B cell:Glial cell interactions and contribution to CNS-compartmentalized inflammation in Multiple Sclerosis

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Abstract

Growing evidence suggests that B cells play important roles during disease relapses and possibly also during the progressive stages of multiple sclerosis (MS). A number of studies have implicated antibody-independent roles of B cells in the periphery of patients, including presence of abnormally higher proportions of pro-inflammatory B cells (Beff) compared to anti-inflammatory B cells (Breg), which is thought to induce pro-inflammatory T cell responses. In the inflamed central nervous system (CNS) of MS patients, B cells are abnormally fostered and found in different compartments, including the meningeal immune-cell collections closely linked with subpial cortical injury and progressive disease. However, there remain major gaps in our knowledge of B cell persistence and their potential contributions to CNS-compartmentalized inflammation. The aim of my doctoral thesis is to shed light on cellular mechanisms involving B cell:glial cell cross-talk and how these interactions may propagate local inflammation and injury as well as contribute to progressive disease in MS.

In the first part of my thesis (Chapter 2), I demonstrate that human astrocytes support the survival of B cells through soluble factors. These soluble factors derived from pre-stimulated astrocytes not only supported B cell survival but also their activation (increased co-stimulatory molecule expression), which further induced T cell proliferation. I further demonstrate that astrocyte-secreted factors supported survival and activation of MS-relevant B cell subsets derived from relapsing remitting MS (RRMS) and secondary progressive MS (SPMS) patients. In chapter 3, I investigated the bi-directional interaction between B cells and myeloid cells (microglia and macrophages) to determine how these interactions may impact the propagation of CNS-compartmentalized inflammation. First, I show that soluble factors derived from M1-activated microglia supported B cell activation, whereas M2c microglia induced B apoptotic

death of MS-relevant B cell subsets. I further demonstrate that soluble factors derived from pro-inflammatory (Beff) B cells (abnormally implicated in the periphery of MS) substantially increased the secretion of the pro-inflammatory cytokines (IL-12, IL-6 & TNF) and diminished IL-10 production by microglia and macrophage. Interestingly, this secretion of pro-inflammatory cytokines by macrophages was dependent on B cell-derived GM-CSF. Lastly, I demonstrate that supernatants derived from distinct B cell subsets (Beff & Breg) were capable of modulating microglia and macrophage phenotype (expression of CD80 & TREM-2), while reciprocally modulating myeloid phagocytosis of myelin.

My overall doctoral investigations enhances our understanding of cellular mechanisms that may be associated with progressive disease. My findings indicate that B cells and glial cells have the capacity to interact and that they may contribute to cascades of pro-inflammatory events. In related work to which I have contributed, we demonstrated a selective cytotoxic effect to oligodendrocytes and neurons in response to soluble factors of B cells derived from MS patients but B cells from matching controls. If these interactions occur *in vivo* they may propagate CNS-compartmentalized inflammation and subpial injury. Taken together, these findings constitute a conceptual advance pointing to novel cellular mechanisms that may contribute to subpial cortical pathology and progressive MS. Future work will aim to elucidate the molecular mechanisms underlying these B cell:glial cell interactions. I am hopeful that my results will eventually help the development of more targeted therapies that can limit or modulate these interaction in a way that is beneficial for progressive MS – a major unmet clinical need.

Résumé

Les études réalisées dans le domaine de la sclérose en plaques (SEP) ont montré le rôle clé des cellules B au cours des poussées, et probablement lors de la phase progressive de la maladie. Plusieurs travaux montrent le rôle des cellules B, dans la pathophysiologie de la maladie indépendamment de la production d'anticorps. Les patients atteints de SEP présenteraient une proportion plus élevée de cellules B pro-inflammatoires (Beff), en comparaison aux cellules B anti-inflammatoires (Breg). De plus, les cellules Beff sont à même d'induire des réponses T pro-inflammatoires. Dans le système nerveux central (SNC) inflammé, les cellules B persistent anormalement au sein du compartiments des agrégats de cellules immunitaires associées aux méninges. Ces derniers sont étroitement liées aux lésions superficielles du cortex, et à la progression de la maladie. Toutefois, nos connaissances restent limitées quant à la persistance des cellules B et leur potentielle contribution à une inflammation compartimentalisée dans le SNC. Le but de ma thèse est d'apporter quelques éclairages sur les mécanismes cellulaires mis en jeu lors des interactions bidirectionnelles entre les cellules B et gliales, et comment ce type d'interactions propagerait l'inflammation locale, induirait la dégénérescence cérébrale et la progression.

Dans la première partie de ma thèse (Chapitre 2), j'ai pu démontré que les astrocytes humains permettraient la survie des cellules B via des produits solubles. Les produits solubles dérivés d'astrocytes pré-stimulés ont amélioré la survie des B, et augmenté l'activation des cellules B (expression de molécules de co-stimulation). Ces derniers, ont permis d'induire une meilleure réponse proliférative des cellules T. Dans le même sens, j'ai pu démontrer que ces facteurs solubles augmentaient la survie et l'activation de sous-populations de cellules B caractéristiques de la SEP, de patients exprimant la forme récurrente rémittente et progressive

secondaire. Dans le troisième volet de cette thèse, j'ai investigué l'interaction bidirectionnelle entre les cellules B et les microglies/macrophages, et comment ces interactions impacteraient la propagation de l'inflammation compartimentalisée. D'abord, j'ai démontré que les produits solubles des microglies-M1 augmentent l'activation des cellules B, alors que les microglies-M2c induisent la mort cellulaire des différentes sous-populations de B. Par ailleurs, j'ai démontré que les facteurs solubles dérivés des Beff (élevées dans la périphérie de la SEP) ont augmentée de manière considérable la sécrétion de cytokines pro-inflammatoires (IL-12, IL-6 &TNF) par les microglies et les macrophages, de manière dépendante du GM-CSF produit par les Beff. Ces mêmes facteurs solubles ont induit la diminution de la production d'IL-10 par les cellules myéloïdes. Finalement, j'ai démontré que les surnageants de cellules (Beff & Breg) ont le pouvoir de moduler le phénotype (CD80 & TREM-2) des cellules myéloïdes, et de moduler de manière réciproque la phagocytose de la myéline.

