Antibody-independent B cell effector functions in relapsing remitting Multiple Sclerosis: Clues to increased inflammatory and reduced regulatory B cell capacity

SARA J. IRELAND, MONICA BLAZEK, CHRISTOPHER T. HARP, BENJAMIN GREENBERG, ELLIOT M. FROHMAN, LAURIE S. DAVIS, & NANCY L. MONSON

University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA

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Abstract
The pathogenic role for B cells in the context of relapsing remitting multiple sclerosis (MS) is incompletely defined. Although classically considered a T cell-mediated disease, B cell-depleting therapies showed efficacy in treating the clinical symptoms of RRMS without decreasing plasma cells or total immunoglobulin (Ig) levels. Here, we discuss the potential implications of antibody-independent B cell effector functions that could contribute to autoimmunity with particular focus on antigen presentation, cytokine secretion, and stimulation of T cell subsets. We highlight differences between memory and naïve B cells from MS patients such as our recent findings of hyper-proliferation from MS memory B cells in response to CD40 engagement. We discuss the implications of IL6 overproduction in contrast to limited IL10 production by B cells from MS patients and comment on the impact of these functions on yet unexplored aspects of B cells in autoimmune disease. Finally, we contextualize B cell effector functions with respect to current immunomodulatory therapies for MS and show that glatiramer acetate (GA) does not directly modulate B cell proliferation or cytokine secretion.

Keywords: B cell cytokines, B cell proliferation, B cell APC function, B cell co-stimulation molecules, B-T interaction

Introduction
Relapsing remitting multiple sclerosis (RRMS) is an inflammatory, demyelinating autoimmune syndrome of the central nervous system. While T cells are the critical immune component required to induce disease pathology, more recent evidence highlights B cells as central components of the disease. MS patients treated with B cell-depleting monoclonal antibody therapy show significant improvement in the clinical symptoms of MS, with a reduction in total and new gadolinium enhancing lesions, and relapse incidence [1–4]. Studies in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) also demonstrate a role for B cells [5–8]. How B cells potentiate immune responses and why B cell-depletion therapy is effective in a T cell-mediated disease remains unclear. While antibodies are thought to play a role in MS disease pathology, current B cell-depletion therapies do not target immunoglobulin (Ig) secreting plasma cells and total Ig levels remain unchanged during the time when clinical symptoms were diminished [1–4].

Therefore, B cells likely influence MS disease pathology through antibody-independent effector functions. These include antigen presentation, cytokine secretion, and co-stimulation that could impact T cell function (Figure 1). Here we review the capacity for B cell effector functions to modulate immune responses in the context of MS and provide our recent data that highlight intrinsic differences between B cells from MS patients and Healthy Donors (HDs).

Memory B cells
Like T cells, B cells can be divided into naïve and memory populations [9]. Human memory B cells are readily identifiable as CD19 + CD27 + [10]. Mem-
B cells have previously encountered cognate antigen and undergone activation, most typically to T-dependent antigens that require T cell help to achieve fulminant activation and maturation. In contrast to their naïve counterparts, memory B cells secrete more pro-inflammatory cytokines and bear somatically hypermutated B cell receptors (BCR) [12,13]. Human memory B cells are more capable of supporting antigen-independent autologous T cell proliferation than naïve B cells [14]. Supernatants from memory B cells, but not naïve B cells, stimulated through CD40 enhanced T cell IFNγ secretion to polyclonal stimuli [15]. In fact, the percentage of peripheral blood memory B cells also increases during MS relapse [16]. Furthermore, activated and memory B cells are overrepresented in the CSF of MS patients [17] and have greater capacity to migrate towards elevated chemoattractant factor CXCL13 in CSF from MS patients [17,18]. Thus, memory B cells that are reactive to self-antigens are likely to exert effector functions that promote inflammatory disease processes in MS patients through reciprocal B-T cell activation [19] and possibly by priming naïve T cells.

**B cell Proliferation**

Although antigen-specific recall and polyclonal proliferative responses by T cells from MS patients have been widely studied [20–24], a limited number of investigations have reported on B cell proliferation in MS cohorts. It has been demonstrated that chronically activated B cells are found in the meninges of MS patients where ectopic germinal centers reside [25,26]. Given that CD40/40L interactions are critical for germinal center development and chronic activation of B cells, it is reasonable to suggest that B cells from MS patients may have a hyper-proliferative response to CD40 engagement.

