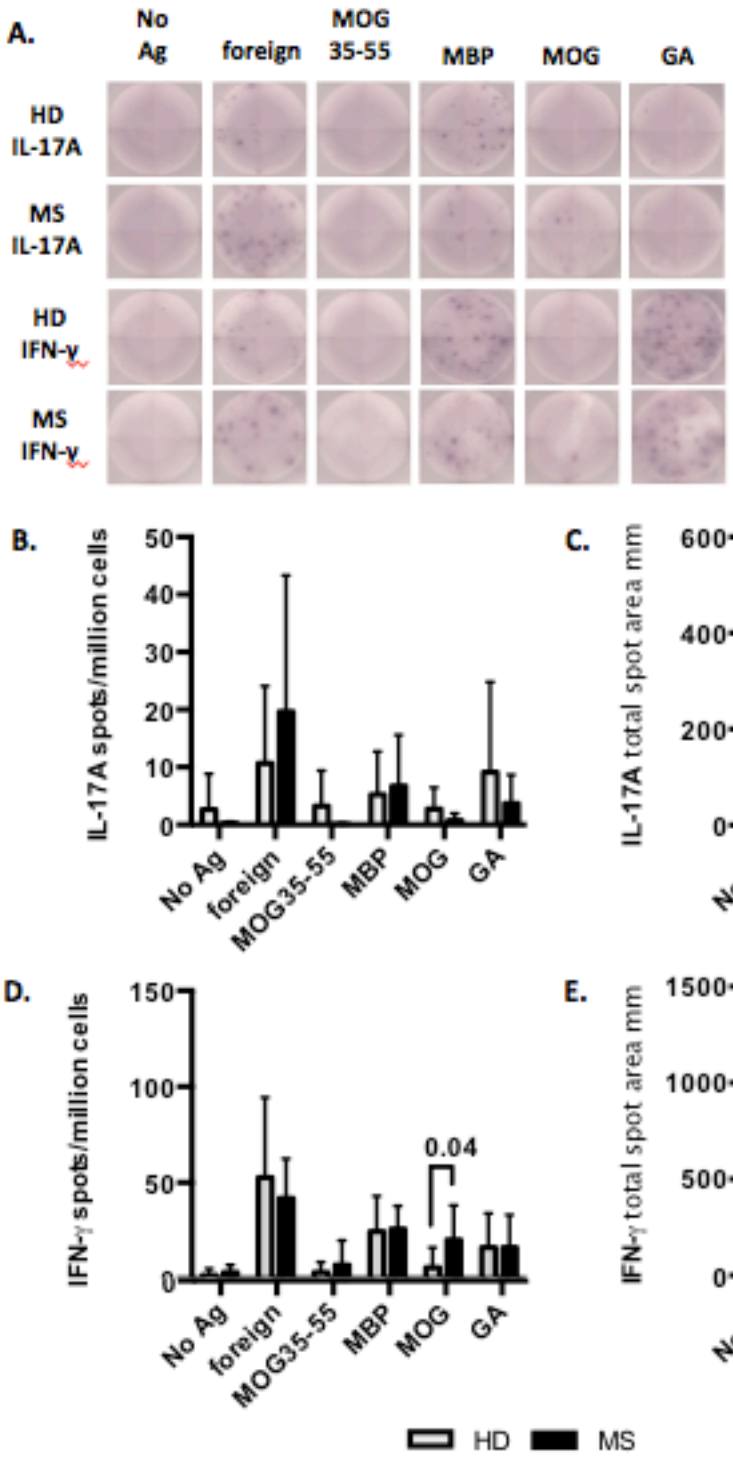


Figure 6-04. ELISpot for IL-17A and IFN-γ showed similar antigen-specific T cell frequency between HD and MS and elevated MOG-specific IFN-γ secreting T cells in MS patients. **A.** Representative ELISpot results from one HD and one MS patient (MS17). Frequency (**B**) and area (**C**) of IL-17-A secreting cells. Frequency (**D**) and area (**E**) of IFN-γ secreting cells.

6.3. Co-culture of T cells with autologous naïve or memory B cells

6.3.1 Proliferation in B-T cultures

To determine whether memory or naïve B cells can influence Th1 or Th17 cells in an antigen specific manner, we incubated total T cells with naïve or memory B cells from the same donor in the presence or absence of antigen. CD4+ and CD8+ T cells were gated according to the strategy in Figure 6-01 after seven days in culture. We extended the culture time from five to seven days to allow for T-cell proliferation before stimulation with PMA/ionomycin for the detection of intracellular cytokines. Proliferation was assessed by CFSE dilution (Figure 6-05A). We identified the response to individual and pooled foreign antigens were approximately additive and the small amount of synergistic activity was similar between HD and TN-MS patients (Figure 6-05B). Therefore, foreign antigens were pooled to conserve the limited amount of memory B cells we could obtain from a PBMC pool from a single donor. In addition, we tested whether foreign antigens that may contain TLR agonists would stimulate B-cell activation by measuring proliferation in the presence of 10ng/mL IL-4 (negative control). We found minimal proliferation to any of the foreign antigens (Figure 6-05A), normalized to negative control (IL-4 only) after five days in culture and no significant differences between HD and TN-MS patients (Figure 6-05C).

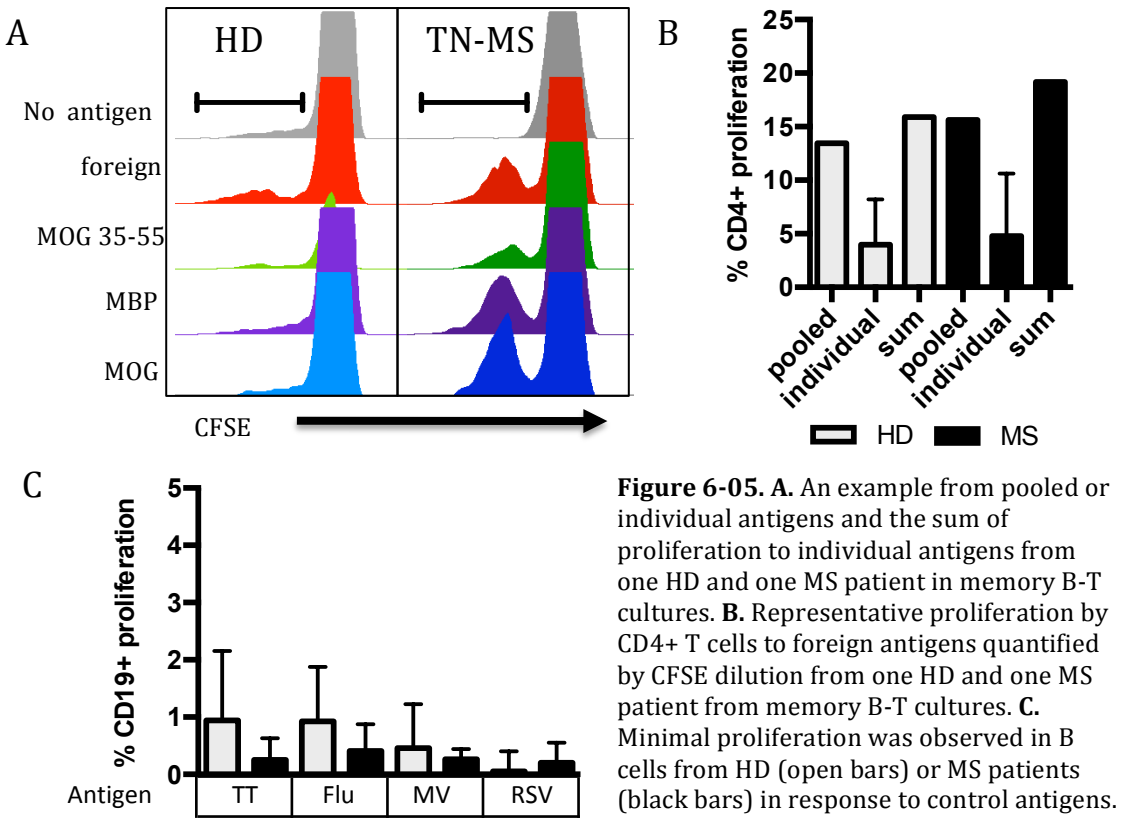


Figure 6-05. A. An example from pooled or individual antigens and the sum of proliferation to individual antigens from one HD and one MS patient in memory B-T cultures. **B.** Representative proliferation by CD4+ T cells to foreign antigens quantified by CFSE dilution from one HD and one MS patient from memory B-T cultures. **C.** Minimal proliferation was observed in B cells from HD (open bars) or MS patients (black bars) in response to control antigens.

We observed no differences between memory B-T cultures without antigen in regard to cell frequency, background proliferation, or cytokine production. Very little proliferation was observed in response to MOG 35-55 in B-T or PBMC cultures (Figure 6-06). While the absence of a response when using B cells as an APC that may not recognize a peptide fragment was predicted, the lack of a response by PBMCs was somewhat puzzling since the APCs in the PBMC pool should most certainly recognize a peptide fragment. We observed IFN-γ and IL-17 responses by ELISpot to MOG 35-55 and the same lot has been used to induce EAE in our laboratory. However, in ELISpot assays, the concentration of MOG 35-55

was much higher, suggesting that responses to this sequence requires a higher concentration compared to recombinant MOG 1-125.

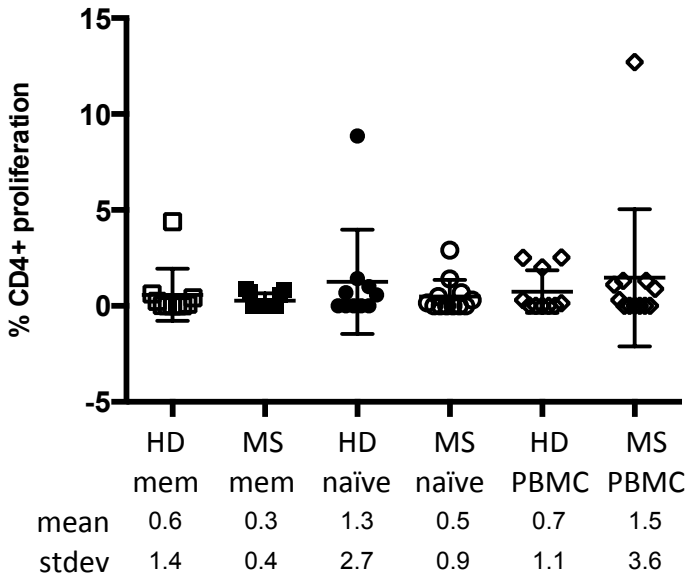


Figure 6-06. Proliferation to MOG 35-55 peptide. Neither memory (mem) or naïve B cells from HD or MS patients supported proliferation by CD4+ T cells to MOG 35-55. Similarly, PBMC cultures from MS patients and HD had minimal proliferation to MOG 35-55

In response to foreign antigens, memory B cells from TN-MS patients displayed similar proliferation compared to HD. In response to neuro-antigens, memory B cells from MS patients supported significantly more CD4+ T cell proliferation compared to HD (Figure 6-07A). In addition the number of responders was significantly elevated in the TN-MS cohort. However, memory B cells supported similar levels of CD8+ T cell proliferation to foreign and neuro-antigens (Figure 6-07B). Of note, we did not find a correlation between proliferation to MOG and the frequency of IFN-γ secreting MOG-specific T cells identified by ELISpot (section 6.2.3).

Naive B cells from TN-MS patients induced more CD4+ proliferation and number of donors that responded by proliferating to foreign antigens compared to HD (Figure 6-07C). Naïve B cells from TN-MS patients induced similar proliferative responses in CD4+ T cells

to neuro-antigens (p=0.08) but the number of donors that responded was significantly increased compared to HD (Figure 6-07C). Surprisingly, naïve B cells from TN-MS patients induced less proliferation by CD8+ T cells in conditions containing both foreign and neuro-antigens, with fewer donors responding to neuro-antigens compared to HD (Figure 6-07D). Of note, while we documented an elevated frequency of MOG-specific IFN-γ+ T cells in TN-MS patients proliferation in response to MOG did not correlate with antigen specific T cell number (p=0.67 and 0.50, respectively).

To investigate whether the observations we made in the B-T cultures were specific to B-T cell interactions, we cultured PBMC in the same conditions. Although the conditions were not optimal for PBMC, we were able to observe proliferation by T cells in response to antigen. Proliferation by CD4+ T cells from TN-MS patients was elevated in response to foreign, but not neuro-antigen (Figure 6-08A). CD8+ T cells showed similar proliferative responses in HD and TN-MS patients (Figure 6-08B). Interestingly, in PBMC cultures, but not in memory or naïve B-T cultures, we observed significantly more CD4+ T cells from TN-MS patients remaining after culture compared to HD (Figure 6-08C). CD8+ T cells did not did not demonstrate this phenomenon (Figure 6-08D). This was true for cultures with foreign and neuro-antigen and is, therefore, unlikely to be the reason we observed more proliferation to foreign antigen by CD4+ T cells from TN-MS patients.

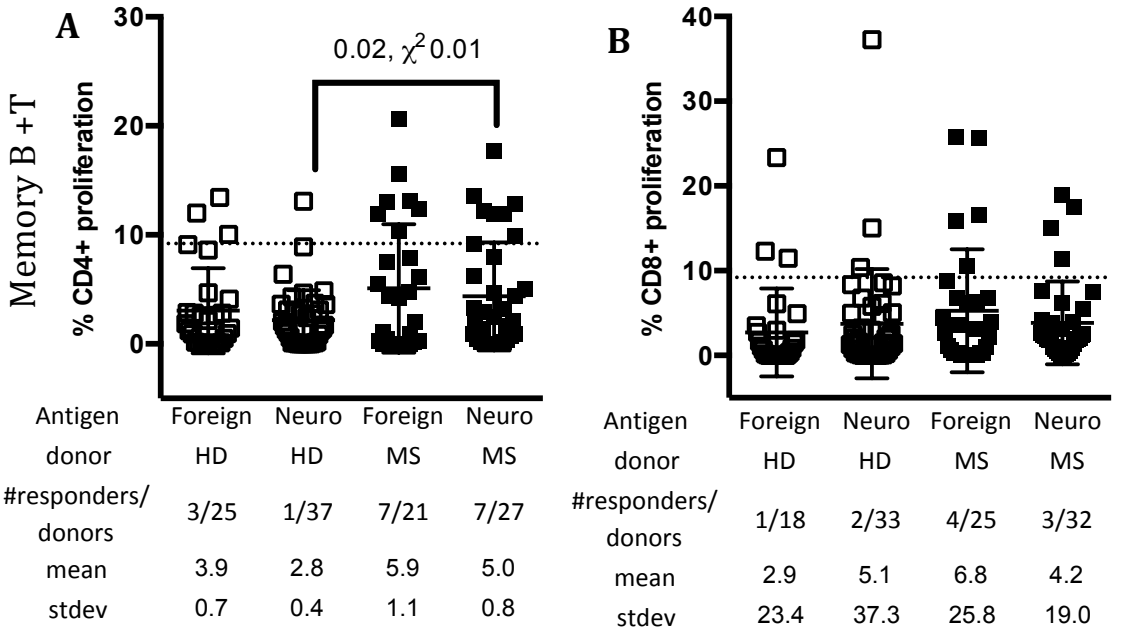


Figure 6-07. Proliferation by CD4+ and CD8+ T cells in memory and naïve B-T co-cultures. **A.** Memory B cells from MS patients induce more CD4+ T cell proliferation compared to HD. **C.** Naïve B cells from MS patients induce more CD4+ T cell proliferation in response to foreign antigens. **B.** No differences were observed between CD8+ responses in cultures with memory B cells. **D.** Naïve B cells from HD include more CD8+ proliferation to both foreign and neuro-antigens compared to MS patients. Dotted lines represent the cutoff for responders, defined as two standard deviations above the mean of HD naïve B cells to neuro-antigens for CD4+ T cells and HD memory response to foreign antigen for CD8+ responses. Populations used to determine responder level for CD4 and CD8 proliferation were naïve HD to neuro-antigen and memory HD to foreign antigen, respectively.