Globalement, mes travaux ont permis l'élucidation de mécanismes cellulaires qui pourraient être associés à la SEP progressive. Mes résultats indiquent que les interactions entre cellules B et gliales pourraient contribuer à la cascade d'évènements pro-inflammatoires. J'ai ainsi démontré que les facteurs solubles de cellules B dérivées de SEP induisaient la cytotoxicité des oligodendrocytes et des neurones. Si ces interactions sont initiées *in vivo*, elles pourraient contribuer à la propagation d'inflammation compartimentalisée dans le SNC, et à la dégénérescence qui mènent aux lésions superficielles corticales. Mes résultats ont contribués a des avancées conceptuelles indiquant l'engagement d'un nouveau mécanisme cellulaire contribuant la progression de la maladie. D'autres travaux axés sur les mécanismes moléculaires apparaissent nécessaires pour générer des thérapies ciblées pour la prise en charge des formes progressive – représentant un besoin critique.

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Finally, I would like to dedicate my thesis to my late aunt Hakima Boukoffa. You are dearly missed and always in my heart. I also wish to dedicate this work to all diseased people, especially those affected by neurological disorders and the children of the world ….

To Hakima (1950-2016)

List of Abbreviations

Beff: effector B cells

Breg: Regulatory B cells

BBB: Blood brain barrier

CIS: Clinically isolated syndrome

CNS: Central nervous system

CSF: Cerebrospinal fluid

DIS: Dissemination in space

DIT: Dissemination in time

EAE: Experimental autoimmune encephalomyelitis

GB: Granzyme B

GML: Gray matter lesion

IBD: Inflammatory bowel disease

IL: Interleukin

LTα: Lymphotoxin alpha

KO: Knock-out

MS: Multiple Sclerosis

NAWM: Normal appearing white matter

OCB: Oligoclonal bands

PPMS: Primary progressive multiple sclerosis

RRMS: Relapsing remitting multiple sclerosis

SPMS: Secondary progressive multiple sclerosis

SLE; Systemic lupus erythematous

TGFβ: tumor growth factor β

Th: T helper

TNFα: Tumor necrosis factor alpha

TREM-2: Triggering molecule expressed on myeloid cells

Chapter 1. Introduction & Review of Literature

1.1. Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) with a presumed autoimmune etiology. Historically, one of the earliest cases demonstrating clinical manifestations resembling MS symptoms is likely the Dutch nun "Saint Lidwina von Schiedham (1380-1422)" [1]. Later in the 19th century, Augustus Frederick d'Este (1794-1848), illegitimate son of the King of England George III, described in his diary and letters symptoms associated with MS clinical manifestations [2]. Today, over 2.3 million individuals worldwide are reported to be affected by MS, which is considered to be a leading cause of non-traumatic neurologic disability in young adults [3]. The prevalence of MS is higher in North America and Europe (over 100 cases per 100 000 people), compared to a lower incidence in East Asia and Sub-Saharan Africa (2 cases per 100 000 population) [4]. Females appear more susceptible to develop MS compared to males, with female:male ratio typically 3:1 [5]. In diseased brains, the typical infiltration of immune cells around post-capillary venules in the white matter is associated with blood brain barrier (BBB) breakdown, activation of resident astrocytes and microglia, oligodendrocyte/myelin loss and axonal injury [6].

1.2. Clinical and epidemiological features of Multiple Sclerosis

1.2.1. Clinical classification of multiple sclerosis

While diagnosis and clinical classification of MS has witnessed substantial evolution since Charcot's first attempt, the early principles require evidence of both dissemination in space (DIS) and dissemination in time (DIT) [7, 8, 9]. MRI can detect subclinical lesions used to fulfill DIS criteria, based on one or more T2 lesions in one or more brain regions (periventricular,

cortical, juxtacortical or infratentorial). MRI can even support DIT criteria based on detection of one or more new gadolinium enhancing lesions or T2 lesions during follow-up MRI, permitting diagnosis prior to the development of new clinical disease activity (relapse) [9]. MS diagnosis may be supported through examination of CSF, including testing for intrathecal IgG synthesis within CSF (oligoclonal bands). The MS disease course has been subdivided into early MS with a single presenting clinical attack, referred to as clinically isolated syndrome (CIS); relapsing remitting MS (RRMS) with evidence of at least 2 or more discrete events (making up 85-90% of the MS presenting forms of MS) and secondary progressive MS (SPMS) for those whose course started as RRMS and then continue to progress without remission; primary progressive MS (making up approximately 10% of MS presentations) refers to patients who progress with no remission from onset [9-11].

1.2.2. Factors associated with susceptibility of MS:

1.2.2.1. Genetic factors

Genetic contribution to MS is polygenic with over 200 susceptibility variants identified, none of which are abnormal per se and each of which contributes only a small amount to risk (as reviewed by [12]). First degree relatives to an MS patient are up to 15 times more likely to develop the disease [13, 14]. Studies investigating gene susceptibility in monozygotic (identical) twins demonstrated a higher concordance rate of 25% - 30%, whereas it appears to be as low as 3% - 5% in dizygotic (non-identical) twins of the same sex [15, 16]. MS preferentially affects Caucasian northern Americans and Europeans, compared to Asian and African populations [4, 15]. Early studies have identified several genes to be associated with MS, including HLA (class I & II), T-cell receptor β, CTLA4, ICAM1 and SH2D2A (as reviewed by [16]). Amongst those

genes the HLA-DRB1 gene, more precisely the DRB1*15:01 allele, was found to be associated with an increased disease incidence and an earlier disease onset in patients who harbor this allele compared to patients who do not present this variant [17].