To test this, we cultured memory and naïve B cells from HDs and MS patients with high or low dose CD40L for 5 days and quantified B cell proliferation by CFSE dilution (Figure 2). Memory and naïve B cells from HDs proliferated similarly to high (57.3%
vs 59.2%, \( p = 0.611 \) and low dose CD40L (9.9% vs 8.5%, \( p = 0.84 \)). In contrast, memory B cells from MS patients proliferated 1.3 fold more than naïve B cells in response to high dose CD40L (64.9% vs 47.7%, \( p = 0.003 \)) and 3.4-fold more than naïve B cells at low dose CD40L (34.9% vs 10.3%, \( p = 0.019 \)). This data suggests that there are unique differences between CD40 stimulated memory and naïve B cells from MS patients as memory B cells appeared to be hyper-responsive to CD40 stimulation.

Given increased expression of CD40L on unstimulated peripheral blood T cells from MS patients [27] and mitogen-stimulated T cells from MS patients [28,29], it is likely that the hyper-responsiveness of memory B cells from MS patients to CD40 engagement that we observed in vitro might even be more profound in vivo. A key challenge then becomes halting B cell mediated pathogenesis and restoring regulation of B cell function in MS patients.

**B cell antigen presentation**

Studies with murine B cells demonstrated that antigen specific B cells are potent antigen presenting cells (APC)[30–33]. In fact, B cells are critical APCs in some mouse models of autoimmune diseases [34,35]. Human B cells are also capable of APC function, as they uptake large soluble and particulate antigens [36–38] similar to the murine B cell’s preference for protein antigen [39]. B cells primarily capture cognate antigen through their BCR, which induces BCR mediated cross-linking and internalization [40,41]. Of note, under certain circumstances, B cells can uptake antigen through pinocytosis [42]. Antigens taken up by B cells are targeted to the endocytic compartment where they are processed and can be subsequently presented to CD4 + T cells in the context of MHC Class II [43].

Initial investigations of human B cells as APC were carried out utilizing Epstein-bar virus (EBV) transformed B cells. Which can process and present particulate exogenous antigen to T cell clones [37]. This methodology proved that B cells can support antigen-specific T cell proliferation to tetanus toxoid (TT) [36]. Others showed that EBV-transformed B cells had the most effective APC function when previously activated T cells were used as responders [44]. 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 IFNγ in response to melanoma antigen [45].

A potential confounding factor in these early human EBV B cell APC assays is that EBV transformation can induce IL10 production in B cells [46], which may
have inhibited T cell proliferation and pro-inflammatory cytokine production in these previous studies.

The next approach used to study human B cell APC function was to activate total human B cells in vitro with CD40L and IL4 (CD40-B). This is a means to increase expression of co-stimulatory molecules, factors known to be important in the generation of T cell responses [47–52] and chemotactic molecules on B cells important for secondary lymphoid organ homing [53]. Murine antigen-specific B cells activated in a similar manner rival the APC capacity of dendritic cells (DC) [54]. This is likely related to the observation that antigen-experienced B cells expressed increased levels of co-stimulatory molecules (CD80, CD86 and CD40) and MHC Class II molecules [11,55].

Initial studies demonstrated that peripheral healthy donor B cells expanded using the CD40-B system were effective APC in the generation of antigen-specific CD4 + T cell lines [30,45]. In addition, CD40-B cells from HDs or cancer patients expanded viral-specific memory, autoantigen/tumor and neoantigen specific CD8 + T cell responses [42]. Human peripheral blood mononuclear cells (PBMC)-stimulated with CD40L and IL4 to activate B cells were highly effective in generating neuro-antigen specific autologous CD4 + and CD8 + T cell clonal proliferation and IFNγ secretion [47]. In our laboratory, we 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 [56]. 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 autoantigen, including neuro-antigens.

More recently, our laboratory investigated APC capacity of peripheral blood memory or naïve B cells from MS patients and HDs that were not stimulated prior to culturing with autologous T cells [57]. We found that memory and naïve B cells from HDs and MS patients are equally able to induce autologous CD4 + T cell proliferation in response to foreign antigen (TT or mumps, Figure 3A). In contrast, proliferative responses of CD4 + T cells to the neuro-antigens myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) were virtually nonexistent in HDs, with only one responder from the naïve B cell compartment (defined as two standard deviations above the mean proliferation in cultures with no antigen).

Strikingly, nearly one-third of all MS patients had memory and/or naïve B cells that induced proliferation of autologous CD4 + T cells in response to neuro-antigens (Figure 3B). From these results, we conclude that B cells from a subgroup of MS patients harbor the ability to stimulate T cell proliferation to neuro-antigens, which are thought to be key in the perpetuation of MS disease pathogenesis.