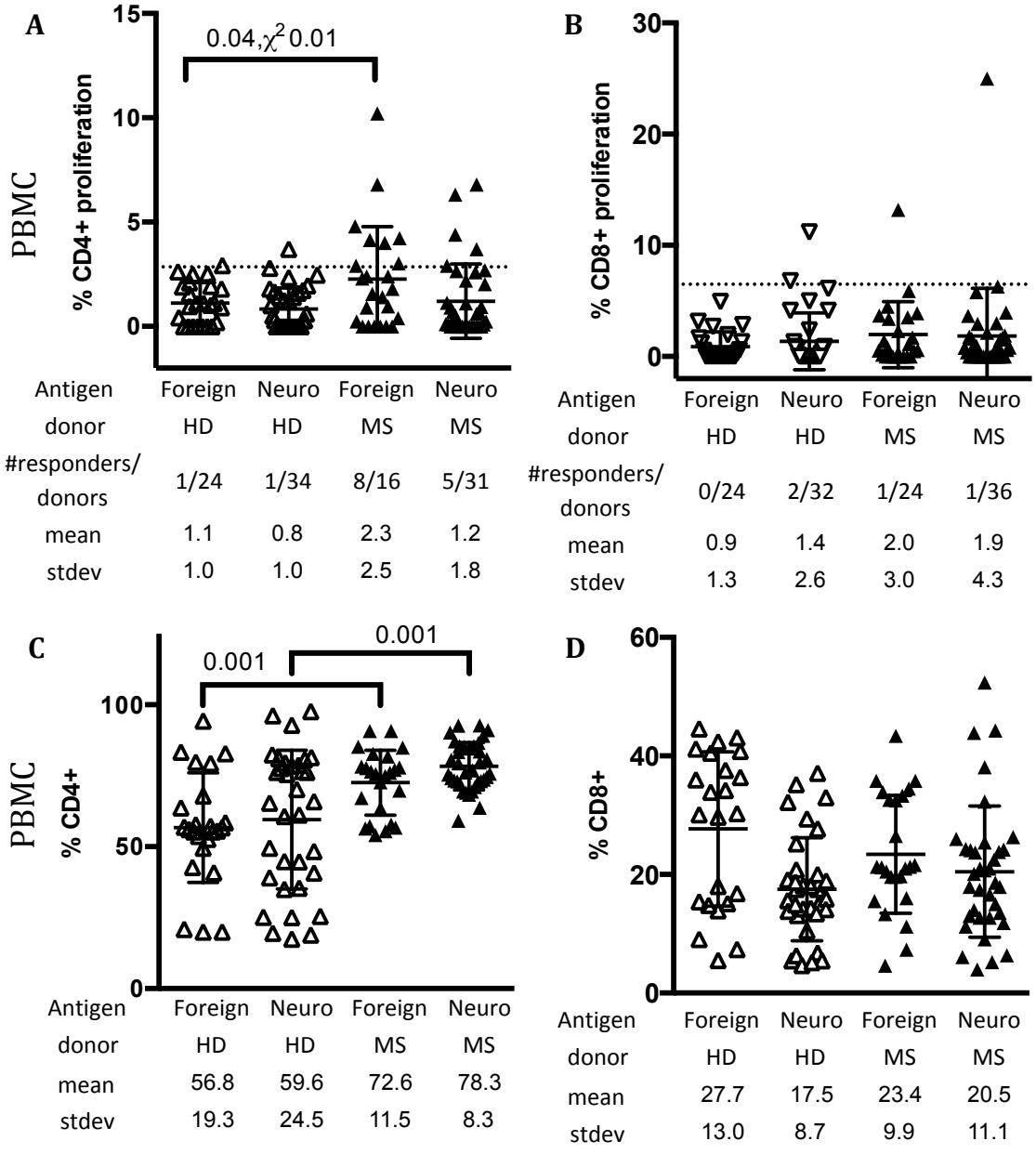
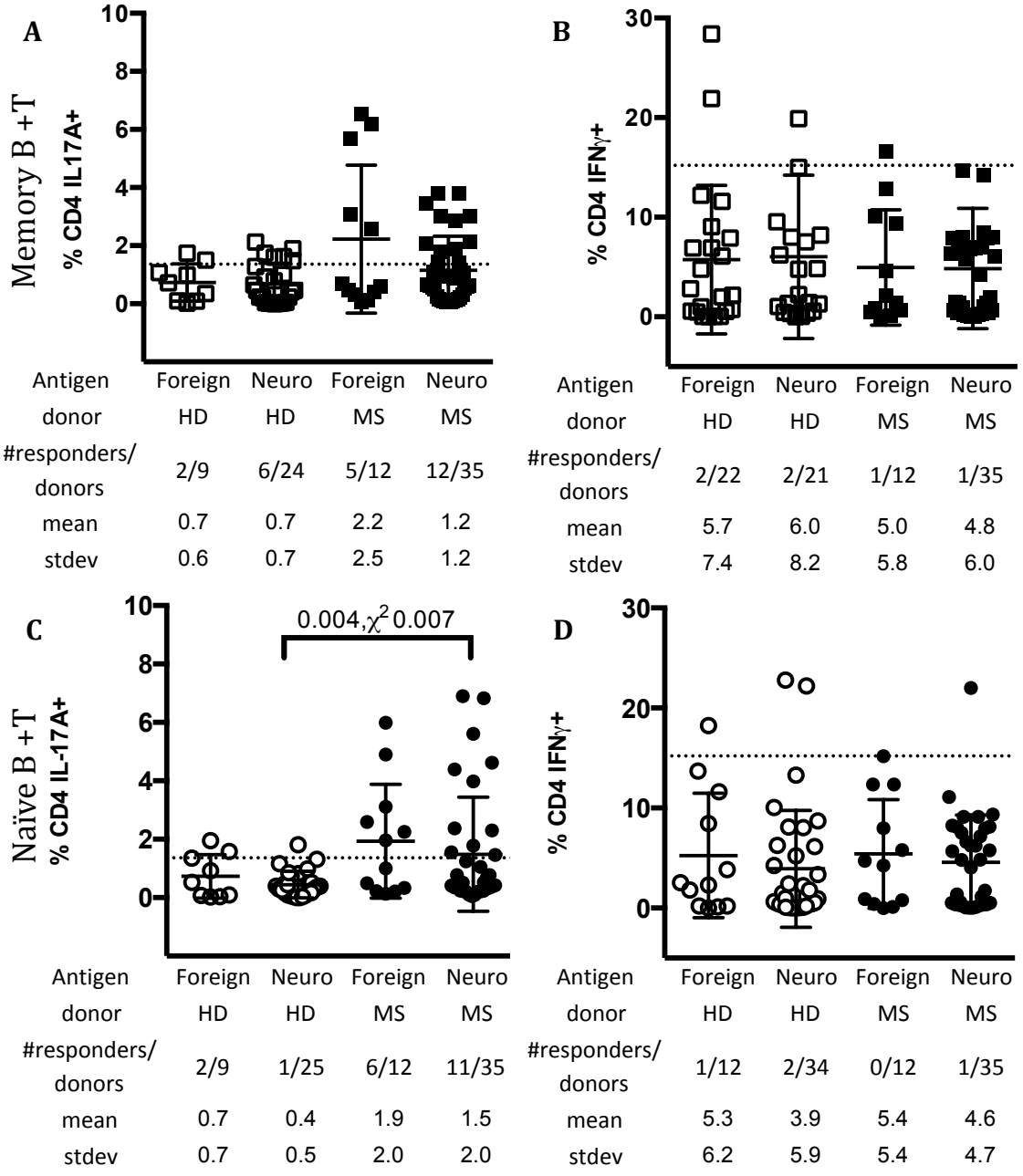


Figure 6-08. PBMC proliferation in cultures. **A.** Proliferation and the number of responders from MS patients were elevated in response to foreign antigen. **B.** Proliferation by CD8+ T cells was similar between HD and MS patients, irrespective of antigen stimulus. **C and D.** The percentage of CD4+ and CD8+ T cells on day seven. HD with neuro-antigen was used to define responders for chi-squared analysis.

6.3.2. Detection of Intracellular Cytokines in B-T cultures.

In addition to measuring proliferation, we also measured production of IFN-γ and IL-17A by intracellular staining. We observed a similar frequency of CD4+ T cells that expressed IL-17A from memory B-T cultures (Figure 6-08A). However, when we examined naïve B-T cultures, we found a significant elevation in the frequency of IL-17A+ CD4+ T cells in TN-MS patients responding to neuro-antigen (Figure 6-08B). We detected no significant differences in the frequency of IFN-γ+ CD4+ T cells from memory or naïve B-T cultures (Figure 6-08 C and D). Similarly, we found no differences in the percentage of IFNγ+ CD8+ T cells (Figure 6-09A and B).

Next we examined the fraction of CD4+ T cells that had undergone proliferation (CFSE-low) in culture for expression of IFN-γ or IL-17A. In response to foreign antigens, the CFSE-low fraction contained similar frequencies of IFN-γ+ or IL-17A+ CD4+ T cells in cultures from TN-MS patients and HD (data not shown). In contrast, memory and naïve B-T cultures from TN-MS patients harbored significantly more CFSE-low cells that expressed IFN-γ in response to neuro-antigens (Figure 6-10A and B). Both naïve and memory B cells from TN-MS patients supported more CD4+ T cells that proliferated and expressed IL-17 in response to neuro-antigens compared to HD (Figure 6-10C and D).



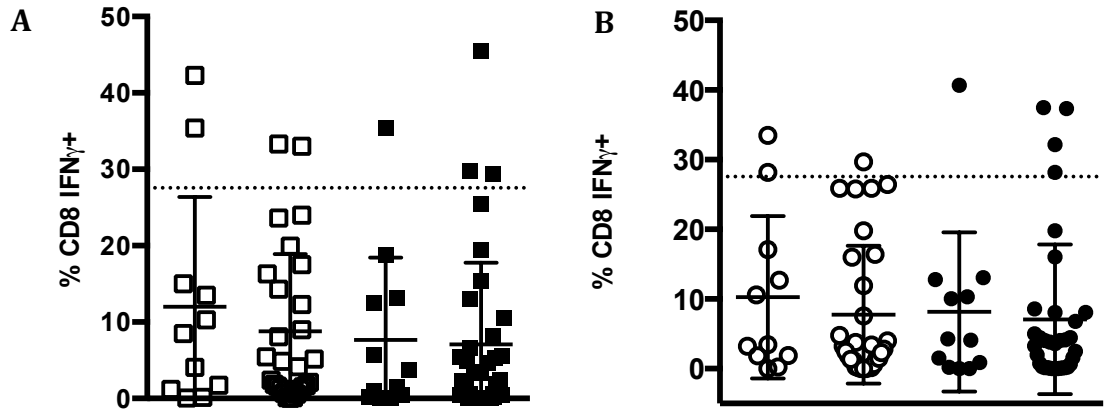
Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	2/9	6/24	5/12	12/35
mean	0.7	0.7	2.2	1.2
stdev	0.6	0.7	2.5	1.2

Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	2/22	2/21	1/12	1/35
mean	5.7	6.0	5.0	4.8
stdev	7.4	8.2	5.8	6.0

Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	2/9	1/25	6/12	11/35
mean	0.7	0.4	1.9	1.5
stdev	0.7	0.5	2.0	2.0

Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	1/12	2/34	0/12	1/35
mean	5.3	3.9	5.4	4.6
stdev	6.2	5.9	5.4	4.7

Figure 6-09. Intracellular staining for IFN-γ and IL-17A in CD4+ T cells revealed an enhanced IL-17A response provoked by naïve B cells from MS patients to neuro-antigens. A**.** IL-17A+ cells were more prevalent in MS memory B-T co-cultures but were not statistically different from HD. **B.** Naïve B cells from MS patients elicited a higher frequency of IL-17A+ and number of patients with elevated IL-17A CD4+ T cells compared to HD. **C and D.** Neither memory nor naïve B-T co-cultures harbored different frequencies of IFNγ+ CD4+ T cells. Naïve B-T cultures with neuro-antigen were used to define responders (dashed line) for chi-squared analysis.



Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	2/12	2/28	1/12	3/33
mean	12.0	8.8	7.7	7.1
stdev	14.4	10.1	10.8	10.7

Antigen	Foreign	Neuro	Foreign	Neuro
donor	HD	HD	MS	MS
#responders/ donors	2/12	5/31	1/12	4/35
mean	10.3	7.8	8.2	7.1
stdev	11.7	9.9	11.4	10.8

Figure 6-10. Intracellular staining for IFN-γ and IL-17A in CD8+ T cells. Memory **(A)** and naïve **(B)** B –T cultures from HD and MS patients supported similar levels of CD8+ T cells that expressed IFN-γ. Naïve B-T cultures with neuro-antigens, were used to define responders (dashed line) for chi-squared analysis.

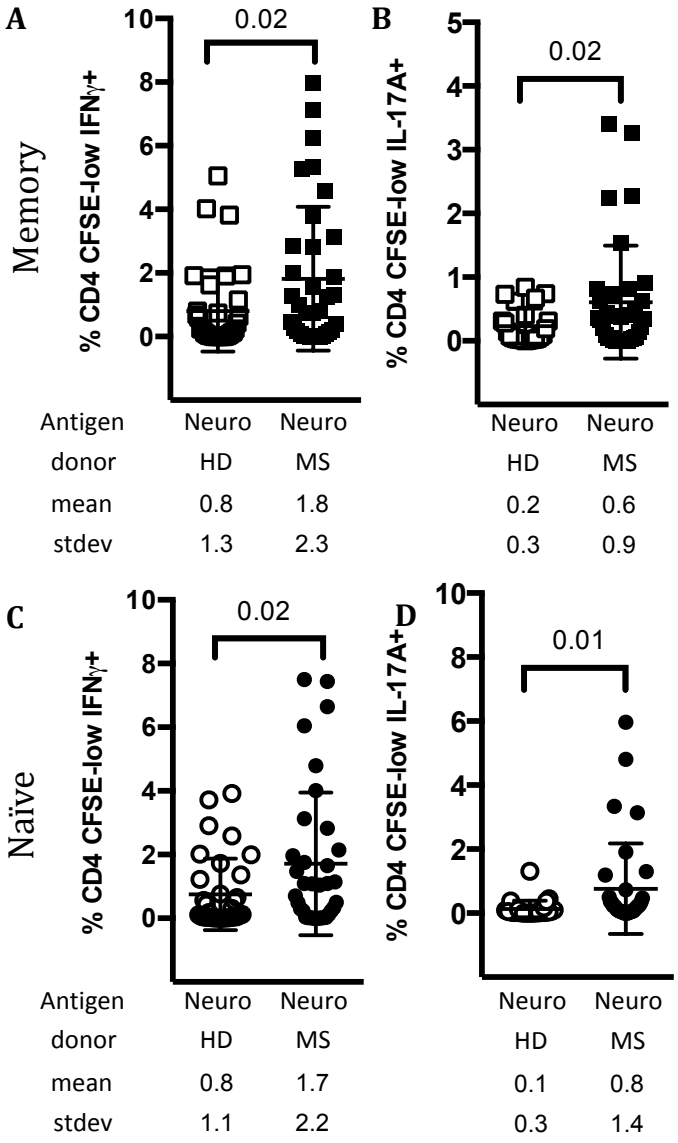


Figure 6-11.Intracellular detection of IL-17A and IFN-y in proliferated CD4+ T cells from B-T co-cultures. CD4+ T cells that had undergone proliferation in naïve **(A)** and memory **(B)** B-T cultures from MS patients contained an elevated frequency of IFN-γ+ cells. The same was true for IL-17A expression in the proliferated fraction of naïve (**C)** and memory **(D)** B-T co-cultures.