1.2.2.2. Environmental factors:

1.2.2.2.1. Sunlight exposure, Vitamin D and MS:

The incidence of MS is more prevalent in higher latitude regions [18], and further associated with a decreased sun exposure consequently leading to a reduced vitamin D production [19, 20]. While this non-random distribution of MS may in part reflect migration patterns of individuals with similar genetic backgrounds, an important role of environmental factors is also thought to be at play. The lack of UV exposure may represent an important MS environmental risk factor through its effects on converting Vitamin D in the skin to its active form [21]. Studies have demonstrated that adequate UV-B exposure during childhood and sufficient vitamin D levels in early life are associated with reduced MS risk and reduced long-term MS activity and progression [20, 22]. However, the exact role of vitamin D in modulating MS risk and/or activity remain unknown. It is speculated that vitamin D has the capacity to modulate the pro-inflammatory vs. regulatory immune functions in MS [23, 24].

1.2.2.2.2. Viral infections and MS:

Viral infections represent another well recognized category of environmental factors that may impact MS risk. Some migration studies proposed that viral infections during a window of exposure risk in early life may trigger the development of MS [21]. People migrating from low MS-risk countries to high-risk countries and before the age of 15, appear to develop a

comparable risk as natives of the country to which they migrated [25, 26], suggesting that infections contracted at a young age may predispose to a later occurrence of MS. The main virus associated with an increased risk of MS is Epstein Barr virus (EBV); a double-stranded DNA virus and part of the herpes virus family known to cause infectious mononucleosis [27]. It is still hard to claim that EBV infection directly induces MS; however, pediatric MS cohorts have the potential to help us address this question given the early window in time. In fact, a study comparing children with pediatric MS and age and sex-matched children with monophasic events of inflammatory CNS demyelination demonstrated a higher prevalence of EBV seropositivity and a higher rate of EBV shedding into the saliva in kids with MS [28, 29].

1.3. General pathophysiology of Multiples Sclerosis

Multiple sclerosis is a complex autoimmune disorder. Despite the major advances the field has witnessed, we still have a limited knowledge regarding the key series of events leading to pathological manifestations associated with MS. A fundamental pathological feature associated with MS is the presence of focal demyelinating plaques within the white matter, gray matter and spinal cord of patients [30, 31]. Those focal demyelinating lesions are characterized by an active destruction of myelin sheaths in close contact with macrophages engulfing disrupted myelin, a loss of oligodendrocytes [32], a degree axonal damage [33], astrocyte and microglia activation [34] and inflammation defined by immune cell infiltration. Involvement of gray matter in this pathology clearly demonstrated back in 1962 [35] has been revisited in more recent years and described in more detail below [36].

1.3.1. Staging and classification of MS lesion pathology

1.3.1.1 Active/acute lesions

Acute plaques are pathologically characterized by loss of myelin and presence of myelin fragments within phagocytic cells, together with injured axons, microglia and astrocyte activation. The borders of demyelinated areas of active/acute lesions may not be well-demarcated while, in chronic phases, edges of lesion tend to appear better defined. The cellular composition of active lesions typically includes a dense phagocytic population. Many of such macrophage and microglia appear activated (MHC II^+) [34, 37] as reviewed in [38] and seem to be preferentially distributed at the lesion edges and periplaques [39]. Lymphocytes (T and B cells) are enriched around blood vessels, forming perivascular cuffs [40]. Early acute lesions may preferentially include $CD4^+$ helper T cells, while $CD8^+$ suppressor, cytotoxic T cells are found in late acute lesions [14, 41, 42]. Such lesions include oligodendrocytes loss which is prominent in the lesion center compared to lesion edges [43, 44], and axonal damage is reported in particular in early lesions [33, 45].

1.3.1.2 Chronic active or subacute plaque

Chronic active lesions exhibit a combination of features of acute and chronic plaques. These plaques are composed of an inactive hyper-cellular gliotic core, carrying characteristic of chronic silent plaques with surroundings displaying some activity with myelin phagocytosis. Demyelination may be accompanied by remyelination, an increased cell number of oligodendrocytes, astrocytes, mononuclear cell infiltrates around the lesions and late markers of macrophage activity [46, 47].

1.3.1.3 Chronic/inactive plaque

Chronic inactive plaques are the most abundant MS lesions to be reported within autopsy material obtained from patients with a prolonged disease course. Such plaques can be found in the white matter or in a smaller size in the cortex and other gray matter structures. The histological features of inactive plaques include myelin loss with a clear demarcation between demyelinated areas and normal tissue [38]. Oligodendrocytes form a rim around inactive lesions, while their cell death has been reported within the center of lesions along with a loss of microglia and hypertrophic astrocytes in addition to a low rate of axonal injury [38, 48]. Inflammation is much less prominent compared to active and chronic active lesions, as pathologically only the occasional perivascular presence of mononuclear cells including T cells and B cells has been reported [42].

1.3.1.4 Remyelinating/shadow plaque

Similar to inactive plaques, remyelinated shadow plaques can be distinguished due to their sharp demarcation from the normal-appearing white matter. Newly derived oligodendrocytes from progenitor cells appear to be responsible of remyelination [49]. Nonetheless, efficient remyelination would require the presence of some healthy axons and some mature oligodendrocytes that survived myelin loss [50]. Interestingly, inflammation could play an important role in repair as T cells, B cells and macrophages (including their soluble products) have been implicated in the remyelination process [51, 52].