Peripheral B cells from MS patients are also able to induce autologous CD4 + T cell IFNγ production in response to neuro-antigens [57], suggesting that B cells have a direct impact on Th1 responses. Others have shown that T cells from MS patients were unique in their requirement for B cells to achieve maximal proliferative and IFNγ responses to polyclonal

Figure 3. CD4 + T cell proliferation provoked by B cell subsets in the presence of antigen. Total peripheral blood T cells were cultured with autologous memory or naïve B cells and antigen for 5 days; proliferation was quantified by CFSE dilution (background proliferation with no antigen was subtracted). A. In response to foreign antigen (tetanus toxoid or mumps virus, plotted together), HD and MS B cells induce similar proliferation by CD4 + T cells. B. Memory and naïve B cells from MS patients induce significantly higher CD4 + T cell proliferation in cultures containing self antigen (myelin oligodendrocyte glycoprotein 1–125 (rMOG) or myelin basic protein (MBP), plotted together) compared to HDs. There were also a significantly greater number of responders to neuro-antigen in the MS cohorts Statistical analysis on the percentage of proliferation was performed using a student’s unpaired t-test. The number of responders by proliferating was defined as two standard deviations above the mean proliferation in cultures with no antigen and compared using χ² analysis. P-values less than 0.05 were considered significant.
activators (αCD3)[58]. B cell-derived cytokines (LTα, TNFα and IL12) are critical for optimal T cell proliferation and differentiation into IFNγ producing cells [15,58]. However, the requirement for these cytokines in antigen specific B-T interactions is not known. It is also unknown how B cells operating as APCs impact particular CD4+ T cell subsets, although it is clear that B cells possess all the required elements for proficient antigen presentation.

The potential impact of cytokines produced by B cells

B cells are capable of producing a wide variety of proinflammatory and regulatory cytokines and growth factors [59–61] that have long been appreciated for their potential to influence B and T cells. Yet, how and the extent to which B cell-derived cytokines impact immune responses and autoimmunity remains incompletely defined.

It is clear that B cells directly stimulate IFNγ secretion and proliferation of TH1 CD4+ T cell in an antigen-dependent fashion [57] and/or through provision of cytokines [62]. Demonstrations of altered cytokine production in B cells from MS patients provoke questions of the contribution of B cell cytokines to autoimmune disease and immune responses to pathogens [59]. Here, we focus on cytokines produced by B cells that have the potential to impact Th17 responses and B cell regulatory function.

TH17 responses

Little is known about how B cells might impact T cell populations, including TH1, TH17, T central or effector memory or even naïve T cells (Figure 1). TH17 cells from HDs produced inflammatory cytokines including IL17, IL21, and IL22 in response to infectious agents. However, TH17 cells are increasingly thought to play an important pathogenic role in autoimmune disorders including MS, Rheumatoid Arthritis (RA), and Inflammatory Bowel Disease (IBD). Few studies have addressed the connection between B cells and TH17 responses. There has been no formal demonstration of the direct impact of B cells on CD4+ TH17 cells in humans. However, several recent pieces of data strongly, and surprisingly, implicate B cells as potent modulators of TH17 responses in HDs and patients with autoimmune disorders.

For example, in HDs, the frequency of memory B cells in the peripheral blood positively correlates with ex vivo IL17 production by CD4+ T cells [63]. In contrast, patients with the primary immunodeficiency, X-linked agammaglobulinemia (XLA), who have few B cells, have a reduced frequency of peripheral IL17- and IL22-producing T cells when stimulated ex vivo with the potent cytokine inducers, PMA and ionomycin [63]. 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+ IL17 producing T cells is negatively correlated with the number of immature B cells and B cell HLA-DR (MHC II) expression [63]. 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 IL17 (~50%) and IL22 (~20-30%) production, yet IL17 and IL22 production by PBMCs from XLA patients were unaffected by B cell depletion [64]. Human TH17 responses to C. albicans in vitro require memory T cells and APC [65]. 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. Separate evidence from Mycobacterium tuberculosis (MTB) infected individuals gives further credence to the link between B cells and TH17 cells [66].

Studies of autoimmune disorders revealed that B cell depletion also decreased TH17 responses in mice [8] and in humans [58,67,68]. RA patients treated with B cell-depleting therapy show decreased IL17 and IL22 production, as well as decreased expression of RORγT (TH17 transcription factor) but not of IFNγ or TNFα in the synovial tissue [68]. One case study showed similar reductions of TH17 cytokines in the peripheral blood of RA patients [67]. 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 IFNγ or IL17 upon ex vivo polyclonal stimulation [58].