6.3.3 Cytokine Production in B-T Co-cultures

In addition to measuring cytokine expression by flow cytometry, we also measured protein levels of IFN-γ, IL-17A, IL-17F, IL-21, IL-22, IL-25, IL-33, TNF-α, sCD40L and IL-10 in the supernatant of B-T cultures on day three, and six of culture. No significant differences were observed in memory B-T cultures without antigen, foreign antigen, GA, or MOG 35-55 (data not shown). These are in line with our observations that memory B cells from HD and

MS patients induce similar proliferation by CD4+ and CD8+ T cells to those antigens. We measured elevated levels of IFN-γ, IL-17A, IL-17F, IL-21, IL-22, IL-25, IL33, IL-33, IL-10, and sCD40L in cultures from MS patients on day 3 (Figure 6-12A-I). On day six, we did not observe differences; except that cultures from HD on day six contained significantly more IL-4 compared to MS patients (Figure 6-12J). No significant differences were observed in memory B-T cultures for IL-31, TNF-α, IL-23 or IL-6 (Figure 6-11K-N)

Naïve B-T cultures from MS patients had significantly higher levels of IL-33 (30±34.9pg/mL) compared to HD (3.2±5.6pg/mL) on day three (p=0.03; data not shown). In the same cultures on day five, levels of IFN-γ were significantly higher in HD cultures (71.8±102.9pg/mL) compared to MS patients (8.8±17.8pg/mL; p=0.04; data not shown). Cytokine responses to MOG 35-55 and GA were similar between HD and MS patients. In response to foreign antigens, levels of IL-21, -25 and -33 were elevated in cultures from MS patients compared to HD (Figure 6-13A-C). In addition, we also observed significantly more IL-6 on day five from MS patients (Figure 6-14 N).

Similar to the response observed in memory cultures with neuro-antigens, we observed elevated cytokine levels in naïve B-T co-cultures for IFN-γ, IL-17A, IL-17F, IL-21, IL-22, IL-25, IL-33, sCD40L and IL-10 (Figure 6-14A-I) on day three. In addition, we also observed significantly more IL-31, TNF-α, and IL-23 in cultures from MS patients (Figure 6-13K-L). On day five, we observed a similar elevation in IL-22 (p=0.04), TNF-α (p=0.02), and sCD40L (p=0.02) in cultures from MS patients compared to HD (data not shown).

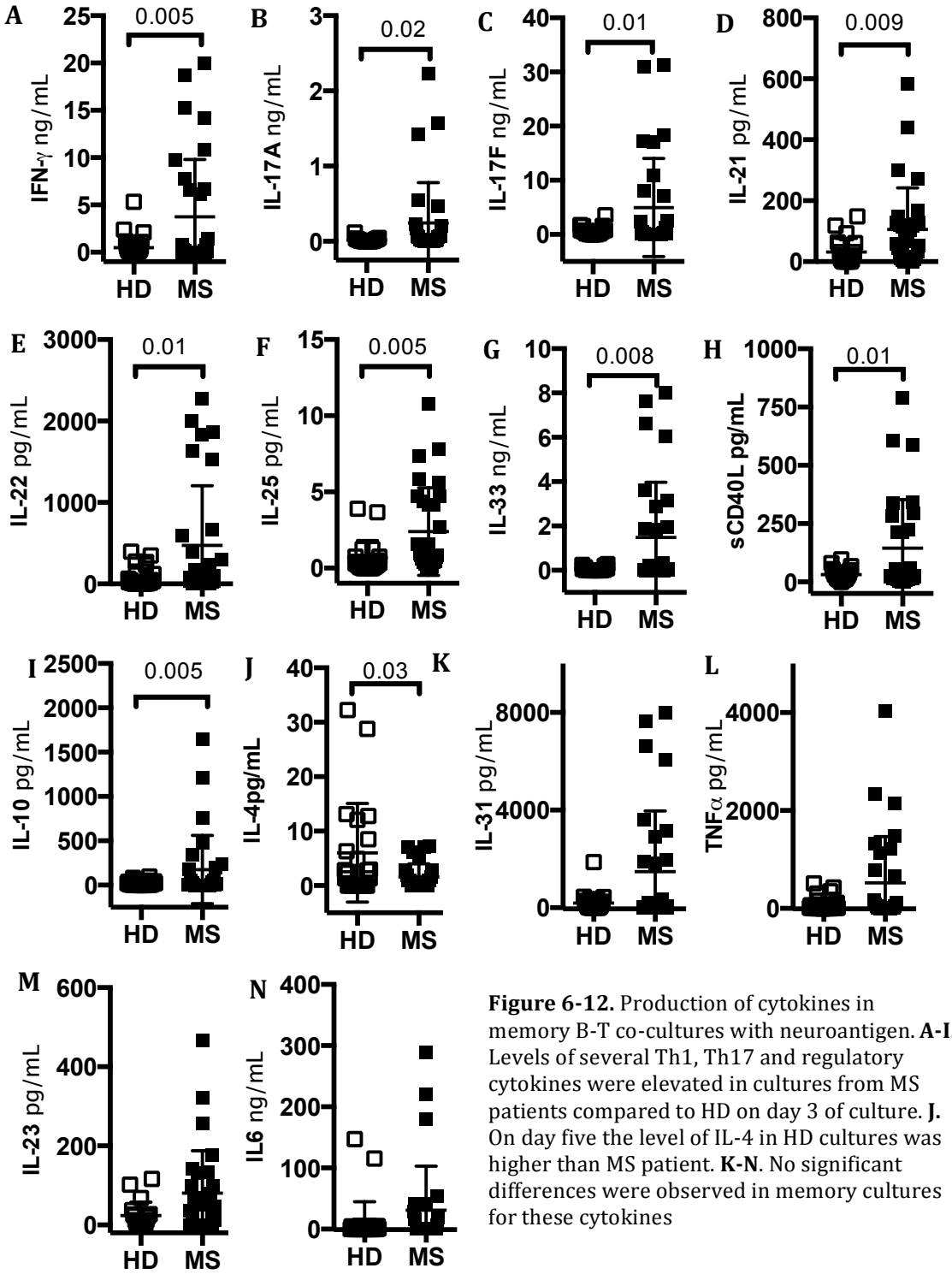


Figure 6-12. Production of cytokines in memory B-T co-cultures with neuroantigen. **A-I.** Levels of several Th1, Th17 and regulatory cytokines were elevated in cultures from MS patients compared to HD on day 3 of culture. **J.** On day five the level of IL-4 in HD cultures was higher than MS patient. **K-N**. No significant differences were observed in memory cultures for these cytokines

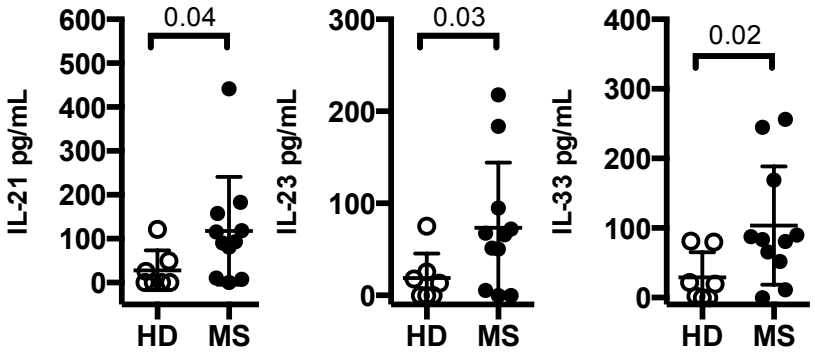


Figure 6-13. Cytokine production in naïve B-T co-cultures with foreign antigens. A-C. Levels of IL-21, -23, and -33 were elevated in cultures from MS patients.

In PBMC cultures with no antigen, the level of IL-33 was significantly elevated in cultures from MS patients (68.5±64.2) compared to HD (17.3±24.6, p=0.04) on day three (data not shown). There were no differences in PBMC cultures in response to MOG 35-55. In response to neuro-antigens, we found a significant elevation in IL-33 production by MS patients (39.9±31.4) on day three, but this was significantly less than the response to no antigen in MS patients (p=0.02). Also on day three, sCD40L was elevated in cultures with HD PBMC (66.7±36.1) compared to MS patients PBMC (41.9±25.7, p=0.1). On day five, levels of IL-17F were elevated in MS patients (400.4±578.8) compared to HD (151.2±153.5, p=0.03). There were no other differences in cytokine production in PBMC cultures.

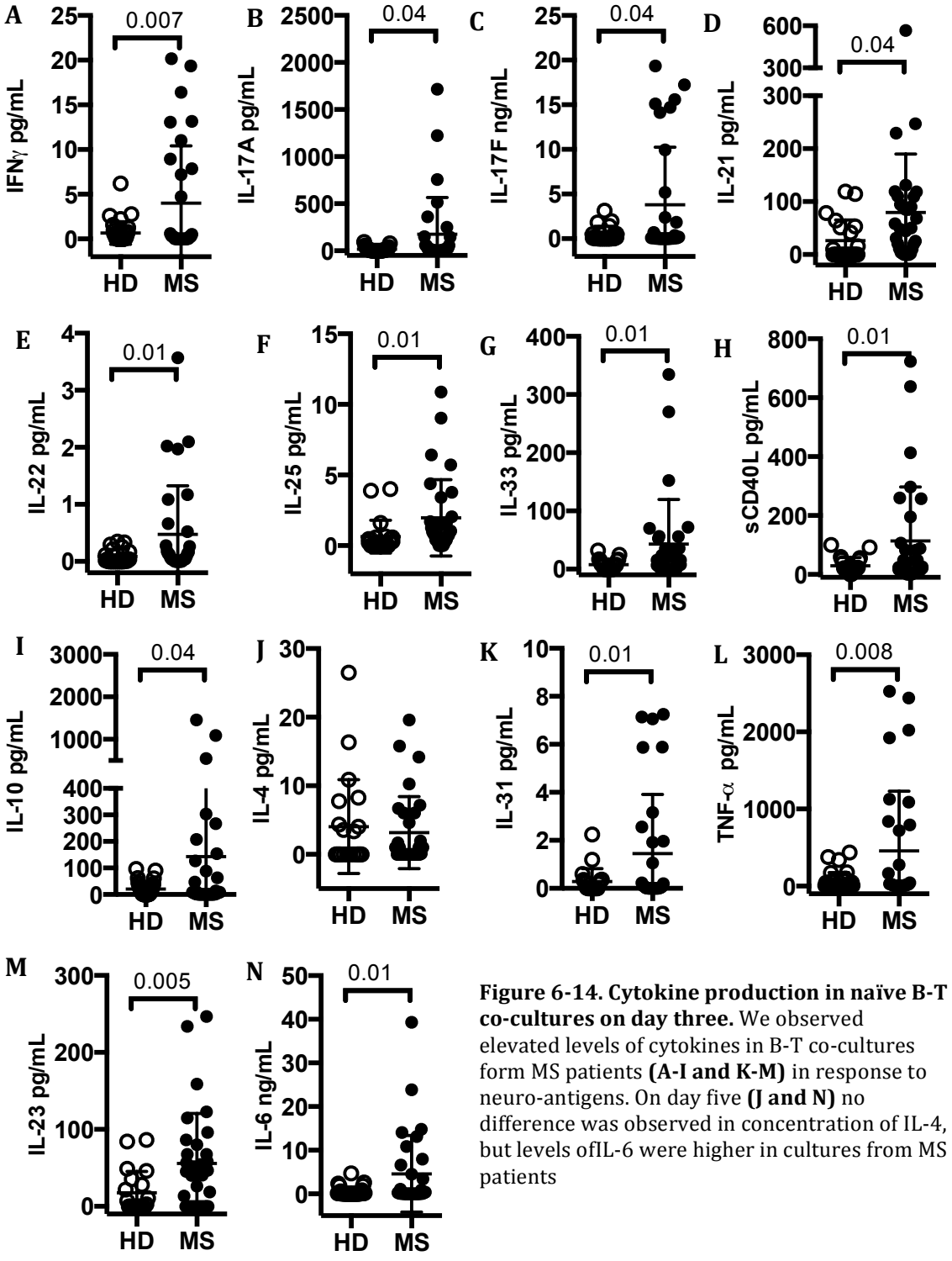


Figure 6-14. Cytokine production in naïve B-T co-cultures on day three. We observed elevated levels of cytokines in B-T co-cultures form MS patients **(A-I and K-M)** in response to neuro-antigens. On day five **(J and N)** no difference was observed in concentration of IL-4, but levels ofIL-6 were higher in cultures from MS patients

6.4. The Impact of GA on B-T co-cultures

6.4.1 Glatiarmer acetate does not modulate proliferation to neuro-antigens in B-T cultures

To address whether GA could modulate interactions between B and T cells in our culture system, we first confirmed our previously published results that GA induced similar proliferation by CD4+ and CD8+ T cells from MS patients and HD (Figure 6-15; CD8 data not shown) in a subset of donors (9 HD and 10 MS patients). Next, B-T cultures were stimulated with MBP and MOG with/without GA. Proliferation by CD4+ and CD8+ T cells was altered in some donors by the addition of GA (Figure 6-16). However, there was not a consistent trend of inhibition or enhancement of proliferation in any of the conditions we tested for any of the donor groups.

6.4.2. The addition of GA to B-T cultures does not impact cytokine production

Secretion of cytokines in memory or naïve B-T cell cultures with MBP plus MOG and GA were similar to paired cultures containing MBP plus MOG within the same donor group. Next, we compared cytokine levels in MBP plus MOG and GA cultures to all other cultures with neuroantigens (which includes MBP plus MOG) in the same donor group and, again, we observed no significant differences (data not shown). Similarly, we observed no changes in cytokine production by PBMC cultures in the presence or absence of GA in paired comparisons. When we compared PBMC cytokine production in the presence of MBP plus MOG and GA we found no significant differences except for an elevation in IL-17A in MBP plus MOG and GA (26.5±10.7pg/mL) compared to all neuroantigens (14.7. ±11.8pg/mL, p=0.02) from HD. This data strongly suggests that the addition of GA to cultures containing

neuroantigen had no impact on cytokine production in PBMC, memory or naïve B-T co-cultures of MS patients.

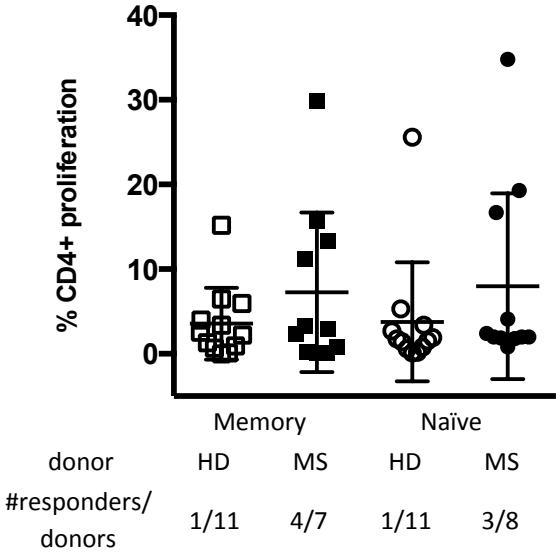


Figure 6-15. Proliferation to GA in B-T cultures. We found similar proliferation to GA by CD4+ T cells from HD and MS patients in memory and naïve B-T co-cultures.

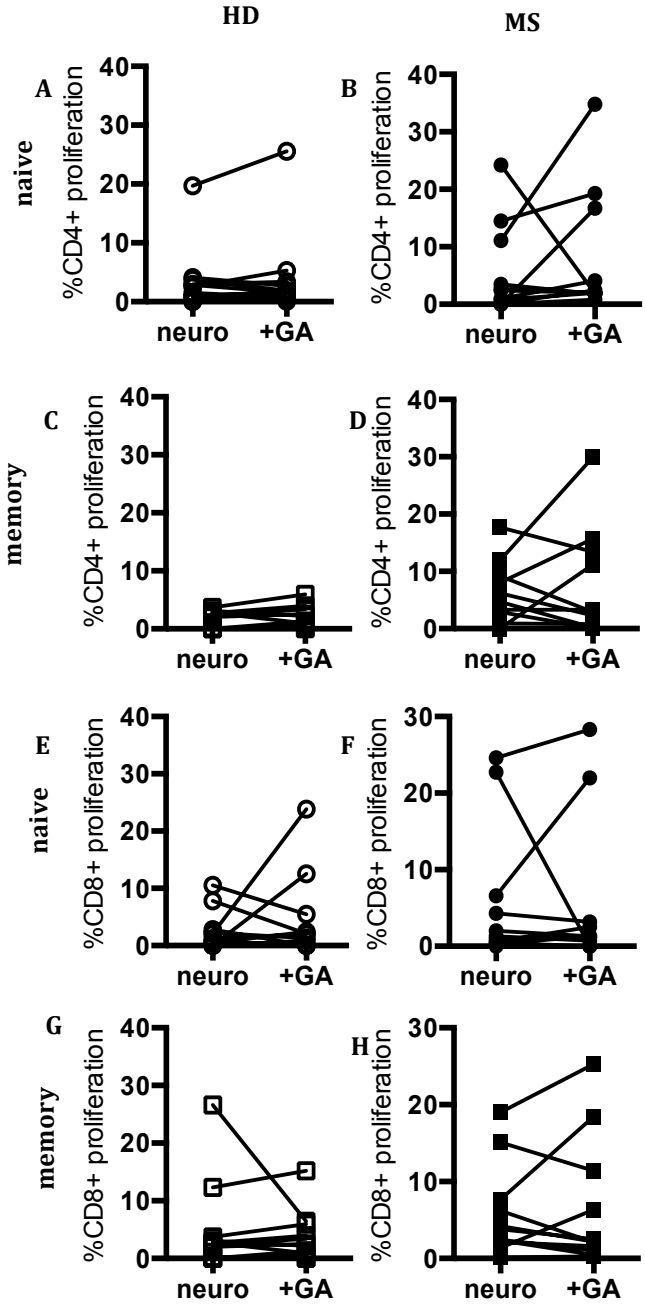


Figure 6-16. GA has a variable impact on T cell proliferation to neuro-antigens. A-D. CD4+ T cell proliferation with or without GA. **E-H.** CD8+ T cell proliferation in the presence or absence of GA.