1.3.2. Gray matter pathology

MS pathology was previously regarded as a white matter disease, despite some studies invoking the potential involvement of gray matter [35, 53]. The importance of gray matter involvement in disease progression is now well established [54]. The extent of cortical pathology in MS tissue correlates with the degree of cognitive and physical disability [55]. The increase of disability and brain atrophy correlates positively with the area of cortical lesions, suggesting that the gray matter lesion (GML) burden represents a good therapeutic target to limit disease progression [56, 57]. Bo et al. identified three major different types of cortical lesions (Figure 1.1) [54]. Type 1 (leukocortical) lesions are observed at the corticomedullary junctions in continuity with peri-vascular white matter lesions, while type 2 (perivascular cortical) lesions are reported within the cortex itself [54]. Finally, type 3 (subpial) cortical lesions represent the most abundant form of cortical demyelination and are found juxtaposed to the pia, forming a ribbon extending from the outer surface of the cortex into deeper cortical layers [54, 58]. It is the development of this subpial cortical lesion burden that is thought to be most relevant for progressive disease manifestations. Elucidating the mechanisms contributing to this injury may lead to the development of novel therapies aimed at limiting or halting progressive MS.

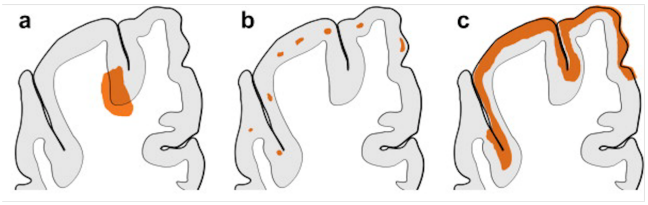

Figure1.1: Adapted from: Ranjun Danton & Bruce Trapp, Prog Neurol 2011, [59]

1.3.2.1 Meningeal inflammation

A relatively recently appreciated feature of MS pathology involves presence of immune cells within the meninges. B cells are found enriched in these immune cell collections compared to typical peri-vascular lesions. Such B cell rich meningeal immune cell collections were initially thought to be late features of disease based on their identification in post-mortem studies of patients who died with long-standing progressive forms of MS; however biopsy material obtained earlier in the course of MS patients suggests that meningeal inflammation including B cells can occur in early RRMS stages as well [36, 60, 61]. More recent imaging studies have captured leptomeningeal enhancement in the brain of individuals with MS [62]. Such structures were mostly found in the deep sulci of the temporal, cingulate, insula, and frontal cortex, which corresponds to the areas where pathological studies have identified predilection for more dense and structured immune cell collections [63]. Interestingly, cases exhibiting meningeal inflammation presented clinically with a more aggressive disease form and a greater extent of tissue injury in association with cortical lesions. The meningeal inflammation has been associated with the subpial form of cortical injury, which in turn leads to demyelination with oligodendrocytes loss and what appears to be a gradient of a neuronal loss and microglial

activation with the most severe injury in the most superficial cortical layers [64]. Meningeal inflammation represents a biology of interest in this thesis, and the enrichment of B cells suggests the possibility that B cells may contribute to the subpial cortical injury and consequently to progressive MS.

1.4. T cells and multiple sclerosis

MS was traditionally considered a T cell-mediated disease mainly based on findings from the commonly used EAE mouse model, but results did not completely translate to patients affected by MS. The prevailing model has been that autoreactive effector $CD4^+$ T cells get activated in the periphery and migrate to the CNS where they are thought to react to self-antigen(s) and initiate an "outside-in" disease process, while the ''inside-out" hypothesis' considers that disease is triggered inside the CNS and is further propagated in the periphery (reviewed in [65]). Different T cell subsets have been implicated in EAE and MS, including effector T cells (CD4 & CD8) and regulatory T cells. While CD4 T cells have received more attention than CD8 T cells in MS (in part driven by the predominant role of CD4+ T cells in EAE), typical MS perivascular lesions tend to have more CD8 T cells than CD4 T cells [66-68]. Potential antigen-presenting cells to T cells include microglia, infiltrating macrophage and B cells. T cells can be found not only in typical deep white matter lesions, but also in the CSF and within meningeal immune cell collections [36, 60, 69]. In EAE, the proportion of $CD4^+$ Th1 and Th17 subsets have been shown to be enhanced in the periphery, and during disease exacerbation as levels of IFNγ and IL-17 were found to be enhanced [70-73]. Additionally, Th17 have been found to efficiently cross the blood brain barrier and infiltrate the CNS [72]. MS patients have been reported to harbor abnormal circulating myelin-reactive T cells, either present at increased

frequencies or producing higher levels of pro-inflammatory cytokines, compared to healthy controls [74, 75]. Circulating CD8+ T cell implication in MS disease has mainly considered cytotoxic functions of CD8+ T cells (for example through production of granzyme B), as the latter have been reported to positively correlate with axonal injury in EAE [76]. CD8+ T cells can also produce granzyme, IFNγ and IL-17 and efficiently transmigrate across blood brain cells [77]. While not all attempts to translate EAE-based findings to human disease have been successful, studies in EAE continue to help us better understand T-cell biology, mechanisms of peripheral activation and regulation, CNS trafficking, and T cell-CNS interactions.

1.5. B cells and multiple sclerosis

1.5.1. B cell targeting therapies in MS

With MS traditionally viewed as a T cell driven disease, most immunomodulating treatments in MS were developed based on how they may target T cell responses. It is notable that all approved MS therapies thought to modulate T cell responses, also impact B cell functions; such therapies include: IFNβ, Fingolimod (FTY720), natalizumab, alemtuzumab and tecfidera [78-81] as summarized in the review by [103].

For instance, IFNβ based therapy decreased the frequency of $CD80^+$ B cells in the periphery of MS patients, consequently limiing T cell activation [82]. Other studies conducted by Shubert and colleagues indicated that IFNβ therapy required B cells for improved treatment's efficacy by increasing the numbers of peripheral transitional B cells [83]. Treatment with fingolimod, which targets S1P receptors, reduced circulating B cell numbers and shifted the B-cell cytokine-response profile from memory pro-inflammatory towards transitional anti-

inflammatory [80, 84, 85]. While the effects of natalizumab on cytokine responses of B cells remain unknown, we know that natalizumab increases memory and marginal zone B cells in the circulation [86], yet displayed beneficial effects that likely reflect blocking migration of multiple immune cell types into the CNS by blocking VLA4 integrin [87]. Our group has recently demonstrated that tecfidera (dimethyl fumarate) treatment not only affects T cell subsets but also impacts B cells (both *in vivo* and *in vitro*) as it reduces B cell pro-inflammatory cytokine (GM-CSF, IL-6, TNFa) responses, resulting in a significant shift of B cells responses away from a pro-inflammatory profile [88]. The highly effective humanized monoclonal antibody alemtuzumab depletes CD52 expressing T cells, B cells, monocytes and NK cells [89, 90].