In the same study, TNFα and LTα 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 [58]. Although these studies provide evidence that the presence of B cells is required for fulminate IL17 responses, the absence of B cells is distinct from direct B cell effects on TH17 cells.

There are several ways in which B cell effector functions could directly impact TH17 cells. First, B cells might act in concert with non-T immune cells and contribute to a yet unknown, but important aspect of TH17 responses. Second, B cells could present antigen, secrete cytokines and provide co-stimulation to incite memory Th17 cell responses.
Finally, B cells could play a role in polarizing naïve T cells to a TH17 phenotype. In the context of human autoimmune disease, it is not known whether B cells reactivate memory T cells or, perhaps, initiate activation and expansion of clonally ignorant naïve T cells.

In humans, TGFβ, in combination with IL6 [69–71], IL1β [72] and/or IL23 [73] is thought to be critical for TH17 polarization. Human B cells from HDs can produce both TGFβ [60] and IL6 [61,74]. In fact, IL4 alone induces IL6 production from human CD19+B cells that is enhanced in a dose dependent fashion by the addition of BCR cross-linking agents [75]. HD memory B cells, and to a lesser extent naïve B cells, produce IL23 upon stimulation through the BCR alone or in combination with CD40 ligation [15]. Human B cells from HDs also produce robust amounts of IL1β upon BCR stimulation [59].

B cells can produce TNFα and LTα, which, in addition to IL23 [76], are important factors for potentiating TH17 responses. Although it is true that HD B cells generally make lesser amounts of these polarizing cytokines than their myeloid APC counterparts, the expression of such cytokines by B cells has not been thoroughly investigated in the context of autoimmune disease.

IL6 is a pleiotropic cytokine produced by B cells, other APCs, and a variety of non-immune cells. IL6 impacts the survival, proliferation and activity of immune cells and acute inflammatory responses. IL6 is important for B cell maturation and impacts CD4+T cells [77]. Moreover, IL6 can cross the blood brain barrier [78]. There is some evidence of increased IL6 levels in the periphery and cerebrospinal fluid of MS patients [79,80]; however, these findings are not consistent in all studies [81]. In contrast, IL6 is known to correlate with increased disease activity in RA[82] and IL6 blockade appears to be of therapeutic benefit in RA patients [83]. These reports, along with the potential impact on B and T cell responses prompted us to investigate the potential for B cells to produce IL6 in MS patients.

We stimulated the naïve and memory B cell subsets from HDs and MS patients with conditions known to induce cytokine production (see methods), sampled supernatants at either day 3 or 5 of culture and assayed for cytokine levels by ELISA. We were able to detect IL6 in all culture conditions tested for both naïve and memory B cells from MS patients and HDs (Table I). IL6 production by naïve and memory B cells from MS patients was more robust than IL6 production by HDs, and even rivaled that of mitogen-stimulated myeloid APC [84].

As shown in Table I, naïve B cells from MS patients stimulated with high dose CD40L plus IL4 produced the largest quantity of IL6 (1263 pg/mL) that was significantly more than HD naïve B cells (p = 0.0019). Similarly, naïve B cells from MS patients produced more IL6 than HD in response to CD40L plus IL2 and CpG (p = 0.0031). Memory B cells from MS patients stimulated with high dose CD40L plus IL4 produced only 43 pg/mL of IL6, but when BCR stimulation was added, IL6 production was increased 18.2-fold (789.5 pg/mL) and was significantly greater than that of HD memory B cells in the same condition (p = 0.0007). Stimulation of memory B cells from MS patients with CD40L, IL2 and CpG induced significantly more IL6 than from memory B cells of HDs (p = 0.016). These data provide evidence that B cells from MS patients produce more IL6 than their HD counterparts to a variety of stimuli. The underlying mechanism of this exaggerated IL6 production is a topic of active investigation in our laboratory. This is potentially linked to both T cell responses in MS and hyperproliferation of B cells.

Further studies are necessary to elucidate whether IL6 produced by B cells (or some other aspect of B cell function) impacts TH17 polarization, T cell or B cell function. Of note, anti-IL6 receptor (α-IL6r) therapy is approved for treating RA and Castleman’s disease (a rare B cell malignancy). Anti-IL6r therapy has been

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Table I. Memory and naïve B cell cytokine production after 3 or 5 days of culture with polyclonal stimuli from HD and MS patients.