6.5 Involvement of IL-6 in B-T cultures

6.5.1. Expression of the IL-6 and IL-10 receptors on Lymphocytes

We characterized dysregulated proliferation and cytokine production by memory and naïve B cells from MS patients in chapters four and five. However, we do not know if altered cytokine production by B cells is able to impact T cell function. Based on previous studies [268]**,** we hypothesized that CD4+ T cells from MS patients would have elevated levels of IL-6R compared to HD and that increased proliferation in the BT-cultures might be due to overexpression of IL-6R on T cells from MS patients.

We were able to clearly discriminate between cells that expressed IL-10R, IL-6R and gp130 and those that did not (Figure 6-17A-D). The overwhelming majority of CD4+ T cells from HD (94.8±2.3) and MS patients (95.2±1.9) expressed gp130. On average, less than ten percent of CD8+ T cells from both donor groups expressed gp130 (HD 7.1±1.9, MS 9.0±2.2). Compared to CD8+ T cells, CD4+ T cells from HD and MS patients had a higher frequency of cells that expressed gp130 (HD $P=5.3x10^{-25}$ and MS $1.6x10^{-22}$) and higher levels of gp130 expression (HD $p=2x10^{-8}$, MS $p=4x10^{-6}$). There were no differences between donor groups with regard to gp130 expression. The frequency of CD4+ and CD8+ T cells that expressed IL-6R and IL-10R were similar between the groups. However, the level of IL-6R and -10R on those cells that expressed these receptors was elevated on CD4+ and CD8+ T cells from MS patients when compared to HD (Figure 6-17 G-J).

Total CD19+ B cells from MS patients had similar frequencies of cells that expressed IL-6R (mean HD 55.3±2.0, MS 55.3±2.6), IL-10R (mean HD 53.0±3.4, MS53.0±2.7), and gp130 (mean HD 27.6±2.8, MS 28.0±2.8). The total frequency of memory B cells from that

same cohort expressed higher levels of gp130 (HD $p=3x10^{-5}$, MS p=0.003) and IL-10R (HD p=0.005, MS p=0.02) compared to naïve B cells. The percentage of memory B cells that expressed IL-6R compared to naïve B cells was higher in HD ($p=9x10^{-4}$), but not MS patients. Of those cells that expressed IL-6R or IL-10R, we found significantly higher levels of all receptors on total, naïve and memory B cells from MS patients compared to HD (Figure 6-18). Total CD19+ B cells and memory B cells from MS patients expressed higher levels of gp130 compared to HD (Figure 6-18C and F). However the naïve B cell gp130 expression level was similar, which suggests the differences observed in total B cells were a result from over-expression of gp130 by memory B cells.

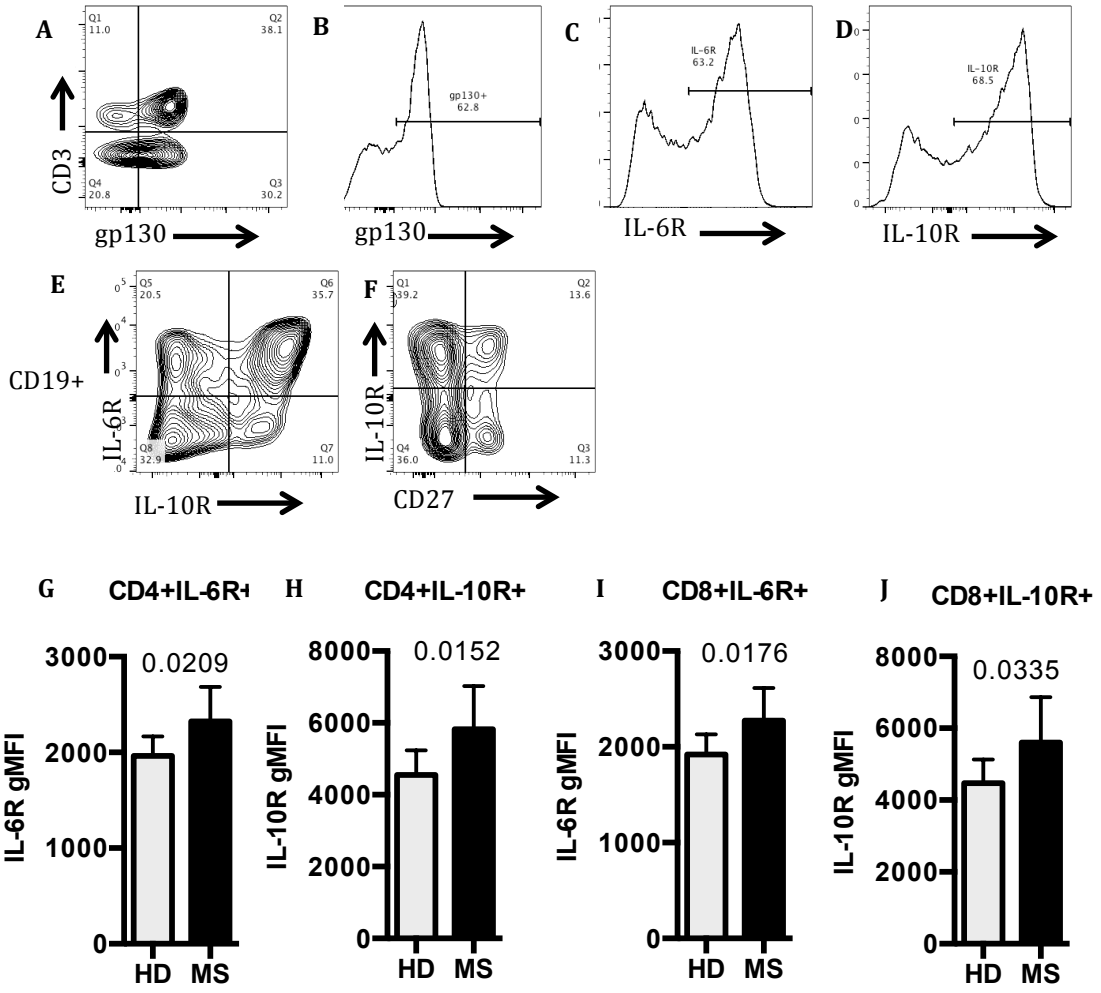


Figure 6-17. **Expression level of IL-6R, IL-10R and gp130 on T cells from HD and MS patients**. **A –D.** Representative plots for the expression of gp130, IL-6R and IL-10R gated on lymphocytes. **E and F.** Representative expression of IL-6 and IL-10 on CD19+ B cells. **G-H** CD4+ and CD8+ T cells from MS patients express higher levels of IL-6R compared to HD.

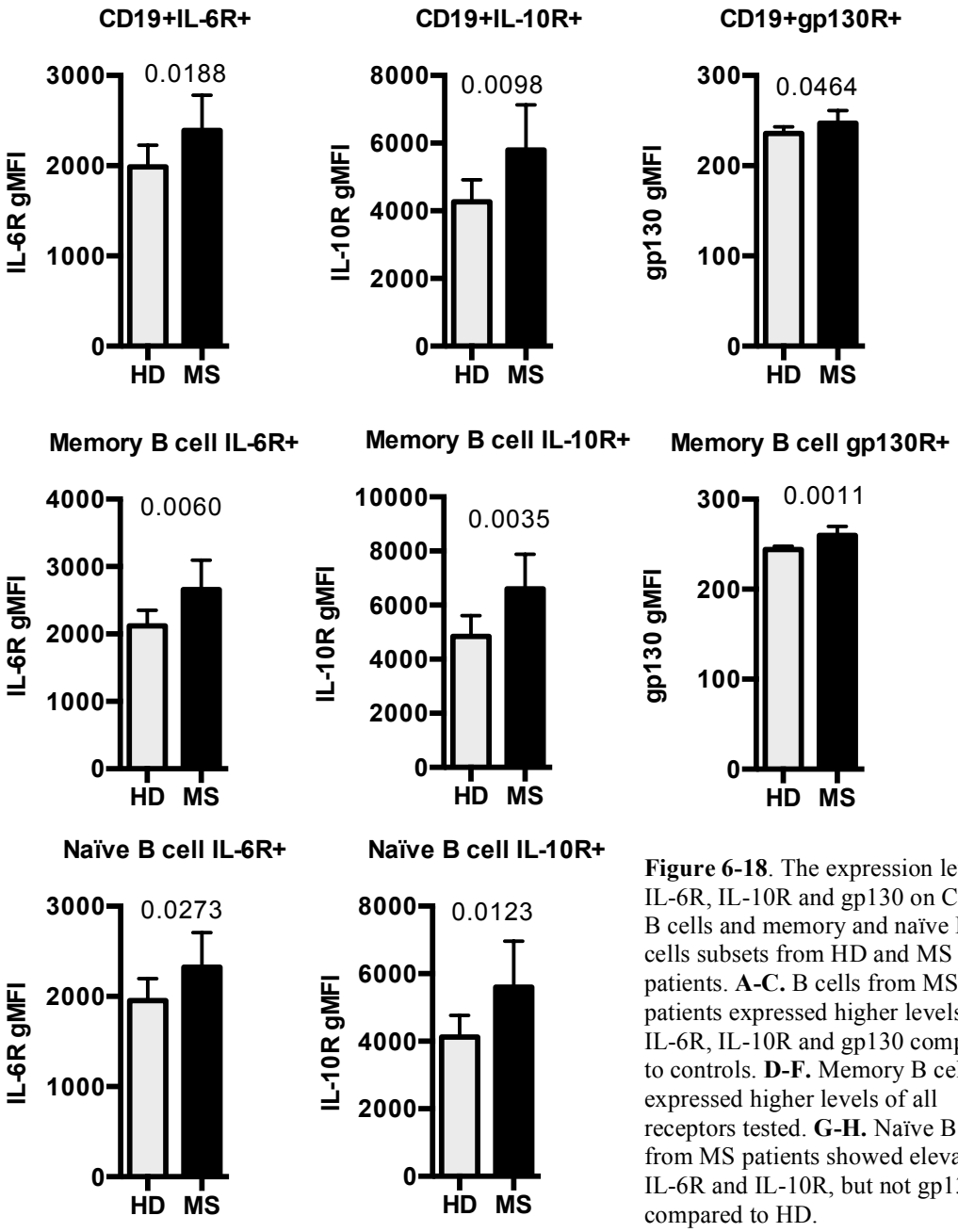


Figure 6-18. The expression level of IL-6R, IL-10R and gp130 on CD19+ B cells and memory and naïve B cells subsets from HD and MS patients. **A-C.** B cells from MS patients expressed higher levels of IL-6R, IL-10R and gp130 compared to controls. **D-F.** Memory B cells expressed higher levels of all receptors tested. **G-H.** Naïve B cells from MS patients showed elevated IL-6R and IL-10R, but not gp130 compared to HD.

6.5.2. IL-6 supports T cell proliferation in HD and MS patients in B-T cultures

We identified over-production of IL-6 by naïve and memory B cells from MS patients and an elevated level of IL-6R on T cells from MS patients. We hypothesized that IL-6 production by B cells might stimulate T cell proliferation in B-T co-cultures. To test this hypothesis, we added an antagonistic IL-6R monoclonal antibody (α-IL-6Rα) to B-T cultures on day zero and day three of a five-day culture to inhibit IL-6R responses.

We found that a blockade of IL-6R signaling inhibited CD4+ T cell proliferation in most donors from both HD and MS patients from memory and naïve B-T cultures to varying extents (Figure 6-19A and C). The same was true for proliferation by CD8+ T cells (Figure 6-19B and D). Although we observed inhibition of proliferation by α-IL-6R in the majority of subjects, the response was heterogeneous and there were no significant differences between CD4+ or CD8+ T cell proliferation in cultures with and without α-IL-6R. Furthermore, the inhibition we observed was not confined to neuro-antigen or foreign antigen conditions, which supports a generalized role for IL-6 in promoting T cell proliferation that is not specific to MS patients or the potential for MS B cells to produce more IL-6 in B-T co-cultures.

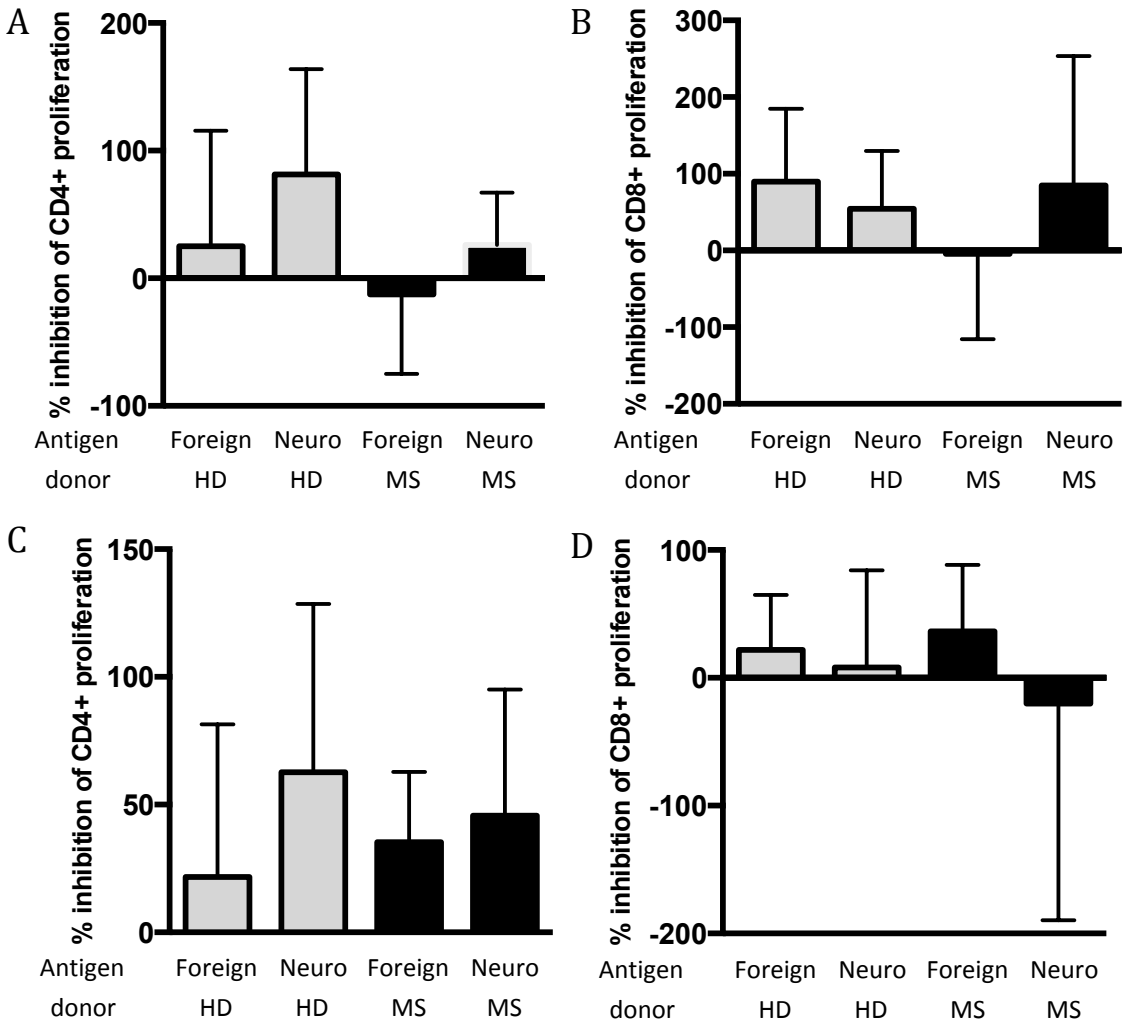


Figure 6-19. Blockade of IL-6 signaling in B-T co-cultures. The percent inhibition of CD4 **(A and C)** and CD8 **(B and D)** T cell proliferation in naïve and memory B-T co-cultures cultures with a neutralizing IL-6R.