The most compelling implication of B cells in MS pathophysiology comes from the demonstration in recent years that selective B cell depletion with anti-CD20 antibodies substantially decreases new MRI and clinical evidence of relapsing disease activity [91, 93, 94]. The phase II and III clinical trials of the anti-CD20 therapies (including rituximab, ocrelizumab and ofatumumab) demonstrated decreases of about 90% in new gadolinium enhancing lesions compared to controls [91-94]. Anti-CD20 B cell depletion with ocrelizumab has also demonstrated a modest effect on limiting disease progression in patients with primary progressive MS, which represents a significant advancement in the field as this has become the first approved treatment for patients with this previously untreatable form of MS [95]. Not all B cell targeting treatments have been beneficial for MS patients. Atacicept (composed of the fusion of TACI receptor and the Fc fragment of IgG), directed against naïve and plasma cells or plasmablasts , while effective for systemic lupus erythematous (SLE) patients [96] and despite promising results in EAE mice models, actually exacerbated relapsing disease activity for MS

patients [97, 98]. The precise mechanism behind this observation remains to be elucidated though, it may reflect atacicept's lesser effect on memory B cells, which are increasingly thought to be the pro-inflammatory disease-mediating B cells relevant in MS. Regardless of the mechanism behind MS disease exacerbation by atacicept, these observations further point to modulation of B cell subsets as having the potential to modulate MS disease activity. Together, the different clinical trials targeting B cells reinforce the concept that, in MS, B cells can have opposite functions depending on their subset, maturation stage and response profiles, and hence can differentially participate in the MS disease process. It is plausible that disease may be exacerbated if depleted B cell subsets had down-regulating functions, while the remaining memory B cells play a pro-inflammatory role. In keeping with this, the same B cell depleting therapy in EAE mice models can result in opposite effects depending on the type of antigen used for immunization and the timing of B cell depletion. The depletion of B cells in EAE induced with MOG_{35-55} worsened disease activity, while B cell depletions in EAE-induced with recombinant MOG reduced disease activity [99]. Additionally, the timing of B cell depletion (before or after) immunization appeared to have an impact on outcomes [100].

1.5.2. B cell functions and implications in MS

In addition to their capacity to differentiate into antibody producing plasmablasts and plasma cells, B cells are recognized as having the potential to be antigen presenting cells and produce both pro-inflammatory and anti-inflammatory cytokines, which may be relevant to aberrant activation of T cells and other cells in MS [101-103]. B cells may also contribute to the formation of lymphoid aggregates through both soluble and contact-dependent mechanisms. These diverse functions of B cells may be relevant both in the periphery and the CNS of patients.

1.5.2.1. B cell in the periphery

1.5.2.1.1. Distinct B cell subsets

The success of B cell depleting therapy limiting new MS relapses without impacting elevated levels of antibodies present in the CSF indicates antibody-independent contributions of B cells to relapsing disease activity [91, 94, 95, 104-106]. A number of groups have demonstrated that distinct cytokine profiles can be secreted by different B cell populations in a context dependent manner. The field has now advanced and we are currently able to distinguish pro-inflammatory from anti-inflammatory B cells according to their cytokine profile, as explained in the section below and summarized in Table 1.1.

Table 1.1: Summary of human Beff and Breg subsets

	Beff	Breg
Maturation status	Mature memory B cells	Mature naïve B cells (and immature Transitional B cells)
Phenotype	$CD27^+$ memory	$CD27^-$ (and $CD24^{high}$ $CD38^{high}$ $CD27^-$)
Induction	CD40, BCR, IL-4R	TLR 9 and CD40
Functions	Up-regulate Th1/Th17 Cells Induce pro-inflammatory myeloid cell responses	Down-regulate Th1 responses
Implication in MS	- Enhanced in MS	-Deceased in MS, compared to Beff -First subset that replenishes in periphery after anti-CD20 therapy

1.5.2.1.1.1. Anti-inflammatory cytokine producing B cells

The capacity of B cells to downregulate immune responses independently of antibody production has been known for some time [107]. Regulatory B cells (Breg) are characterized by their capacity to secrete a higher proportion anti-inflammatory cytokines. In humans, $IL\text{-}10^+$ Breg are of an immature transitional phenotype: $CD24^{high}$ $CD38^{high}$ $CD27^-$ IgD^{low} IgM^{high} [108, 109]. Our lab demonstrated human naïve B cells ($CD27^-$) but not memory B cells ($CD27^+$) are capable of producing IL-10 upon single stimulation with CD40L [110], a function that was abnormally diminished in B cells of MS patients [105]. IL-10 producing B cells can indirectly down-regulate pathogenic T cell responses with the help of dendritic-cells [111]. Rodent B10

cells can be induced within the memory ($CD27^+$) pool to produce IL-10 through TLR4&9 stimulation using LPS and CpG [112, 113]. The selective knock-out (KO) of IL-10 producing B cells in mice, resulted in a more severe EAE [114], while when they induced IL-10 producing B cells *in-vitro* and adoptively transferred them, EAE severity diminished in an IL-10 dependent manner [100, 111, 115]. Moreover, IL-10 producing B cells suppressed TNFα production by monocytes via IL-10; and further diminished TNFα production by antigen-specific $CD4^+$ T cells independently of the IL-10 produced by B cells [113] .

In mice, B cells have also been demonstrated to secrete transforming growth factor β (TGFβ); a cytokine known to control cell differentiation and proliferation. Initially, Caver and colleagues demonstrated that after stimulation mouse B cells can produce TGFβ1 [116], which appeared to inhibit autoimmune disease exacerbation and further promote allograft tolerance, perhaps through the modulation of the balance between effector and regulator T cell [117, 118]. It still remains unknown whether human B cells are capable of TGFβ secretion.