<table>
<thead>
<tr>
<th></th>
<th>IL6 production Day 3 (mean, in pg/mL)</th>
<th>IL10 production Day 5 (mean, in pg/mL)</th>
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<tr>
<td></td>
<td>CD40L IL4</td>
<td>CD40L IL4 BCR</td>
</tr>
<tr>
<td>HD naïve</td>
<td>75</td>
<td>165</td>
</tr>
<tr>
<td>HD memory</td>
<td>62</td>
<td>57</td>
</tr>
<tr>
<td>MS naïve</td>
<td>1263b</td>
<td>568</td>
</tr>
<tr>
<td>MS memory</td>
<td>183</td>
<td>43</td>
</tr>
</tbody>
</table>

Supernatants were assayed for IL6 and IL10 by ELISA, results are presented as the mean for each group in pg/mL. Naïve B cells from HC tended to produce more IL10 than MS patients and significantly more in cultures with CD40L, IL2 and CpG. Memory B cells from MS patients and HDs secrete similar amounts of IL-10; however, the number of HD memory B cells that responded by producing IL10 was significantly greater than MS patients in cultures with CD40L and CD40L plus IL4 and BCR cross-link. Student’s unpaired t-test was used to compare HD to MS production of cytokines within memory or naïve subsets for each stimulation, the number of responders (defined as two standard deviations above the mean of MS B cells with CD40L only) was compared using chi-squared test.; * ≤ 0.05 t-test.; b ≤ 0.01 t-test.; c ≤ 0.001 t-test.; d Chi-squared ≤ 0.05.
tried in a variety of autoimmune and B cell malignant disorders, but has not been tested in MS [85] though multiple reports in mouse models of MS support a role for IL6 in disease pathology [86–92]. It is tempting to speculate that α-IL6r therapeutics would show efficacy in MS. However, anti-IL23/12p40 [93] and TNF-α [94,95] therapies, designed to block TH17 expansion in vivo, do not have predictable outcomes in MS patients.

**B cell-derived IL-10 and regulatory B cells**

Interleukin 10 is another pleiotropic cytokine capable of acting on both B and T cells, which is of great interest to regulatory B cell effector functions [96]. Recent investigations primarily focused on IL10 as a regulatory and anti-inflammatory cytokine, although more historical observations defined the important role of IL10 as a survival and maturation factor with the ability to potentiate autoimmune diseases involving B cells [97,98]. Human B cells secrete IL10 to a variety of stimuli including CD40L, BCR cross-link [46], and CpG alone [74] or in combination with CD40L [99]. IL10 acts in an autocrine or paracrine fashion on B cells to increase antibody production [55]. In fact, both IL10 and IL6 are important for supporting antibody secretion [46]. Exposure of B cells to IL10 tends to lead to B cell survival and differentiation into plasma cells [100].

The most well-known role for IL10 is that of an anti-inflammatory molecule and IL10 is secreted by regulatory B and T cells, but also by macrophages [101] and by some subsets of dendritic cells [102,103]. IL10 dampens immune responses in several ways. First, IL10 limits antigen presentation through down-modulation of MHC Class II co-receptors (CD80 and CD86) expression on APC [96,97,104,105]. IL10 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 [104], as well as increase T and B cell sensitivity to apoptosis [106].

Thus, it became of interest to determine the impact of B cell derived IL10 on inflammation associated with MS. Initial reports that serum titers of IL10 in MS patients were elevated [107] further intensified the pursuit. However, the first indication of a prominent role of IL10 production by B cells in controlling disease was established in mouse models of MS [108–111]. More recently it has been shown that IL10 producing B cells are critical for the induction of FOXP3+T regulatory cells and for limiting TH17 and TH1 T cell responses in a mouse model of arthritis [112]. B cell production of IL10 plays a role in controlling disease severity in mouse models of MS [108–111]. Such data is highly suggestive of a central role for IL10 producing B cells in the regulation of MS.

However, the impact of IL10 production by B cells in MS patients is in its advent of being understood. Previous studies in our laboratory and others showed a distinct deficit in IL10 production by B cells from MS patients [17,56,58,113] and suggest that the lack of IL10 producing B cells allows for progression of the inflammation associated with the disease. However, it is known that helminth infection can overcome the deficit in peripheral B cell production of IL10 in MS patients and restore it to that of HD [114].