6.6 Allogeneic B-T Co-cultures

6.6.1. B-T cultures require autologous B and T cells to support T-cell proliferation

Despite similar frequencies of antigen specific T cells from MS patients and HD (MOG is an exception) we have documented perturbations in the cytokine secretion by and phenotypic distribution of B and T cells from TN-MS patients. The differences in cell populations input

to B-T cultures could impact the outcome. For instance, in this cohort, we found significantly more CD4+ T central memory cells and fewer CD4+ T cells that secreted IFN-γ spontaneously in MS patients. To address this issue, we cultured allogeneic CD19+ B cells with T cells from two HD and two MS patients.

After the data was normalized to no the antigen controls several of the responses were negative, indicating very little proliferation in allogeneic B-T cultures (Figure 6-19). These results were not unanticipated, as we have previously observed that approximately 80% of CD4+ and 30% of CD8+ T cell proliferation is impaired in B-T cultures by blocking HLA-DR interactions [192]. We did not genotype donors used in these experiments to determine similarity in the HLA locus and these results likely reflect an inability of T cells to respond to an HLA-mismatched APC. This further supports our hypothesis that B cells are acting as an APC for autologous myelin-specific T cells.

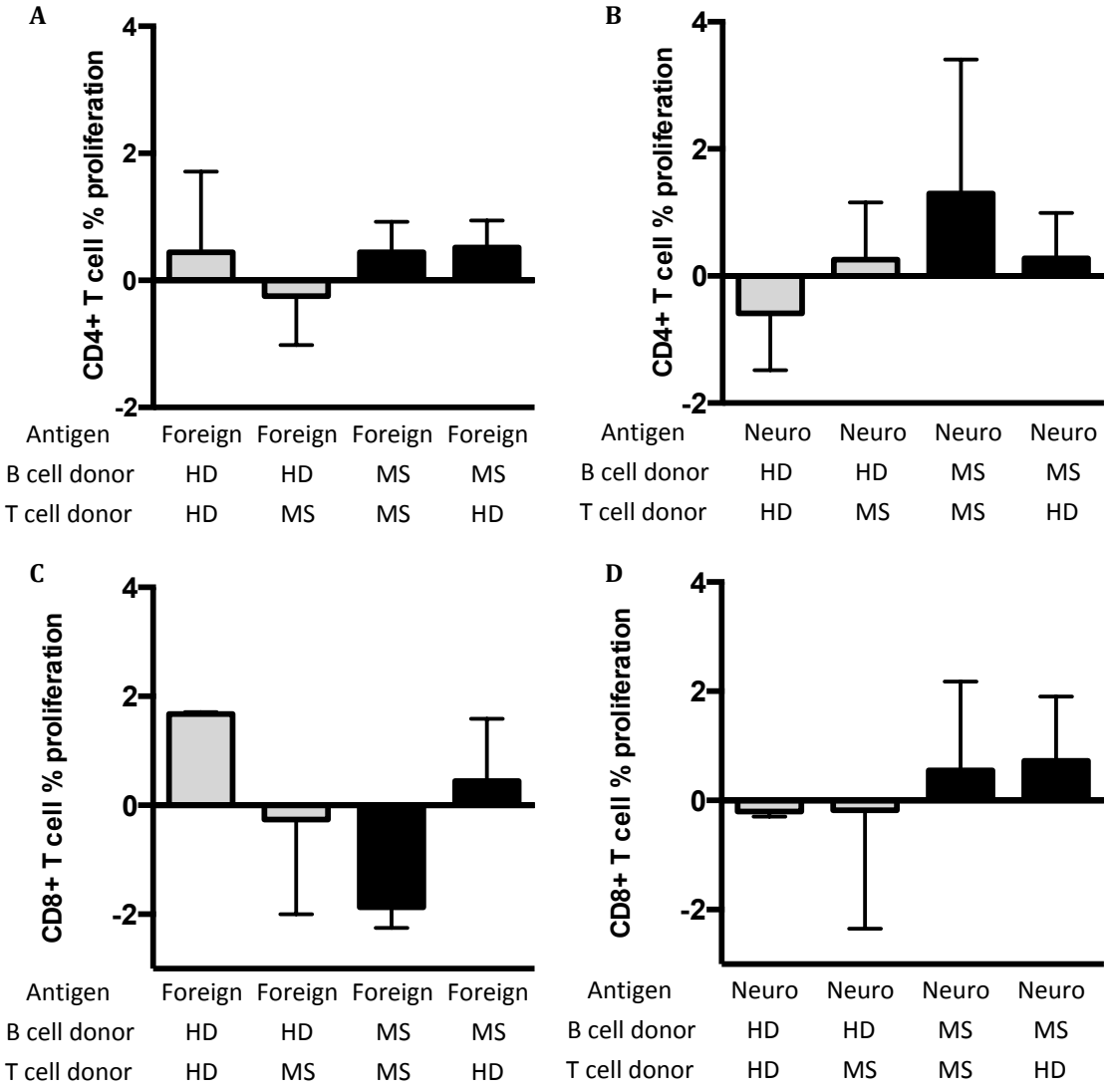


Figure 6.-20. Allogeneic B-T co-cultures. Little proliferation by CD4+ **(A and B)** or CD8+ **(C and D)** was observed in B-T co-cultures from allogeneic donors in response to foreign **(A and C)** or neuro-antigens **(B and D).**

CHAPTER SEVEN
Conclusions and Discussion

Introduction

7.1. Observations in these studies

The data presented here support an antibody-independent role for B cells in MS. We found a defect in the ability of B cells to secrete IL-10 that is associated with a blockade in the regulatory response in the context of autoimmunity. We also identified a striking elevation in the secretion of IL-6 by B cells from MS patients. IL-6 directly supported antigen-specific T-cell proliferation in both HD and MS patients and the impact of B cell derived IL-6 appears to underpin an inflammatory role for B cells in EAE. We also found a hyperactive memory B cell proliferative response to CD40-engagement that could support germinal center formation and B and T cell activation. We investigated whether glatiramer acetate would modulate B-cell function directly or in patients receiving GA therapy. While GA had no impact on B cells directly *in vitro*, GA therapy restored IL-10 production and transiently diminished IL-6 secretion by B cells. We also found that GA therapy remodeled the distribution of B cells and T cells and induced a profound impact on immunoglobulin secretion *in vitro.* Moreover, we identified a role for human B cells from MS patients to support autologous Th1 and Th17 responses to neuro-antigens. To our knowledge this is the first direct demonstration that human B cells directly influence Th17 responses in humans. Taken together, these data demonstrate a role for B cells in a T cell-driven autoimmune disorder that was not previously

appreciated. These observations also raise important questions for evaluating the impact oftherapies that modulate B-cell function.

7.2 Dysregulation of B-cell Proliferation by Naïve and Memory B cells

We hypothesized that B cells from MS patients would exhibit dysregulated proliferation to stimulation *in vitro*. Given the presence of clonally expanded memory B cells, plasmablasts and plasma cells in the CSF of MS patients, it is possible that proliferation by B cells occurs within the intrathecal compartment. CD40-CD40L interactions could impact reciprocal B and T cell activation and likely play a role in the formation of ectopic follicles in the CNS that have been described in SPMS [127, 234]. Indeed, we observed perturbations in the proliferative responses of B cells from MS patients.

Stimulation in low-dose CD40L conditions revealed hyperactive proliferation by memory B cells from MS patients compared to naïve B cells from MS patients and memory B cells from HD. This data suggests that memory B cells from MS patients have a low threshold for CD40-mediated activation, and has been noted in SLE as well [269]. In addition, mutations in the CD40 locus are associated with MS [31], although it is not known whether these alter the function of CD40 as has been reported in other patient populations [270]. Ongoing investigations in our laboratory are addressing the possibility of enhanced CD40 signaling in B cells from MS patients.

In low-dose and high-dose CD40L conditions, HD memory B cells responded to IL-2/CpG more strongly than naïve B cells and memory B cells from MS patients. This is not surprising because naïve B cells express less TLR9 and require stimulation to induce

expression [271]. In addition, one report found that memory B cells from MS patients express less TLR9 [151], but this does not explain why we also observed less proliferation by naïve B cells when stimulated with CpG, as well. It may be that naïve B cells from MS patients are defective in inducing TLR9 expression in these culture conditions compared to HD.

In high-dose CD40L conditions, we observed less proliferation by naïve B cells from MS patients compared to HD, which is consistent with the observations we made in low-dose conditions. In contrast, we observed similar proliferation between memory B cells from HD and MS patients to CD40L, alone, and with IL-4. In fact, memory B cells from MS patients failed to proliferate to a greater extent on high-dose CD40L when any additional stimulation was added to the culture. It is not clear if this is a result of memory B cells failure to integrate multiple signals, or if negative regulatory factors are induced in high-dose CD40L conditions [272].

CD40-CD40L interactions are a critical component of immune cell activation and perturbations in CD40 signaling in B cells is a common feature of autoimmune disorders [260, 269, 273]. Modulation and targeting of CD40-CD40L are of great interest as pharmacological targets in autoimmunity [274, 275]. Of note, the expression of CD40 on total or naïve and memory B cells is the same between HD and MS patients (unpublished observations). However, T cells from MS patients have elevated levels of CD40L *ex vivo* [239] as do mitogen-stimulated T cells from MS patients [240, 241]. This over expression on un-manipulated T cells combined with the hyper-activation of memory B cells from MS patients highlights the importance of CD40-CD40L interactions in MS.

7.3 Cytokine Production by Memory and Naïve B cells

B cells are capable of secreting a wide variety of cytokines; we chose to focus our efforts on those that may impact T cell responses or the inflammatory environment in MS. We surveyed supernatants from memory and naïve B cells for LT-α, TNF-α, IL-12p70, IL-10 and IL-6. We identified very little difference between memory and naïve B cells from HD and MS patients in their ability to secrete TNF-α. Others found an increase in TNF-α production by B cells from MS patients [138]. We tested TNF-α because this cytokine is notable for its role in germinal center formation. However, TNF-α neutralization was not beneficial for MS patients. In fact, several patients in the TNF-α trial experienced adverse events .

We did not observe any clear differences in LT-α production between MS patients and HD by naïve or memory B cells. However, we did find that total CD19+ B cells from MS patients secreted more LT-α. This latter observation is in agreement with another report showing elevated LT-α production by B cells from MS patients [243]. We also measured IL-12 from B cell cultures. Human B cells are quite capable of secreting IL-12; one study found that total B cells stimulated though CD40 and TLR9 produced on average 20pg/mL IL-12p70 in HD [193]. Unfortunately we were not able to quantify the level of IL-12 in the majority of memory and naïve B cell cultures due to the low sensitivity of the ELISA. IL-12 is a potent cytokine and is generally secreted in a low concentration compared to other cytokines. This is unfortunate given our goal of understanding the potential for B cells to influence on Th1 responses.

We identified a paucity of IL-10 production by both memory and naïve B cells from MS patients. These results were most evident on day five of culture in high-dose CD40L conditions where both memory and naïve B cells from MS patients failed to produce IL-10 to the same extent as the HD cohort. Previous reports documented diminished IL-10 production by total B cells when stimulated with through BCR followed by CD40L [138, 243] or CpG [123] irrespective of remission or relapse [151]. However, our lab previously reported that IL-10 was increased in naïve B cells from MS patients in this same two-step stimulation compared to HD [194]. This study used concentrations of CD40L-expressing cells that were three to thirteen-times greater than the dose of CD40L used in the studies presented here.

We titrated the concentration of CD40L-expressing cells based on B cell proliferation, but it is quite possible that we were also able to detect a more sensitive integration of signals because we used a lower dose of CD40L. Many of the observations we made for cytokine production in high-dose CD40L conditions were trends in low-dose CD40L but did not meet statistical significance. It is not known whether the threshold for signaling to induce proliferation is lower than that of cytokine secretion but our data would suggest that in order to observe maximal secretion of cytokines, a higher level of CD40-engagment is required. This may have contributed to the differences we observed. In addition, most other studies have only measured cytokines during the first 48-72 hours of culture. We found that maximum IL-10 production occurred after three days.

The most surprising observation we made was the excessive production of IL-6 by B cells from MS patients. We observed elevated IL-6 production by both memory and naïve and total B cells from MS patients when activated *in vitro*. In particular, naïve B cells

secreted high levels of IL-6. Another report simultaneously confirmed our results [253]. In the same study, Barr *et al* found that re-emerging B cells from patients that had undergone B cell depletion therapy had normalized levels of IL-6 secretion and lower levels of IL-17, but not IFN-γ, production *in vitro* [253]. While stimulation of B cells with CD40L combined with other stimuli, CpG alone induced similar levels of IL-6 secretion by B cells from HD and MS patients and but less IL-6 from MS patients' B cells during exacerbation [123]. This suggests that over-production of IL-6 by B cells from MS patients requires the integration of signaling from B-cell activation cues.

The exact mechanism(s) of this deficit in IL-10 production and enhanced IL-6 secretion in patients with MS is not clear. There are many possible biological processes that could be involved including RNA degradation, preferential activation of transcription factors, or even epigenetic regulation. However, the selective induction of IL-6 and IL-10 production has not been studied extensively in human B cells. Recently, one study found that IL-10, but not IL-6, production was triggered by TLR stimulation leading to activation of signal transducer and activation of transcription (STAT) 3 and ERK [276]. MicroRNA (MiR) 132 is overexpressed in B cells from MS patients, blocking MiR 132 reduced LT-α and TNF-α levels to normal levels but did not change IL-6 levels [277]. Of note, this dysregulated axis of IL-10 and IL-6 is also observed in B cells from patients with Bachet's disease following stimulation with B cell activating factor (BAFF) [152].

7.4 Implications of IL-6 and IL-10 production by B cells, Lessons from EAE

IL-6 and IL-10 are pleiotropic cytokines that are produced by B cells, other APC, and, for IL-6, a variety of non-immune cells. IL-6 impacts the survival, proliferation and activity of immune cells and plays an important role in mediating the acute inflammatory response. While IL-10 has gained acclaim in recent years for its anti-inflammatory effects, it cannot be ignored that IL-10 acts to bolster autoimmune conditions such as SLE [278].

Most studies supporting the roles of IL-10 as protective and IL-6 in promoting disease were carried out in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Both MS and EAE involve Th1 and Th17 cells; the discovery that IL-6 promotes Th17 development prompted further investigation on the role of IL-6 in EAE and MS. Indeed, IL-6-deficient mice do not develop EAE [249, 250] because they fail to induce CNS-specific Th1 and Th17 cells [279]. Treatment of EAE mice with a neutralizing antibody to IL-6 diminished EAE severity, but increased IL-6 levels in the central nervous system (CNS) [251]. Furthermore, IL-6 deficient T cells cannot induce EAE upon adoptive transfer to wild type mice [280]. However wild type dendritic cells loaded with CNS antigen rescued susceptibility to EAE in IL-6-deficient recipient mice [281]. These studies highlight the importance of IL-6 in the establishment of EAE, although the role of IL-6 in ongoing EAE or MS is less clear. B cell-specific MHCII and IL-6 knockout mice have a decreased disease severity in a B cell-dependent EAE model [282], suggesting a role for APC function and IL-6 production by B cells in establishing disease and propagating disease. While another study found this B cell IL-6 effect in peptide MOG 35-55 EAE [253], another showed that it was specific to the recombinant MOG EAE model, which supports the notion that B cells are pathogenic in this mode [282].