Additionally, B cells can produce IL-35, an anti-inflammatory cytokine and part of the IL-12 cytokine family [119]. IL-35, known to be produced by regulatory T cells [120, 121], has also been found to be produced by murine B cells [122]. Interestingly, IL-35- producing B cells displayed plasma cell phenotypic surface markers indicating plasma cells may harbor a previously unappreciated antibody-independent role [122]. IL-35 producing B cells played a critical role during recovery from EAE and EAU, and the IL-35 produced by the B cells could directly inhibit immune activation or do so indirectly via the induction of IL-10 secretion by B cells [123].

1.5.2.1.1.2. Pro-inflammatory cytokine producing B cells

B cells are now well recognized for their capacity to secrete pro-inflammatory cytokines in both rodent- and human-derived systems. Tumor necrosis factor alpha (TNFα) and lymphotoxin alpha (LTα) were among the first cytokines from the TNF cytokine family to be identified from a B cell source and are able to promote inflammation in the context of pathogen invasion [124, 125].

Stimulation of human B cells through CD40 and BCR induces the production of TNFα and LTα [126]. Our lab previously demonstrated that, upon stimulation, MS-derived B cells produce significantly higher levels of TNFα and LTα compared to B cells derived from age- and sex-matched healthy individuals. TNFα and LTα from B cells was implicated in subsequent T cell activation in MS [105, 110]. The micro-RNA (miR)-132:SIRT1 axis has been implicated in modulation of B cell secretion of TNFα and LTα and in the aberrant production of these cytokines by MS patient B cells [127]. After B cell-depleting therapy in patients, the reconstituting B cells expressed lower levels of TNFα and LTα and higher levels of IL-10 [110]. It is noteworthy that, in mice, $CD138^{+}$ IgA^{+} plasma cells producing both TNFα and iNOS have been described [128], again highlighting the potential for even fully differentiated plasma cells to contribute to local immune environments through antibody-independent mechanisms.

Amongst other pro-inflammatory cytokines, IL-6 produced by B cells is also implicated in MS [129]. IL-6 has previously been ascribed both pro- and anti-inflammatory roles [130] though, in MS, the capacity of IL-6 to induce the differentiation of naïve T cells into Th17 cells [131] while inhibiting regulatory T cells generation and function [132] [133] may be particularly relevant. At

the same time, IL-6 has the capacity to induce IL-10-producing regulatory B cells as well as regulatory myeloid cells [134, 135]. B cells derived from MS patients secrete abnormally increased levels of IL-6 compared to matching controls [129], and the selective knock-out of IL-6 diminished the severity of EAE and Th17 responses [136].

Additionally, IL-15, a member of the four α-helix cytokine family, may be produced by different types of cells [137]. IL-15 has been abnormally implicated in MS pathology as higher levels of IL-15 have been reported in serum and CSF of patients compared to matching controls [138]. Activated MS-derived B cells can produce higher levels of IL-15 compared to matching controls, and the activation of mouse B cells via CD40 and BCR induced IL-15 secretion, promoting CD8+ T cell CNS recruitment and mediated oligodendrocytes cytotoxicity [139].

While granzyme B (GB) secretion is usually associated with cytotoxic T cells and NK cells [140-143], Hagan and colleagues were able to demonstrate that human B cells (but not murine B cells) produce GB [142, 143]. Subsequent characterization of GB-producing B cells indicated that naïve B cells produce significantly more GB in comparison to memory B cells after stimuli inducing BCR signaling [142]. GB-producing B cells seem to play a dual role as it is beneficial in the context of tumor and viral immune response. While it is deleterious during autoimmunity where it induces injury within tissue of target organs, it exacerbates disease activity by directly killing regulatory T cells [143-145]. For example, GB produced by B cells alters the epithelial barrier in patients with inflammatory bowel disease IBD [146], and, in SLE, GB-producing B cells limit the proliferation and differentiation of own subset [145].

Finally, the granulocyte macrophage-colony stimulating factor (GM-CSF) has been studied as a growth factor for myeloid lineage cell development and function. It is secreted by both immune and non-immune cells during infection and autoimmune disease [147] and has also been implicated in CNS inflammation. In EAE models, Th17 T cells are reported as key producers of GM-CSF as the selective KO of GM-CSF from Th17 cells fail to induced passive EAE [148, 149], whereas GM-CSF KO mice are resistant to active EAE induction [150]. In humans, both Th17 and Th1 are capable of GM-CSF production, the latter cell subset being increased in the CSF of MS patients [151-153].

GM-CSF-producing B cells have been generated from B1a murine cells, also called ''innate response activator'' (IRA) B cells, and reported to play an important protective role during infections but a deleterious role during atherosclerosis [154]. Our lab recently identified a human B cell subset producing GM-CSF, and in contrast to murine IRA cells the human GM-CSF$^+$ B cells, belong to the CD27$^+$ memory pool and co-express high levels of IL-6 and TNFα [155]. The proportion of GM-CSF producing B cells was significantly higher in the periphery of untreated RRMS patients and further increased upon activation compared to B cells derived from matching healthy controls [155]. Our group was further able to demonstrate that GM-CSF$^+$ B cells increased myeloid pro-inflammatory responses in a GM-CSF-dependent manner, whereas the depletion of B cells from MS patients resulted in the decrease of such myeloid pro-inflammatory responses consistent with a pathogenic role of this B cell population *in vivo* [155]. To date, there has been no unique cell surface marker attributed to either pro-inflammatory or anti-inflammatory cytokine-producing B cells, and specific transcriptional markers have also not been identified.