There are also multiple reports of putative regulatory B cells (Breg) in MS patients. Peripheral B cells from MS patients stimulated with CpG produce less IL10 than HDs, and the frequency of CD27+ memory B cells from MS patients that secrete IL10 is significantly less than HDs or MS patients in remission [115] indicating the importance of frequency and fluctuations in regulatory or inflammatory B cell populations. A separate study found that the frequency of peripheral B cells (CD27hiCD24hi), thought to harbor Breg, was increased in MS patients compared to HD when stimulated with CD40L plus CpG or lipopolysaccharide; however several patients were receiving immunomodulatory therapy at the time of blood draw [116]. A third study found that the number of IL10 producing B cells from total peripheral blood B cells from MS patients is similar to HD in unstimulated cultures, yet was decreased in MS patients stimulated with CpG alone [113].

The differences in assay conditions, cohorts and methods for quantifying B cell IL10 production led us to investigate whether B cells from our treatment naïve RRMS patient cohort were competent IL10 producers. We also reasoned that naïve and memory B cells may have different capacities to produce IL10, considering the historical understanding that the impact of IL10 on activated B cells is different than the impact of IL10 on naïve B cells [100]. Thus, we cultured naïve or memory B cells from HD and MS patients with polyclonal stimuli and asked whether B cells from MS patients and HDs were equally capable of producing IL10.

Our results indicate that naïve B cells from HDs stimulated with CD40L alone produced significant amounts of IL10 (average = 113.3 pg/mL), which was enhanced with the addition of IL4 + BCR (134.4 pg/mL) or IL2 + CpG (156.7 pg/mL)(Table I). However, none of these conditions induced robust IL10 production by either naïve or memory B cells from treatment naïve MS patients (Table I). Thus, although both memory and naïve B cells from MS patients are capable of producing IL10, the production is minimal compared to that produced by naïve and memory B cells from HDs. In separate studies, memory B cells secreted robust amounts of IL10 [61,117]. Further investigation is needed to understand the role of IL10 producing B cells in MS.
patients with careful attention to culturing conditions, types of B cells in the cultures, clinical status of the patients in the cohorts, and surface marker combinations used to identify IL10 producing B cells.

**Ratio of IL6 and IL10 expression**

The deficit in IL10 production by B cells from MS patients combined with excessive production of IL6 by B cells from MS patients has the potential to impact the balance of regulatory and inflammatory signals in this disease. Of particular interest is that B cells from patients with Behçet’s disease produce increased IL6, with limited IL10 production in response to B cell activating factor (BAFF) stimulation [116], just as we have observed in our MS cohort. Moreover, reports have shown that increases in the ratio of IL6 to IL10 were prognostic of poor outcomes in systemic inflammatory response syndrome [118] and infant sepsis [119]. In addition, the overabundance of IL-6 in RA patients is known to block inhibitory effects of IL10 on CD4+ T cells [120].

This led us to compare the IL6/IL10 ratio produced by B cells in HDs and MS patients. The IL6/IL10 production ratios by memory and naïve B cells from HDs and MS patients are presented in Figure 4. These data demonstrate that memory and naïve B cells from MS patients are more likely to secrete IL6 in response to a broad spectrum of stimuli, while memory and naïve B cells from HDs are more likely to secrete IL10 in response to a broad spectrum of stimuli.

These data highlight the intrinsic dysregulation of proliferation and cytokine production by B cells from MS patients. Our observations and other studies have identified a defect in IL10 production by B cells from MS patients. Whether this decreased ability to secrete IL10 impacts the regulatory function of B cells in MS patients remains unknown. In addition, the increased expression of IL6 in B cells from MS patients we identified here could impact not only B and T cell function, but also contribute to tissue inflammation. In the context of APC capacity, increased IL6 expression might influence T cell polarization or function.

Finally, the altered ratios of cytokines secreted by B cells to polyclonal stimuli indicate an intrinsic predisposition to or initiation of an inflammatory program that is unique to B cells from MS patients. Future studies are needed to address the mechanism of this skewed cytokine production and how immunomodulatory therapies might restore balanced cytokine secretion in B cells.

**Impact of immunomodulatory therapies on B cells: Type 1 interferons**

Both IFNβ-1b and IFNβ-1a are FDA approved therapeutics for treating RRMS. While their effects on T cell responses and other myeloid cells are the subject of numerous investigations [16,121], there are few reports on the impact of IFN therapies on B cells, particularly with respect to IFNβ-1a.