IL-10-deficient mice develop more severe EAE compared to wild type mice, whereas mice overexpressing IL-10 are resistant to EAE [283, 284]. However, the ability of recombinant IL-10 or IL-10-transduced cells to control disease once disease is established have not been met with consistent results [285-288]. Yet, the adoptive transfer of IL-10-expressing B cells, at the onset of disease, or T regulatory cells reduced EAE severity through mechanisms involving IL-10 [289-292]. It is worthwhile to note that in addition to IL-10 production, regulatory cells utilize cell contact dependent-dependent mechanisms [141, 293] including CD40 and IL-21 [141], and other cytokines, such as IL-35 [140] to suppress EAE. These results suggest that it is not simply the presence of IL-10 that limits disease, but rather regulatory cells that express IL-10 are the key to controlling aberrant immune responses.

The role of IL-10 producing B cells in EAE is complicated by several factors. First, most B cells in mice are capable of producing IL-10 upon stimulation with LPS or CpG, including lymph node, follicular, marginal zone and peritoneal B1 B cells [294]. In fact, the mycobacterium adjuvant used to induce EAE activates TLR2/4 to trigger expression of IL-10 in B cells [294]. In the MOG 35-55 EAE model, B cells play a protective role. In fact, depletion of B cells by CD20 prior to EAE results in increased disease severity, but depletion of B cells after induction of EAE reduced disease [290]. In contrast, recombinant MOG 1-125 induces EAE [295] that is B cell dependent [296]. In this model, depletion of B cells by CD19 [297] or CD20 [195] reduces disease. In line with these observations, the MOG 1-125 EAE model, but not MOG35-55 EAE model requires expression of activation induced cytedine deaminase (AID) which mediates SHM and class switching to achieve maximum

disease and infiltration of B cells into the brain and spinal cord [298]. However, while B cells contribute to EAE in the MOG model, they are not sufficient to induce EAE upon adoptive transfer [299].

7.5 IL-6 and IL-10 in MS

The role of IL-6 and IL-10 in MS has been studied study in humans, as well. Most studies focused on the potential impact of IL-6 and IL-10 on immune cells, but resident central nervous system cells are also capable of secreting and responding to cytokines. The interaction between the immune system and nervous system is not well understood, however several studies in EAE have identified links between the two. For example, human astrocytes are capable of secreting IL-6 [300] and IL-10 [301]. In one study, Colombatti *et al* found that myelin basic protein (MBP)-specific T cells induced IL-6 expression in an astrocyte cell line *in vitro* [302]. Intranasally transfer of microglia exposed to IL-10, IL-4 and TGF-β secreted less IL-6 and dampened EAE severity [303]. IL-10 in rats can regulate neuronal stem cells [304]. Moreover, IL-6 is linked to other neurological disorders (as reviewed by [305, 306]). The complex interactions between the immune system and central nervous system are active areas of study.

A multitude of studies have focused on serum, plasma, or cerebrospinal fluid (CSF) cytokine levels to predict conversion to MS, or MS relapses. Some studies have identified elevated IL-6 in the CSF and elevated IL-10 in the serum of MS patients compared to controls; however these results have not been consistent [307-313]. In one study, IgG correlated with IL-10, but not IL-6, levels in the CSF [314]. Moreover, IL-6 can cross the

blood brain barrier [315] and is sufficient to drive Ig production by CSF derived B cells [316].

The CSF gives researchers a window into the CNS compartment of MS patients, but may not be representative of the site of demyelination in multiple sclerosis lesions. In order to determine whether IL-6 and IL-10 are features of MS lesion pathology post mortem brain tissues can be stained by immunohistochemistry or interrogated to determine gene expression. IL-6 transcripts were significantly increased in MS lesions compared to non-MS brain tissue; whereas IL-10 transcripts were not detected in either MS or control tissues [317]. However, in another study, IL-10 and IL-10R were localized both to astrocytes and macrophages in MS lesions [318]. In a separate study, IL-6 co-localized with GFAP+ astrocytes and in a few cases with CD68+ macrophages [319]. Interestingly, in another study, IL-6+ cells increased with the severity of demyelination, but was more prevalent in inactive lesions and those lesions with less oligodendrocyte loss [320]. These observations collectively suggest that IL-6 and IL-10 are present in MS lesions and that cells at the site of demyelination are capable of producing IL-10 and IL-6 and sensing IL-10.

7.6 Production of IL-6 and IL-10 by other immune cells in MS

B cells are not the only cell type in MS that exhibits dysregulated IL-10 production. Myeloid cells act as APC and secrete a broad spectrum of cytokines that influences T cell polarization, B cell responses and inflammation. What is perhaps most interesting about innate immune cells in MS is the differences in cytokine production driven by conserved elements through TLR. It is not entirely clear how the autoimmune environment influences innate immune

cells, but multiple studies supporting the existence of subtypes of monocytes and dendritic cells that are programmed to secrete specific cytokines, much like the Th subtypes, may play a role in the differential cytokine expression observed in these cells.

Several studies in humans have focused on the cytokine profiles of myeloid cells that can exert profound effects on T cell polarization. Monocytes are a major source of IL-6 and can produce IL-10. In PBMC cultures, monocytes produce more IL-6 when stimulated in the presence of MBP and polyclonal stimulation in MS patients, but IL-10 was not evaluated in this study [321]. CD14+CD16+ monocytes are preferentially expanded in MS patients and express higher levels of activation markers compared to HD [322]. In the same study, both CD14+CD16+ and CD14+CD16- classical monocytes from MS patients secrete more IL-6 compared to HD when stimulated with LPS, but CD14+CD16+ monocytes secreted significantly more IL-10 compared to HD [322]. This is surprising because the CD16+ cells are largely thought to be a pro-inflammatory monocyte subset that produces less IL-10 than the classical CD14+CD16- monocytes [323].However, in another study, monocytes from MS patients expressed less TLR7 and secreted less IL-6 upon TLR7 stimulation [324]. This suggests that monocytes may produce imbalanced levels of IL-6 and IL-10, but is dependent upon the stimulation conditions.

Dendritic cells (DC) are highly potent APC that exert profound effects on T cell polarization. In MS patients the frequency of DC producing IL-6 is significantly elevated, while those producing IL-10 are similar [325]. However, spontaneous secretion of IL-6 and IL-10 was lower in MS patients' plasmacytoid dendritic cells (pDC) particularly after stimulation with double strand DNA compared to HD [326]. This may be attributed to the

observation that, despite the lowered pDC cytokine production, the frequency of myeloid dendritic cells (mDC) that secrete IL-6 following TLR7 stimulation is increased in MS patients, but not elevated in pDC [327]. mDC incubated with GA produce less IL-12, more IL-10 [221] and induce more Th2 cells [328].

There have been limited studies specifically focused on Tr1 cells in MS. The induction of Tr1 cells and IL-10 secretion is reduced in T cells from MS patients that have been stimulated with anti-CD3 and anti-CD46 [88]. Similarly, Treg expansion measured by the percentage of IL-10+ cells and secretion of IL-10 induced by *in vitro* stimulation with anti-CD28, -CD3 and -CD46 is defective in MS patients [89]. In helminth-infected MS patients, there is an increase in MBP-specific T cells that secrete IL-10, and the absolute frequency and percentage of CD25+ T cells is elevated [329]. Treg are expanded in the CSF of helmnith-infected individuals [330]. Together these studies showed that *in vitro* differentiation of Tr1 is defective in MS patients. It is unknown whether these *in vitro* defects are reflected by impaired Tr1 function *in vivo* [331], could be an effective therapeutic approach for MS.

Although T cells secrete IL-6, reports on T cell derived IL-6 are limited as the focus has largely been on the ability of IL-6 to influence Th17 development. In one study, researchers found that *ex vivo* stimulation of MS patients' T cells with concanavalin A (ConA) or anti-CD3 produce less IL-6 [332]. In contrast, more recent studies found that CD4+ and CD8+ T cells from MS patients produced more IL-6 when stimulated in the presence of MBP and polyclonal stimulation [321]. Not only are the levels of IL-6 elevated but a separate study found that the temporal control over IL-6 production was also altered

MS patients. CD4+ and CD8+ T cells produce IL-6 at an accelerated rate in MS patients, after only a few hours of anti-CD3 stimulation, while T cells from HD do not make detectable IL-6 until 24h after stimulation [333].

7.7 The Impact of Glatiramer Acetate on B-cell Proliferation

Glatiramer acetate was the first therapeutic approved for the treatment of MS. Despite its long history, the impact of GA on B cells has not been investigated in humans beyond the induction of GA-specific antibodies in patients on GA therapy. GA directly influences monocytes, macrophages and dendritic cells. One study found that in monocytes, GA induces PI3Kδ, AKT and MEK/ERK activation, likely in an HLA-DR-independent manner [334]. PI3Kδ [335], AKT [336] and MEK/ERK [337] are important proximal signaling intermediates in B cell activation and induction of proliferation.

Purified human B cells are capable of binding GA. The majority of B cells bind low molecular weight GA <7kDA (unpublished observations), while a subset of memory B cells exclusively binds high-molecular weight GA [194]. In another study, fractions of B cells that bound GA at a high level did so via the BCR [284]. We hypothesized that GA might influence activation of memory B cells by altering BCR signaling. However we found that Glatiramer acetate had no direct impact on memory or naïve B cell proliferation *in vitro.*

When we examined proliferation by total B cells in HD, TN-MS and GA-treated MS patients we found that high-dose CD40L stimulation alone or combined with IL-4, IL-4/BCR or IL-2/CpG increased proliferation by HD B cells. We found this same result in total B cells from MS patients except in the case of IL-2/CpG. In contrast, B cells from GA-MS patients

failed to increase proliferation when high-dose CD40L was combined with other stimuli. It stands to reason that GA therapy may alter pathways that are important for integrating these activation signals and other key immunological genes that are modulated by GA therapy [338, 339]. However, GA- and TN-MS patients showed similar defects in their response to high-dose CD40L combined with other stimuli compared to HD. This was similar to the trend we observed in memory B cell cultures from MS patients so it appears that while GA therapy has profound effects on B cell function, GA does not completely normalize B cell proliferation.

7.8 Modulation of B-cell Cytokine Production by Glatiramer acetate

We hypothesized that GA therapy would modulate cytokine production by restoring the ability of B cells to secrete IL-10 and diminishing the ability of B cells to secrete IL-6. We found no direct modulation of IL-10, IL-6, TNF-α, or LT-α production when purified memory or naïve B cells from MS patients or HD were exposed to GA *in vitro.* In contrast, one recent study found that exposure to GA in three of 6 patients reduced levels of TNF-α and IL-6 secreted by total B cells stimulated with through CD40 or CD40 plus BCR [340].

Next we tested whether B-cell cytokine production was altered in patients receiving GA therapy. In contrast to the results using sort-purified memory and naïve B cells, we found that total B cells from TN-MS patients produced significantly elevated levels of LT-α, which is in agreement with previous observations [138, 194]. While the level of LT-α was significantly higher in GA treated patients in response to CD40L alone, B cells from the same donors failed to induce LT-α production when CD40L was combined with other

stimuli and secreted levels similar to HD. LT-α plays an important role in some EAE models, but its role in MS is unknown [341]. The differential temporal regulation observed here suggests a more nuanced regulation than previously described.

We found that IL-10 production was restored to a level similar to HD in all patients on GA. While we observed GA-MS patients' B cells produce more IL-10 after 3-5 days of *in vitro* activation, irrespective of the length of GA therapy, the frequency of B10 and $B10_{PRO}$ cells detected by flow cytometry was not significantly different than TN-MS patients at *ex vivo* or after 48 hours post-stimulation. In fact, we found that in stimulated cultures, GA-treated patients had fewer $B10_{PRO}$ cells compared to HD. IL-10 production is the defining feature of B10 and $B10_{PRO}$ cells and human IL-10 producing B cells are not uniquely confined to any phenotypic population [160]. We are not aware of studies that have directly compared the ability to secrete IL-10 with intracellular detection of IL-10 by flow cytometry in the same donors. Our findings suggest that there is a discrepancy in these two detection techniques and such a study would be quite informative.

We also found that GA therapy transiently suppressed IL-6 production by B cells in patients on therapy for less than 32 months. Those patients on GA therapy for more than 32 months secreted more IL-6 than untreated MS patients. Detection of cytokine-producing B cells by flow cytometry revealed a higher frequency of IL-6+ cells from HD *ex vivo* compare to TN-MS and GA-MS patients. After a 48-hour stimulation; however, we found that TN-MS patients had a higher frequency of IL-6+ cells compared to GA-MS patients and a higher intensity of IL-6 staining. This supports the notion that dysregulated integration of B cell signaling pathways results in high levels of IL-6.

The role of IL-6 in GA therapy is much less clear because IL-6 can also support TH2 differentiation in T cells by inducing expression of IL-4 and blocking IFN-γ signaling [342, 343]. This makes GA a particularly interesting therapy to study the dual role of IL-6 in supporting both TH2 and TH17 responses in MS. In fact, some GA-specific T cell clones secrete IL-6 [344]. Whether these alterations can be used to guide therapeutic options remains to be seen. A larger longitudinal study is warranted to assess the correlation between B cell cytokines, immunoglobulin production and the effects of GA as a disease modifying therapy.

We sampled two patients who experienced exacerbations while on GA therapy during relapse. These patients harbored B cells with a severe deficit in IL-10 production. No IL-10 was detectable in the culture supernatants from these patients. The sample patients produced high levels of IL-6 *in vitro* similar to untreated MS patients. Whether this represents a failure of B cells to respond to GA would require a larger cohort and longitudinal follow-up. However, of the patients sampled for this study, 18 are still on GA therapy and have not experienced exacerbations or new MRI activity in the 1-2 years since samples were acquired. This includes the patients with high levels of IL-6 production by B cells. This would suggest, at least, that high IL-6 production, by itself, is not indicative of failure on GA therapy. Of the 21 patients in this study, three of them are no longer on GA therapy, but have switched to new therapies. None of these three patients had high levels of IL-6 production in our assay. Of those three patients who are no longer on GA therapy, one progressed to SPMS, another showed Gd+ lesions and is now on Tysabri (an α4 integrin neutralizing antibody), the last

experienced new symptoms but was negative for MRI and is now on Tecfidera (a fumaric acid). Three additional patients were lost at follow-up.

7.9 Modulation of IgG secretion by Glatiramer Acetate Therapy.

Most surprisingly we identified a dramatic enhancement of IgM and IgG secretion by B cells from patients on GA therapy when stimulated *in vitro*. These results were not reflected in the serum of the same patients, who had similar levels of immunoglobulin compared to HD and TN-MS patients. Both IL-6 and IL-10 are important for supporting plasma cell development and one report indicated that IL-10-producing B cells in mice went on to become IgM and IgG secreting plasmablasts and plasma cells and produce significant levels of serum Ig.[161]. We reasoned that IL-10 production might correlate with Ig secretion.

We observed a significant correlation between IL-10 production and IgG in some, but not all, conditions. For example, levels on day five of culture with high-dose CD40L and IL-2/CpG, there was a significant positive correlation between IL-10 and IgG (R^2=0.88, p=0.0003). We did not evaluate whether blocking IL-10 or IL-6 in cultures with GA-MS B cells would inhibit the enhanced Ig production we observed, nor did we measure other factors such as IL-21 or BAFF that might enhance differentiation into antibody secreting cells (ASC). We were not able to measure whether the antibodies produced by B cells from GA-MS patients were reactive to GA or if they were of a specific Ig subclass, such as IgG2 or IgG4, that have been associated with GA therapy. These questions regarding Ig production

are particularly interesting because IgG4 production is enriched in IL-10 producing B cells [153] and IgG4 is prevalent during GA treatment.