1.5.3. B cells in the central nervous system

The initial implication of B cells in MS is based on the long-standing observation that the CSF of patients typically exhibits an increased Ig synthesis rate and elevated Ig levels that produce oligoclonal bands (OCB) when run on electrophoretic gel [156-158]. Several studies have described B cells, plasma cells and plasmablasts, in various CNS compartments including the CSF, the parenchyma and the meningeal immune cell collections (as reviewed in [101]). These B cells are thought to be the source of the elevated CSF Ig levels and synthesis rate as well as the OCB [159-162].

1.5.3.1. Cerebrospinal fluid B cells

Early studies investigating the cellularity of CSF in patients with MS revealed that higher B cell/monocyte ratios were associated with disease progression and severity while lower B cell/monocyte ratios were associated with slower disease progression [163]. Complementary findings indicated that B cells found in the CSF of MS patients are mainly class-switched IgD^- IgM^- memory B cells [164] and short-lived plasmablasts [165, 166]. A more recent study confirmed previous observations using deep repertoire sequencing of IgG variable heavy chain (IgG-V_H), which further revealed that B cells persisting within the CNS shared some common IgG-V_H compared to those from the periphery, indicating that perhaps only few B cells were able to successfully access (and/or persist in) the CNS [167].

The elegant studies based on somatic hypermutation analysis of the heavy chain immunoglobulin (Ig V_H) variable region of CSF B cells in comparison to circulating B cells (both purified from MS patients at the same time) consistently revealed that the MS CSF was

enriched in clonally expanded B cells compared to the periphery. These findings suggest that the accumulation of B cells in the CSF of patients may be associated with an antigen driven process as the CDR3 region possessed a number of mutations. Other recent studies utilizing IgM-chain gene analysis within CSF-derived B cells demonstrated not only extensive hypermutations but also clonal expansion in IgM^+ B cells [168].

In other related work, Ritchie and colleagues pre-sorted total $CD19^+$ and $CD138^+$ plasma cells/plamsablasts from the CSF of MS patients and amplified the IgG variable region, which demonstrated that both subsets displayed somatically mutated expanded clones [169]. The analysis further indicated that the plasmablasts/plasma cells displayed a more exclusive repertoire, despite some overlapping total B cells [169]. Whether B cells infiltrate the CNS as activated memory B cells or as naïve B cells that then undergo maturation locally due to a favorable intrathecal milieu that supports germinal-center like reactions remains unclear.

1.5.3.2. Parenchymal B cells

Histopathological studies examining the parenchyma of deep white matter perivascular demyelinating lesions typically indicated the presence of relatively few B cells and plasma cells compared to higher number of myeloid and T cells [44, 58, 170]. Interestingly, type II demyelinating lesions (described above) exhibited important Ig and complement deposition [44, 58]. Moreover, a study looking into 11 relatively early MS cases and in particular around areas of complete demyelination (active demyelinating lesions) demonstrated an increased number of not only T cells and myeloid cells but also B cells and IgG^+ plasma cell [170, 171]. This data

indicates the possibility that B cells and plasma cells are involved in early lesion evolution, a feature that most autopsy series (typically capturing chronic active or inactive lesions) would not be able to identify.

1.5.3.3. B ells within meningeal immune cell collections

The presence of meningeal immune cell collections were reported over a decade ago in both progressive MS tissue and early relapsing MS cases [36, 60, 61], some of which have been found to be enriched in B cells and are commonly named ‘’follicle like structures’’. A deeper characterization of meningeal immune cell aggregates in MS patients revealed enrichment of proliferating ($Ki67^{+}$) $CD20^{+}$ B cells in addition to the presence of some plasma cells/plasmablasts, $CD8^{+}$ and $CD4^{+}$ T cells, and CXCL13-producing $CD35^{+}$ cells with follicular dendritic cell phenotype [64].

Studies investigating somatic hypermutation of B cells and plasma cells within different CNS sub-compartments have indicated shared ancestors between the CSF and parenchymal B cells [161] and also between the parenchyma and the meningeal cells based on autopsy tissue [172]. Shared clonally expanded B cells and plasma cells were also found populating the CNS and cervical lymph nodes of the same MS patient, suggesting bidirectional B cell trafficking occurs in MS between the CNS and the periphery [167, 173, 174]. It remains unclear whether formation of meningeal immune cell collections occurs from early on or only in late disease stages and whether or not it is transient.

1.6. Glial cells and multiple sclerosis

1.6.1. Astrocytes in MS

Very early pathological reports described abnormalities of astrocytes in MS; however, until recently astrocytes have largely been regarded as secondary players and received relatively little interest. The last few years witnessed a surge of interest in astrocytes in both health and disease. The attributed positive marker for astrocytes is the glial fibrillary acidic protein (GFAP), which defines the cytoplasmic cell body and lacy processes [47]. Physiological functions of astrocytes include regulation of local blood flow, immune regulation, tissue homeostasis through cytokines and chemokines secretion (TNFα, IL-6, CXCL10 & NO synthetase), and maintenance and stabilization of neurons and axons [47, 175-177]. Additionally, astrocytes can express major histocompatibility complex (MHC) class II, known to be required for antigen presentation [178]. Morphology and astrocytic responses may vary according to the disease stage and type of lesion. Briefly, active plaques include hypertrophic astrocytes with gemistocytic cytoplasm, intermediate filaments, voluminous nuclei and abnormally swollen possesses that appeared to phagocytose myelin [47]. In contrast, astrocytes in inactive plaques were characterized by a small soma and nuclei with long thin processes. Distribution of astrocytes within chronic active plaques included gliotic astrocytes in the center of lesions. Reactive astrocytosis is also reported in normal appearing white matter (NAWM) and cortical lesions but to a lesser extent. Astrocytes may contribute to regional differences in inflammatory reactivity as differences in microRNA expression profiles have been documented in lesional white matter versus non-inflamed tissue [179]. Astrocytes can play both direct and indirect roles in mediating tissue injury. Previously, our lab has implicated a deleterious role of human derived-astrocytes *in vitro* as astrocytes exposed to Th1 conditioned media mediated oligodendrocyte progenitor cytotoxicity in a

CXCL10-dependent manner [180]. In addition, IL-15 released by astrocytes can increase NKG2D (Natural Killer Group 2 D) expression of cytotoxic $CD8^+$ T cells, further increasing subsequent cytotoxic functions of $CD8^+$ T cells [181]. As I describe in Chapter 2, human astrocytes under inflammatory conditions supported MS B cell survival and activation through soluble factors [177]. Direct evidence of complex functions of astrocytes in CNS inflammatory injury and repair is seen in EAE where depletion of reactive astrocytes during the acute phase of the disease worsened EAE, while their depletion during progressive disease course led to a significant improvement of disease [182].