Recently the impact of IFNβ-1b on B cells was shown *in vitro* where activated B cells exposed to IFNβ-1b had decreased CD40 and CD80 costimulatory molecule expression [76,122], and decreased IL23 and IL1β secretion but increased IL10, IL12 and IL27 production [76]. Supernatants from IFNβ-1b exposed B cell cultures inhibited IL-17

![](image.png)

**Figure 4.** B cells from MS patients secrete altered ratios of IL6 to IL10. Comparison of the IL6 on day 3 and IL10 on day 5 production from individual cultures in response to polyclonal stimuli. A. Naïve B cells from MS patients exhibit exaggerated IL6 production that is disproportionate to IL10 production compared to HD in response to CD40L high plus IL4. B. Memory B cells from MS patients also have an IL6-skewed cytokine profile in response to CD40L high plus IL4 and BCR cross-link. Mean IL6: IL10 ratios are provided below the panels. Students unpaired t-test used to identify statistical differences between ratios defined as a p-value at or below 0.05.
secretion by PBMC [76]. IFNβ-1b also reduced the ability of B cells to induce proliferation and IFNγ secretion by T cells [76,123]. The frequency of CD27+ peripheral memory B cells is diminished with IFNβ therapy, as well as expression of CD86 [16,17]. In the same study, CCR5 expression was also decreased on total CD19+ and naïve B cells, but not memory B cells [16]. Although IFNβ-1a receptor is expressed on B cells and they respond to the cytokine, few studies have addressed the effect of this IFN treatment on B cells. It is known that IFNβ-1a does induce inhibitory proteins in B cells [124]. Collectively, these observations demonstrate that the existing IFN therapies can impact effector functions of B cells that include co-stimulation, cytokine production and APC activity.

Impact of immunomodulatory therapies on B cells:
Glatiramer acetate

Glatiramer acetate (GA) is a random polymer composed of amino acids in a similar ratio to a myelin basic protein (MBP), a candidate auto-antigen in MS, and is an FDA approved immunomodulatory therapy for the treatment of MS. Antibodies isolated from the CNS of some MS patients bind MBP [125–128] and B cells from peripheral blood can bind MBP or GA directly [56,57]. Although GA can bind directly to HLA molecules [129], it is possible that MBP-specific B cells bind GA via the BCR and are processed to HLA in a specific manner. In fact, our laboratory found that FITC labeled MBP or GA can directly bind purified B cells. While GA was considered to modulate T cells by binding directly to MHC II and interfering with self-antigen presentation, some recent evidence showed that myeloid APC treated in vitro with GA had increased IL10 expression and decreased IL-12p70 [130] and TNF-α [131]. These effects of GA have not been assayed in human B cells.

Most interestingly, B cells from mice treated in vivo with GA produced more IL10 than controls, with a concomitant decrease in the co-stimulatory molecules, CD80 and CD86 [109,110]. Adoptive transfer of in vivo GA exposed B cells confers resistance to the mouse model of MS, shown by a reduction in IFNγ and IL17 responses [110]. In the same study, B cells were required for the mechanism of GA activity in vivo. These studies prompted us to ask whether direct exposure of human B cells to GA in vitro alters their cytokine production profile or proliferative capacity.

To test this hypothesis, we incubated memory or naïve B cells from HDs and MS patients with polyclonal stimuli in the presence or absence of GA. We found that the presence of GA in B cell cultures did not alter proliferation (Table II) or cytokine secretion by B cells (Table III). These data show in vitro treatment of purified B cells with GA does not directly impact the B cell functions tested here. Future studies are necessary to determine whether human B cell activity is altered by therapeutic treatment with GA similarly to what was observed in mice. Furthermore, it is possible that in both mice and humans, altered B cell activity is not resultant of a direct interaction between B cells and GA.

### Table II. Direct exposure of B cells to GA does not alter proliferation.

<table>
<thead>
<tr>
<th>CD40L high</th>
<th>CD40L low</th>
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<tbody>
<tr>
<td>Naïve HD</td>
<td>0.95 0.25</td>
</tr>
<tr>
<td>Memory HD</td>
<td>0.27 0.45</td>
</tr>
<tr>
<td>Naïve MS</td>
<td>0.72 0.42</td>
</tr>
<tr>
<td>Memory MS</td>
<td>0.72 0.63</td>
</tr>
</tbody>
</table>

Values in the table represent p-values comparing B cell proliferation under the stimulation combination in the absence of GA to B cell proliferation under the same stimulation combination in the presence of GA.