7.10. GA therapy modulates the distribution of B cells

To better understand how GA might influence the B cell compartment, we undertook studies to phenotype B cell populations from GA-MS patients. We found that GA therapy reduced the total CD19+ B cell frequency, plasmablasts ($CD27^{hi}$), and class-switched memory B cells. These results are somewhat unexpected in the context of the elevated Ig secretion we found after *in vitro* culture. This suggests that GA might influence the ability of B cells to differentiate into ASC from a naïve or memory state. In contrast to our results, Haas *et al.* described no changes in B cell subsets in patients on GA therapy [123]. While the gating strategy for identifying IgD- and CD27-expressing subsets class was similar, we utilized CD19 to identify B cells as the parent gate for all other subsets while Haas *et al.* used CD20. In addition both cohorts contained 10-15 patients in the GA-treated groups and could be reflective of heterogeneity in human subjects. Additionally, the distribution of B cells is not immediately altered by GA therapy as it is for some populations of T cells within 12 hours of initiating therapy [213]. There is an inverse correlation between the frequency of class-switched memory B cells in the peripheral blood of MS patients compared to the CSF in treatment-naïve MS patients [123]. Whether B cell frequencies that are affected by therapies in the periphery modulate the composition of the CNS is not known.

We also reported that class-switched memory B cells from GA-MS patients expressed less HLA-DR compared to TN-MS patients. This is an interesting finding because memory B cells actually express less HLA-DR compared to naïve B cells and are the type of cell that

binds high molecular weight GA [194]. It would be interesting to investigate whether memory B cells from GA-treated patients have an increased capacity to elicit GA-specific responses by T cells.

The memory B cell pool also harbors the majority of B10 and $B10_{PRO}$ cells [152], and since the frequency of CD27+ memory B cells is decreased in GA-MS patient peripheral blood, it is likely that this could account for the similar frequency of IL-10 producing B cells in the GA-MS patients and the control cohorts that we observed by intracellular staining for IL-10. The naïve B cell pool may harbor IL-10 producing B cells, but they likely require longer periods of stimulation than what we did here in order to induce toll-like receptor 9 (TLR9) expression and subsequently respond to CpG [271]. Whether GA-induced IL-10 producing B cells can control the balance of inflammatory and regulatory T cells remains unknown.

7.11 GA Modulates B-cell Function in EAE

At the time these studies began, the impact of GA on B cells was entirely unknown. Since then, several groups have demonstrated a remarkable impact of GA on B cells in mice. Naïve mice treated with GA yield GA-conditioned/primed B cells. These B cells have lower expression of CD80 and CD86 co-stimulatory molecules, but similar expression of MHCII on compared to naïve mice, and secrete more IL-10 when stimulated *in vitro* with CD40 and GA [256]. GA-conditioned B cells induced less proliferation by MOG-specific TCR transgenic T cells from 2D2 mice in response to MOG35-55 compared to ovalbumin-primed B cells [256]. Whether GA has a direct impact on murine B cells is not known, but this data

suggests that even in the absence of an autoimmune condition, GA exerts an influence on B-cell function.

In the context of the MOG35-55 EAE model, administration of GA enhanced IL-10 producing B cells, particularly in the CD5+ B cell pool that are associated with regulatory function [254]. However the frequency of CD5+ B cells was unaffected [255]. B cells isolated from EAE mice treated with GA expressed less IL-6, TNF-α and IL-12 *ex vivo* [255]. In the same study, after *in vitro* stimulation, levels of IFN-γ, TNF-α and IL-6 produced by B cells were diminished while IL-13 and IL-10 were enhanced in GA-treated EAE mice. In fact, one study found that GA treatment required B cells to fully suppress EAE [256]. These data support our findings in humans that GA modulates IL-6 and IL-10 production by B cells when those B cells are exposed to GA *in vivo* and suggest that B cells could contribute to the therapeutic affects of GA.

GA-conditioned B cells reduced EAE severity. Adoptive transfer of CD5+ B cells from GA-conditioned mice reduced EAE scores and delayed disease onset significantly and to a greater extent than CD19+ B cells from a naïve mouse and CD5- B cells from GA-conditioned mice [254]. Adoptive transfer of GA-conditioned B cells reduced CNS pathology, reduced CNS CXCR5 expression, and increased brain derived neurotrophic factor (BDNF) that is associated with protective responses [254]. In addition, IL-10 and IL-13 production was enhanced in the spleen and cervical lymph nodes as was expression of STAT6, a key molecule in the induction of Th2 responses [254].

Adoptive transfer of resting CD43- GA-primed B cells resulted in decreased frequencies of peripheral splenic CD4, CD8, CD11b and CD11c+ cells and decreased MOG-

specific recall responses in the spleen and lymph nodes [256]. MOG-specific recall responses in the spleen and lymph node from animals with adoptively transferred GA-primed B cells showed less IFN-γ and IL-17 secretion and significantly enhanced IL-10 production [256].

Of interest is the impact of GA on B cell survival and differentiation factors. BAFF and APRIL were reduced in the CNS and spleens of GA treated EAE mice, respectively [255]. The level of BAFF-receptor (BAFF-R) on B cells was increased by EAE and normalized by GA treatment, but TACI, BAFF and APRIL receptor was reduced by EAE and showed no difference in GA treated animals [255]. GA-primed B cells induced increased serum α-MOG IgG1 and IgG2a, but decreased IgG2b during EAE [256]. This suggests that GA also influences factors that drive plasma cell development and potentially ties into the observations we made regarding elevated Ig secretion *in vitro*.

7.12 Establishing a Baseline for B and T cell Input for B-T cultures

We did not detect changes in the make-up of the B cell compartment in HD compared to TN-MS patients suggesting that the input of memory and naïve B cells was approximately equivalent. Previous studies in our laboratory found that the expression of CD80, HLA-DR and CD40 are also similar in HD and MS patients' naïve and memory B cells ([192, 194] and unpublished observations). Furthermore we did not detect B cell proliferation to antigens in culture without T cells. The frequency of MOG, MBP, GA, and TT-specific B cells is similar between HD and MS patients, although memory B cells are enriched for GA and MBP-reactive cells [194].

To determine if the input of T cells was similar in B-T cultures and to identify changes in the phenotypic distribution of the T cell compartment, we used flow cytometry to

detect changes in T cell populations. We found that TN-MS and GA-MS patients harbored elevated frequencies of CD3+ T cells and TN-MS patients had more CD4+ T cells. These are not widely supported by findings in other studies. However we detected this increase in samples obtained by leukapheresis (TN-MS) and peripheral blood draws (GA-MS), which suggests that these findings are not the result of different sampling techniques. There were differences between HD and GA-MS patients in a variety of T cell subsets. A previous study found that both CD4+ and CD8+ naïve CD45RA+ T cells were elevated in GA-treated MS patients. Aside from the proportion of CD4+ T cells within the CD3+ T cell subset, we only detected increases in the frequency of memory CD4+ and CD8+ CXCR3+ T cells in MS patients. This suggests that the T cell pool in MS patients is enriched for effector memory cells with enhanced migratory capabilities to sites of inflammation.

Despite the differences observed in memory T cells based on phenotypic data, we observed little difference in the frequency of INF-γ or IL-17A-secreting antigen-specific T cells by ELISpot or ex vivo secretion by flow cytometry. As outlined in the review of the literature, many techniques have been applied to identify the antigen-specific T cell frequency with wide ranges of specificities reported. We chose to assay using ELISpot and intracellular staining for IFN-γ and IL-17A because 1) we used those cytokines as a readout in B-T co-culture assay 2) needed to establish a baseline and their pathogenic relevance to MS and 3) to inform the differences between spontaneous potential and antigen-driven cytokine responses.

Using *ex vivo* intracellular staining revealed that the magnitude of the IFN-γ and IL-10 response by CD4+ T cells was lower in MS patients. However, we observed a significant

elevation in IFN-γ+ MOG-specific T cells from MS patients in this assay. Importantly, proliferation in the B-T co-culture did not correlate with the input of antigen-specific T cells. This suggest that the magnitude of the response in B-T assays is not entirely based on the frequency of antigen-specific T cells because we failed to detect similar proliferation to foreign antigen when using naïve B cells from MS patients as an APC. We also detected similar proliferation to neuro-antigens in PBMC cultures, which indicate that the proliferation we observed in B-T co-cultures was not simply a result of the frequency of antigen specific cells.

7.13 B-T co-cultures

To understand how B cells uniquely support antigen-specific T cell responses we used PBMC as a comparator. We observed that PBMC cultures from HD and MS patients induced similar proliferation to neuro-antigens. We observed a significant increase in the CD4+ T cell proliferative response to foreign antigens. While we confirmed that MS patients' B cells responded similarly to foreign antigens in culture alone, we did not test this in isolated non-B cell APC. While we did not carry out cultures with B cell-depleted PBMC, another study found that in cultures lacking B cells, GA, phytohaemagglutininin (PHA), and α-CD3 stimulation failed to induce the same extent of CD4+ and CD8+ T cell proliferation observed in non-depleted cultures [138]. In the same study, B cells MS patients required B cells were required for maximal proliferation in the PBMC cultures when stimulated with low-dose anti-CD3.

We did not observe robust proliferation to MOG35-55 in PBMC cultures in line with several other studies that found MOG35-55 does not stimulate maximal proliferation by PBMC and that recombinant MOG 1-125 or full length MOG induce more robust proliferation [78, 345]. We anticipated lower proliferation in response to MOG 35-55 than to MOG 1-125, but were surprised to find that the response to MOG35-55 was similarly low, if not entirely absent in most cases, in both PBMC and B-T cultures. By ELISpot, a similar frequency of MOG35-55 and MOG1-125 cytokine secreting cells was observed. However, we used a higher concentration of antigen in ELISpot culture and it may be that the concentration of MOG35-55 in this culture system was too low to drive proliferation.

We confirmed our previous results that memory B cells from MS patients support autologous CD4+ T cell proliferation to neuro-antigens. However, we also found that naïve B cells from MS patients induce more proliferation in response to foreign antigens and that more donors responded to neuro-antigens when cultured with naïve B cells. These differences are likely due to two changes in the methodology. First, cultures with foreign antigen were carried out with a wider variety of antigens that included flu and RSV whereas the first study used tetanus toxoid and mumps virus separately. Second, to allow sufficient time for T cell proliferation and intracellular cytokine detection, assays were carried out in seven instead of five days. We did not evaluate B cell markers on day seven so this latter point may have allowed B cells to develop an activated phenotype and allowed them to more efficiently influence T cell proliferation and activation.

We did not observe enhanced proliferation to neuro-antigens by CD4+ T cells in PBMC cultures. From these results we can conclude that in this culture system, proliferation

is uniquely supported by B cells. This is likely due to the ability of B cells to take up specific antigen via the BCR and present it to CD4+ T cells [346]. In addition, we found that naïve B cells supported significantly more IL-17+ CD4+ T cells in response to neuro-antigen compared to HD. These results are quite surprising given the low level of IL-17A-secreting cells we observed in the spontaneous *ex vivo* assay. The fraction that had undergone proliferation in the neuro-antigen, but not foreign antigen, cultures showed a significant enrichment for both IFN-γ+ and IL-17+ cells in both memory and naïve B-T cultures. Together, these results indicate that the B cells from MS patients do, in fact support antigen-specific Th1 and Th17 responses and are capable of expanding these cells *in vitro*. Given the enrichment of IL-17+ $CFSE^{high}$ cells in naïve B-T cultures from MS patients, it might also be that naïve B cells support Th17 responses that are independent of proliferation.

We observed elevated IFN-γ, IL-17 (A and F), IL-21, and IL-22 in memory and naïve B-T co-cultures from MS patients in response to neuro-antigens. The elevation of these Th1 and Th17-associated cytokines underscore the observations we made by intracellular staining. In addition we observed elevated IL-23, TNF-α and IL-6 in naïve but not memory cultures. It is not clear whether these which play a role in Th17 differentiation support the expansion of Th17 cells in these cultures. It is also important to note that we observed IFN-γ production in these cultures, which can have inhibitory affects on developing Th17 cells. However, we cannot confirm whether the cells that proliferated or secreted cytokines were already of a memory phenotype.

7.14 Potential impact of B cells on Th17 Responses

In HDs, the frequency of memory B cells in the peripheral blood positively correlates with *ex vivo* IL-17 production by CD4+ T cells [347]. In contrast, patients with the primary immunodeficiency, X-linked agammaglobulinemia (XLA), who have few B cells, have a reduced frequency of peripheral IL-17- and IL-22-producing T cells when stimulated *ex vivo* with the potent cytokine inducers, PMA and ionomycin [347]. In patients with common variable immunodeficiency disorder (CVID), who have a dramatic reduction in B cell maturation (memory and plasma cells), the frequency of CD4+ IL-17 producing T cells is negatively correlated with the number of immature B cells and HLA-DR (MHC II) expression by B cells [347]. These correlative data indicate a potential link between B cell memory APC capacity and the generation or maintenance of Th17 cells.

Evidence from infectious disease models more conclusively point to a role for B cells in bolstering Th17 responses. Healthy PBMCs depleted of B cells and incubated with *C. albicans,* a known stimulator of Th17 responses, showed dramatic reductions in IL-17 (~50%) and IL-22 (~20-30%) production, while IL-17 and IL-22 production by PBMCs from XLA patients were unaffected by B cell depletion [348]. Human Th17 responses to *C. albicans in vitro* require memory T cells and APC [349]. This indicates possible B cell involvement with antigen-specific memory Th17 responses; conversely, it does not preclude the notion that B cells indirectly modulate Th17 cells by influencing other immune components, such as macrophages that are important for Th17 responsiveness to *C. albicans*.

Studies of autoimmune disorders revealed that B cell depletion also decreased Th17 responses in mice [195] and in humans [138, 350, 351]. Rheumatoid arthritis patients treated with B cell-depleting therapy show decreased IL-17 and IL-22 production as well as

decreased expression of RORγT (Th17 transcription factor), but not of IFN-γ or TNF-α in the synovial tissue [351]. One case study showed similar reductions of Th17 cytokines in the peripheral blood of RA patients [350]. In MS patients, therapeutic B cell-depletion reduced total CD4+ and CD8+ T cell proliferation and the proliferating fraction of CD4+ T cells that produced IFN-γ or IL-17 upon *ex vivo* polyclonal stimulation [138]. In the same study, TNF-α and LT-α from supernatants of autologous CD40/BCR activated B cells could partially restore T cell proliferation and IFNγ production in B cell-depleted T cell cultures from MS patients [138]. While these studies provide evidence that the presence of B cells is required for fulminate Th17 responses, the absence of B cells is distinct from direct B cell effects on Th17 cells. To our knowledge our data is the first evidence that specific interactions between B and T cells results in the expansion of IL-17+ and IL-17-secreting cells in response to neuro-antigen.

7.15 Detection of Regulatory and Other Cytokines in B-T Cultures

In addition to inflammatory cytokines in the B-T culture supernatants, we identified the production of regulatory factors. We observed elevated levels of IL-10 and IL-25 (IL-17E, a Th2-associated cytokine). We did not examine FOXP3 expression or intracellular IL-10 to determine whether B cells also supported simultaneous expansion of Treg. This is a distinct possibility as the expansion of anergic or regulatory cells is the most common finding when using activated or allogenic B cells as an APC in the absence of specific antigen. However, when cognate antigen is present it appears to change the role of B cells from inducing regulatory responses. For example, B cells from patients who had been infected

with *Leishmania panamensis* could support both regulatory, Th1 and Th2 responses in autologous T cells [352]. Observations in mice using models of autoimmunity and infection have found reasonable evidence that B cells are involved in directly influencing the activation and fine-tuning of the T cell compartment as reviewed by Lund and Randall [263] and point to a role for antigen-specific B cells as APC. This is further underscored by crossing MOG B and T cell transgenic mice [353] that result in CNS pathology and the role for MHC II in MOG 1-125 induced EAE [282].

In addition we detected elevated IL-33 in these cultures. IL-33 is protective in EAE [354], and is also elevated in the serum and PBMC from MS patients and is present MS lesions [355]. However, its role in antigen-specific responses has not been thoroughly characterized. IL-31 was elevated in naïve B-T cultures with neuro-antigen. This cytokine plays an unknown role in MS, although it is related to the IL-6 family and appears to be associated with Th1 responses [356]. Further studies are needed to elucidate the role of these cytokines in the context of MS.