<table>
<thead>
<tr>
<th>IL6 Day 3</th>
<th>IL10 Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve HD</td>
<td>0.46 0.34</td>
</tr>
<tr>
<td>Memory HD</td>
<td>0.65 0.24</td>
</tr>
<tr>
<td>Naïve MS</td>
<td>0.65 0.86</td>
</tr>
<tr>
<td>Memory MS</td>
<td>0.89 0.37</td>
</tr>
</tbody>
</table>

Values in the table represent p-values comparing IL6 or IL10 production by B cells under the stimulation combination in the absence of GA to IL6 or IL10 production by B cells under the same stimulation combination in the presence of GA.
Conclusions

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. Here, we discussed the potential role of B cell APC capacity, cytokine secretion and memory B cells on MS with a focus on how these functions impact T cells. B cells subsets from MS patients have significantly greater propensity to induce autologous CD4 + T cell responses to self antigens than HDs, while maintaining a similar response to foreign antigen. We also showed that B cell subsets from MS patients exhibit increased IL6 production that is not coordinately regulated with IL10 production as it is in memory and naïve B cells from HDs.

Our studies suggest that how currently approved immunomodulatory therapies could impact B cell effector functions, but demonstrated that one therapeu tic, GA, does not directly act on B cells to induce regulatory function via secretion of IL10 or dampen proliferative capacity. Taken together, there are a multitude of B cell effector functions that have the potential to explain the efficacy of B cell-depleting therapies. These B cell functions are just beginning to be understood and they are worthy of future investigations to increase our knowledge of the role of B cells in autoimmune responses, particularly in the context of MS.

Methods

Human samples: Relapsing remitting MS (RRMS) patients were recruited at the University of Texas Southwestern Medical Center according to Institutional Review Board approved criteria. Cells were obtained by leukapheresis. Patients were not on corticosteroid treatment within 60 days prior to leukapheresis and had never received immunomodulatory therapy. All patients had at least one relapse in the 2 years prior, but not within 60 days of leukapheresis. Patient characteristics are provided elsewhere [57]. PBMC from HDs and MS patients were isolated by ficoll paque (GE Healthcare) density gradient centrifugation and cryopreserved at -120°C on the same day in 50% pooled human serum (Gemini Bioproducts), 10% DMSO (Fisher) and 40% RPMI 1640 (Cellgro).

B cell purification and isolation: Total B cells were isolated from PBMC by magnetic activated cell separation (MACS) using α-CD19 microbeads (Miltenyi) according to the manufacturers instructions. Purity was typically above 95%. Total CD19 + B cells were stained with CD19-PECy5, CD27-PE and IgD-FITC (BD Bioscience) and sorted into naïve (CD19 + IgD + CD27−) and memory (CD19 + CD27+) populations on a FACS Aria (BD Biosciences, custom order system).

Cell Culture: Memory and naïve B cell subsets were stained with CFSE as previously described [57]. $1 \times 10^5$ naïve or memory B cells were cultured for 5 days in u-bottom 96-well plates (Corning) in 200 μL minimal media (RPMI 1640, Cellgro; 10% fetal calf serum, Hyclone; 100 μg/mL penicillin 100 U/mL streptomycin and 2 mM L-glutamine, Fisher Scientific). Immortalized Swiss mouse fibroblast cells (NIH-3T3) stably expressing membrane bound human CD40L (CD40L+NIH-3T3) were a kind gift from Gordon Freeman. CD40L+NIH-3T3 cells were grown in minimal media supplemented with 200 μg/mL gentamicin/G418 (Fisher). NIH3T3 cells were sub-lethally irradiated with 10,000 rads from a Cs137 source to prevent proliferation and seeded into 96 well plates at 500 (low CD40L) or 2000 (high CD40L) cells per well. CD40L+NIH3T3 cells were allowed to adhere overnight before the media was removed and B cell cultures added. Further stimulation of B cells was carried out with 10 ng/mL of recombinant human IL–2 (Gibco) or IL–4 (R&D Systems). Goat anti-human IgM/IgG (Jackson Immunoresearch) was added at a final concentration of 0.5 μg/mL and class-B CpG (Invivogen CpG ODN 2006 or Coley Pharmaceuticals CpG ODN 10103, CpG ODN) was used at a final concentration of 5 μg/mL.

Quantification of Cytokines: Culture supernatants were collected on day 3 and replaced with fresh media. Supernatants were frozen at -20°C for batched analysis. IL10, LTα (BD Pharmingen), IL12p70, TNFα and IL6 (eBioscience) were quantified by ELISA. The lowest detection level for IL6 and TNFα was 1 pg/mL, 5 pg/mL for LTα and 9.5 pg/mL for IL12p70.

Statistical Analysis: the unpaired Student’s t-test was performed to compare cytokine concentration and proliferative responses; a p-value less than 0.05 was considered statistically significant. The number of responders for each cytokine was defined as two standard deviations above the mean cytokine concentration of naïve B cells from MS patients stimulated with CD40L alone. Chi-square analysis was used to determine the statistical significance between the number of responders.

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