7.16 CD8+ T cells in B-T co-cultures

We observed less CD8+ T cell proliferation in naive B-T cultures, but not memory cultures. B cells can present antigen to CD8+ T cells to induce regulatory-like and effector cells [357, 358]. We cannot exclude the possibility that CD8+ neuro-antigen specific T cells are acting in a suppressive or inflammatory manner in B-T cultures; however during remission, the CD8+ T cell suppressive capabilities are similar between HD and MS patients[101]. It is also important to note again that in B-T cultures blockade of HLA-DR

also impacts CD8+ T cell proliferation with CD40-activated B cell [192] suggesting that CD8+ T cell proliferation results from the activation of CD4+ T cells in an HLA-DR-dependent manner. If this is the case, the reason that CD8+ T cells did not proliferate may be due to HLA-DR-independent effect of naïve B cells on CD8+ T cells.

7.17. IL-6 and IL-10 Receptor signaling in MS and the impact of IL-6 on B-T cultures

We observed a significant elevation in IL-6 production by B cells from MS patients, particularly from naïve B cells. To determine whether IL-6 preferentially influenced proliferation by T cells in naïve B-T cultures we neutralized binding of IL-6 to its receptor with an anti-IL-6R antibody. First, we evaluated the expression of IL-10R, IL-6R and gp130 on lymphocytes. We found that CD4+ T cells expressed higher levels of IL-6R in line with previous studies [268, 359]. However we also found that IL-6R, IL-10R and gp130 are expressed at a higher level on CD8+ and CD4+ T cells, and B cells in MS patients compared to HD. This suggests that cells from MS patients are poised to respond to both IL-6 and IL-10. Our results contrast previous reports that found IL-10R levels on total PBMC were similar between HD and MS patients [89]. The IL-6Rα antibody used in these studies may be capable of binding IL-6Rα in complex with IL-6 and associating with membrane-bound gp130.

Several observations made in T cells underscore the potential importance of overproduction of IL-6 and a deficit in IL-10. IL-6 binds to IL-6R expressed on the cell surface and associates with gp130; however, both of these receptors are also found in soluble form (sIL-6r and sgp130, respectively). IL-6 in complex with sIL-6R can interact with

membrane-bound gp130 to initiate trans-signaling [360] a process that is antagonized by the presence of sgp130 [361]. IL-6 or IL-6R trans-signaling activates Janus kinase 1 (JAK1), Growth factor receptor-bound protein 2(GRB2), PI3Ks and mitogen activated protein kinases (MAPKs). IL-10 binds to IL-10Rα/β heterodimers on the cell surface to signal though JAK1 and tyrosing kinase 2 (TYK2). IL-6Rin complex with gp130 and IL-10 receptor (IL-10r) signal by activation of Stat3 though phosphorylation (p-STAT3). STAT3 activation induces the expression of suppressor of cytokine signaling 3 (SOCS3), which acts as a potent negative regulator of IL-6 signaling. So despite having overlapping signaling molecules, the program induced by IL-6R and IL-10R are quite distinct.

What is perhaps most surprising is that exposure to IL-6 appears to render T cells from MS patients resistant to Treg/Tr1-mediated suppression. The effect may be compounded by the diminished ability for T, B, and innate immune cells to secrete IL-10 and further preclude naturally occurring regulatory cells from controlling encephalitogenic T cells. This may have a profound affect on the immune response in the context of autoimmunity.

Evidence for IL-6 in resistance to regulatory cell-mediated suppression is first supported by elevated expression of the IL-6R on CD4+ T cells [268, 359], CD8+ T cells, B cells, and monocytes in MS patients compared to HD [333]. The baseline level of pSTAT3 is elevated in monocytes, CD4+ and CD8+ T cells in patients with MS or at high risk to convert to MS and is correlated with disease activity [362, 363]. However, studies have identified elevated SOCS3 transcripts in PBMC from MS patients [268, 364].

To understand how IL-6 impacts T cell responses in MS patients, several studies have isolated T effector cells and incubated them with Treg or Tr1 cells. In one such report, Schneider *et al* found that CD4+ T cells and PBMC from MS patients have elevated p-STAT3 after exposure to IL-6, but not IL-10, compared to HD [268]. The level of pSTAT3 and IL-6R, but not gp130, correlated with the resistance to Treg mediated inhibition in cultures stimulated with anti-CD3 and anti-CD28. This resistance to Treg-mediated suppression was reversed by an inhibitor of STAT3 and unaffected by trans-signaling mediated by soluble IL-6r in complex with IL-6 [268]. Furthermore, MS patients who are resistant to dexamethasone, a cortical steroid, continue to produce IL-6 upon stimulation with LPS with dexamethasone *in vitro* [365]. CD4+ and CD8+ T effector cells from MS patients continued to produce higher levels of IL-6 and proliferate when stimulated by anti-CD3 in the presence of CD4+CD25+FoxP3+ Treg as compared to HD[333].

This resistance to Treg suppression was reversible with the addition of a blocking antibody for IL6R (Toclizumab) or an PKB/c-Akt inhibitor that blocks part of the IL-6R signaling cascade. Interestingly, the presence of IL-6 (provided by MS T effector cells in a transwell assay or exogenous IL-6) induced expression of IL-6R in HD T effector cells that conferred a transient resistance to Treg. There is also an apparent defect, not only in the ability of MS T cells to differentiate into Tr1 *in vitro,* but also to respond to IL-10. T effector cells from MS patients were incubated with supernatants from Tr1 cultures or recombinant IL-10 were insensitive to IL-10 mediated inhibition as measured by proliferation. MS patients' CD4+ and CD8+ T cells failed to induce pSTAT3 to the level of HD after incubation with IL-10 [89]. Despite this reduced response to IL-10, IL-10R and STAT3

transcripts were significantly induced in PBMC after 24h stimulation with IL-10. *In vitro* treatment of cells with GA inhibited p-STAT3 and Th17 differentiation in Th17-polarizing conditions [226]. Interestingly, these studies are similar to IL-6 mediated resistance to IL-10 in RA patients [366]. Taken together these studies highlight the deficiencies in production of and response to IL-10 while the IL-6 response is hyperactive and provide additional rationale for the possible role of dysregulated cytokine secretion by B cells in MS pathoetiology.

We observed significantly more IL-6 in naïve B-T cultures from MS patients in the presence of neuro-antigen. In combination with our results showing that naïve B cells from MS patients secreted high levels of IL-6, we hypothesized that blocking IL-6 signaling in naïve B-T cultures would inhibit T cell proliferation. Neutralization of IL-6 in B-T cultures inhibited proliferation in the majority of cultures, but did not influence cultures from HD or MS patients differently. The affect of neutralizing IL-6 was not unique to either set of antigens and suggests a role for IL-6 in supporting T cell proliferation. Preliminary analysis of cytokine profiles in these cultures did not reveal an imbalance in cytokines in the supernatants. Another study using allogeneic T cells with monocyte-derived dendritic cells to model graph versus host disease found no difference in Treg, Th1, or Th17 cells when incubated with Toclizumab [367]. Studies in mice suggest that IL-6r and IL-6 trans-signaling have different roles for naïve and effector T cell functions [368]. We did not characterize sIL-6R or gp130 or their impact on B-T cultures in the studies presented here. Further study is needed to determine whether IL-6 blockade had a more nuanced affect on neuro-antigen specific T cell responses.

Several have proposed that the IL-6R targeting therapy Toclizumab should be used for treatment of MS. This drug, which showed efficacy in RA, juvenile idiopathic arthritis and a Castleman's disease also has been tried in neuromyelitis optica, a CNS autoimmune disease thought to be driven by antibodies against aquaporin 4. Based on the FDA report, MS has been reported in individuals while on Toclizumab. It is not known how IL-6 plays a role in MS, despite mounting evidence for its role in EAE and presence in MS lesions.

7.18. Allogeneic B-T cell interactions and in response to antigen

In order to determine whether there was a B or T cell intrinsic effect on the proliferation or cytokine production by T cells in B-T co-cultures, we used allogeneic B-T cell pairings in the presence of foreign or neuro-antigens. We observed little to no proliferation in B-T co-cultures using B and T cells from different donors in response to foreign or neuro-antigens. Several studies have used allogeneic B cells in culture with T cells. For example, B cells isolated from the synovial fluid of juvenile idiopathic arthritis patients induced IFN-γ secretion by naïve T cells [369]. Combined with previous observations that approximately 80% of the CD4+ T cell proliferation is HLA-DR dependent, and that IL-6 plays a similar role in MS patients and HD, we can further conclude that the results from the autologous B-T assays are, indeed, antigen specific.

7.19. Concluding remarks and future directions

The results from my experiments reported here further our knowledge about dysregulated functional responses by B cells in relapsing-remitting multiple sclerosis as it relates to

contributions to the inflammatory milieu and aberrant memory B-cell activation in response to CD40-stimulation. The contributions of altered B cell activation to MS are not known but evidence from the mouse model of EAE suggests they are paramount to understanding how fulminant CNS demyelination develops and is propagated. With the advent of B-cell depletion therapies, it is more important than ever to understand the role for B cells beyond that of antibody secretion.

We demonstrated, for the first time in humans, that the immunomodulatory therapy glatiramer acetate influences B cells. Glatiramer acetate remodeled the B cell compartment and restored IL-10 production by B cells but only transiently suppressed IL-6. We also observed a profound impact on the ability of B cells from GA-treated patients to secrete immunoglobulin after stimulation. How these observations might be used to guide therapeutic decisions will require a larger and longitudinal study.

Finally, we report for the first time that unstimulated naïve and memory B cells from MS patients support neuro-antigen specific Th17 responses. These responses were antigen-specific, required autologous B and T cells, and were not modulated by the presence of GA or neutralization of IL-6. Collectively, these results suggest that B cells play a larger role in MS than previously appreciated and may serve to contribute to the overall inflammatory environment in MS and the activation of encephalitogenic T cells.

The studies presented here identified B cells as possible contributors to the neuro-inflammation associated with MS because of their aberrant cytokine production, activation, and neuro-antigen presentation. Whether cytokine production is distinct from effector functions that require antigen specificity, such as APC function or antibody secretion remains

unknown, but is of critical importance to ensure proper targeting of potentially harmful B cell functions while leaving beneficial B cell functions intact. Current therapies for MS either deplete total B cells or systemically modulate the immune system as a whole, thus making the relative contributions of discrete B cell functions difficult to dissect. It is our hope that the data summarized in this document and the accompanying publications will convince the research and pharmaceutical community to develop more refined therapeutics in the future that specifically target these wayward B cell functions so apparent in MS patients.

B cell antigen specificity and dysregulated cytokine production as contributors to the neuro-inflammation associated with MS may be equally important and dependent on each other. B cells that are antigen specific are likely to become activated and traffic to the CNS where both antigen specific and cytokine related functions are required to support neuro-inflammation. In fact, our laboratory has demonstrated a specific pattern of mutations in the antibody genes of CSF B cells that have the potential to predict conversion from clinically isolated syndrome to clinically definite MS [106]. This is highly suggestive that antigen-specific B cells play a role in MS and can serve as a biomarker of disease. Yet, whether the contribution of these auto-reactive B cells is due to cytokine production, APC activity, Ig secretion, or some combination of all three remains to be elucidated. Such experiments are currently being pursued in our laboratory.

The mechanism(s) that support altered B cell activation in MS are an area of ongoing investigation in our laboratory, as well. Understanding the underlying pathways that allow B cells to become activated with a lower threshold of CD40-stimulation and culminates in imbalanced cytokine production is important to understanding how B cells initially become a

participant in autoimmunity and how to intervene with B cell activity once autoimmunity takes hold. If a specific molecule or pathway causes exaggerated B cell activation, it could represent a novel pharmacological target that could be exploited for treatment of MS or other disorders whose B cells are documented to be dysregulated in similar ways.

We found a significantly enhanced ability for naïve B cells from MS patients to support T cell proliferation in response to foreign antigens. This suggests that prior to antigen encounter, B cells from MS patients have an altered capacity to stimulate T cells. However, this effect was not simply due to IL-6 production by the naïve B cells propagating T cell proliferation since blocking IL-6 in the cultures did not completely abolish T cell proliferation. Understanding intrinsic B cell defects or alterations in B cell development that allow them to drive T cell responses that are usually less robust would greatly enhance our understanding of the role of B cells in MS.

Very few studies have addressed the impact of therapies on B cell functions. We found that GA therapy modulated the composition of the B cell compartment, the ability of B cells to proliferate and cytokine production by B cells. In fact, we found that IL-6 was suppressed during the first 32 months of GA therapy. Whether the restoration of IL-6 production by B cells can be used as a biomarker for patients who respond positively to GA therapy is being actively followed. It is also possible that the IL-6 produced by B cells from patients on GA for an extended period of time supports Th2, rather than pathogenic T cell responses. To determine if this is the case, B cells would need to be sampled prior to therapy, during the first 32 months of therapy and after 32 months of therapy and culturing them with T cells from the pre-treatment time point. We would predict that B cells sampled during

short-term therapy would no longer support pathogenic T cell responses, but would instead support Th2 responses. We would further predict that B cells sampled after 32 months of therapy that demonstrated high IL-6 production would once again be equipped to support pathogenic T cell responses.

Comparing the transcriptome signature of B or T cells in such patients during early GA treatment and long-term GA treatment may also provide an avenue for identification of patients who continue to respond favorably to GA therapy. RNA-seq technology would be ideal to identify genes that are differentially expressed in HD, MS and GA-MS patients in CD4+ and CD8+ T cells and naïve and memory B cells. By studying gene expression in individual populations, we hypothesize that we will be able to identify molecular targets that will serve as biomarkers for predicting positive responses to GA therapy.

Curiously, we found a remarkable enhancement of IgG and IgM production by B cells from GA-MS patients. In many autoimmune mediated diseases, such as RA or SLE, enhancing B cell differentiation into antibody secreting cells (ASC) would be extremely detrimental. The mechanism by which GA therapy influences B cells to secrete more Ig, and whether this Ig pool may enhance or modulate the neuro-inflammation of MS is unknown. This observation is even more surprising given the enrichment of naïve B cells and diminished frequency of plasmablasts in the peripheral blood of patients on GA therapy. There are several possible explanations for this. First, the memory B cells from GA-MS patients could more readily differentiate into antibody-secreting cells (ASC). Second, GA therapy could alter naïve B cells in a way that allows for more facile differentiation into ASC, for example by lowering the requirement for IL-21 [370]. Third, the ASC could have a

higher level of Ig secretion on a per cell basis. Fourth, since GA induces antigen-specific B cell responses and is administered on a daily basis it is possible that we captured B cells from the peripheral blood that have encountered GA as an antigen and more easily formed ASC *in vitro* due to an ongoing response to GA.

To address the first two possibilities of heightened Ig responses in GA-MS patients, memory and naïve B cells could be cultured separately. Alternatively, the pattern of somatic hypermutation, which is very low in naïve B cells that differentiate *in vitro* [371], could be analyzed to determine the source of Ig production. It is more likely that the source of Ig is from both memory and naïve B cells since we observed high levels of IgG in as few as three days and that on day five the highest levels of antibodies were of the IgM isotype. To determine whether Ig production is higher on a per cell basis, we would need to determine the absolute number of live cells and the proportions of memory, naïve, and plasma cells after *in vitro* stimulation. Finally, if the antibody response is directed toward GA, it would be possible to enumerate the number of antigen-specific B cells in circulation by flow cytometry [194, 340]. Importantly, IL-10 secretion is associated with plasma cell differentiation and one recent study found that IFN-β therapy enhanced plasmablasts that express IL-10 in MS patients [372]. Thus it is also possible that GA provides some benefit by enhancing ASC differentiation, and consequently, enhanced IL-10 production.

Despite the growing evidence from murine experiments, clinical trials, and studies like this, a singular role for B cells in MS has not emerged. However, the data presented here in this document and the accompanying publications provide a critical foundation to investigate the impact of B cell manipulation in controlling autoimmunity or inducing

regulatory responses. In addition, uncovering the B cell centric pathways leading to autoimmunity might be exploited to enhance the immune response in individuals with primary immunodeficiencies, HIV or enhancing vaccine efficacy.

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