1.6.2. Microglia and infiltrating macrophage in MS

Microglia represent the brain resident myeloid-cells endowed with immunologic properties. Recent work has established that microglia and other distinct tissue-resident macrophages are formed during embryonic development and independently persist and self-restore in their respective organs during adulthood [183-185]. Microglia make up about 6-18% of neocortical cells in the human brain [186] and originate from a mesodermal cell lineage distinct from blood-derived monocytes/macrophages during primitive hematopoiesis in yolk-sac [187, 188]. Both CNS-resident microglia and infiltrating macrophages belong to the mononuclear phagocyte cell group. To date, there are no exclusive markers distinguishing human microglia from infiltrating macrophages in humans; nonetheless, gene sequencing studies have now defined a unique TGFß-dependent molecular and functional signature in microglia [189]. Microglia and infiltrating macrophages are thought to have several functions relevant to MS, including phagocytosis of myelin debris, interaction with T cells including antigen-presentation, and interactions with neurons and oligodendrocytes [190, 191] (Figure 1.2). Microglia and/or

infiltrating macrophages are dominant cell types in both acute and chronic MS lesion and can contribute to changes reported in NAWM (as described above). Active MS lesions harbor microglia with an activation profile and enhanced miR-155 expression profile compared to parenchymal microglia, and miR-155 was further increased in peripheral $CD14^+$ monocytes purified from MS blood [192]. In pathological conditions, microglia and macrophages can be found in close proximity to pathogenic T cells.

1.6.2.1. Resting/quiescent state of microglia/macrophage in physiological conditions

In healthy conditions, resting microglia and macrophages are thought to exist in a steady/quiescent state. They display a largely resting phenotype distinguished by ramified processes and small soma [193, 194]. The phenotype of quiescent microglia/macrophages can be defined by the relative expression of several molecules known as ‘’inhibitory’’ molecules, including CD200R, the triggering molecule expressed on myeloid cells (TREM-2), fractalkine receptor (CX3CR1), macrophage colony stimulating factor receptor M-CSFR (macrophage colony stimulating factor receptor) and CD172 (SIRP1α), which are all thought to play key roles in maintaining the inhibitory/resting profile of microglia(reviewed in [190, 194]). In the EAE mouse model, the absence of CD200 (CD200R ligand) results in a more rapid onset of the disease [195]. The glycoproteinTREM-2 is demonstrated to be a protective molecule and often exhibits anti-inflammatory properties [196]. Bone marrow-derived precursor myeloid cells of mice transduced with TREM-2 ameliorated EAE disease course by increasing phagocytic activity and inducing an anti-inflammatory cytokine milieu [197]. TREM-2 is necessary for efficient phagocytosis of apoptotic cell membranes by microglia, whereas decreased expression

of TREM-2 impairs clearance of apoptotic neurons and increases TNFα and iNOS expression [197, 198]. This indicates there can be a promising role for increasing TREM-2 in order to mediate anti-inflammation and possibly help disease recovery. Microglia also have the potential to express receptors that are essential cellular survival such as M-CSFR which is known to play a major role in the survival and development of microglia and also implicated in survival and differentiation of peripheral macrophage [199]. In fact, mice deficient in M-CSFR displayed a total absence of microglia and macrophage lineage cells [200].

1.6.2.2. Activated state of microglia/macrophage during MS stages

Both microglia and infiltrating macrophage could play an important role in mediating autoimmunity by perpetuating ongoing local inflammation. Upon activation, microglia and macrophage further activate $CD4^+$ T cells, which are thought to attack the myelin sheath and axons (reviewed in [201]). In the MS CNS, microglia and infiltrating macrophages may indirectly damage oligodendrocytes and neurons as they have been reported to produce pro-inflammatory cytokines such as IL-6, and TNF-α, which will sensitize axons to glutamate excitotoxicity [202]. Both microglia and macrophages are capable of myelin phagocytosis *in vitro* and *in situ* in lesions [54, 203]. Functional phenotypes of microglia and infiltrating macrophages continue to be defined based on *in vitro* and *in vivo* studies, indicating that multiple myeloid phenotypes can exist *in vivo* depending on the local milieu. When microglia or peripheral macrophages are activated *in vitro*, they change their morphology by losing their processes and increasing in size to an ‘’amoeboid’ shape, and upregulate a number of cell surface markers such as CD45, CD80, CD86 and MHC-II [204, 205]. Pathological MS studies clearly

demonstrated the predominance of what appear to be pro-inflammatory microglia in normal appearing white matter (NAWM) and active demyelinating lesions characterized by the up-regulation of markers such as CD40, CD86 and CD64 [206]. Studies focusing on meningeal immune cell aggregates enriched in B cells in MS patients revealed a greater extent of tissue injury in the subjacent cortical regions and a gradient of microglia activation juxtaposed to such structures, which diminished moving more deeply into layer II and layer III of the cortex [64].

Most of these studies highlight the importance of the maintenance of the delicate balance between activation and inhibitory microglial functions. The pro-inflammatory or anti-inflammatory milieu of microglia/macrophage likely remains crucial in shaping and defining their contributions. Therefore, elucidating mechanisms by which microglia may be pathologically activated as part of the CNS-compartmentalized could help to develop more targeted drugs that counter the loss of regulation and restore microglia quiescence.

Figure 1.2:

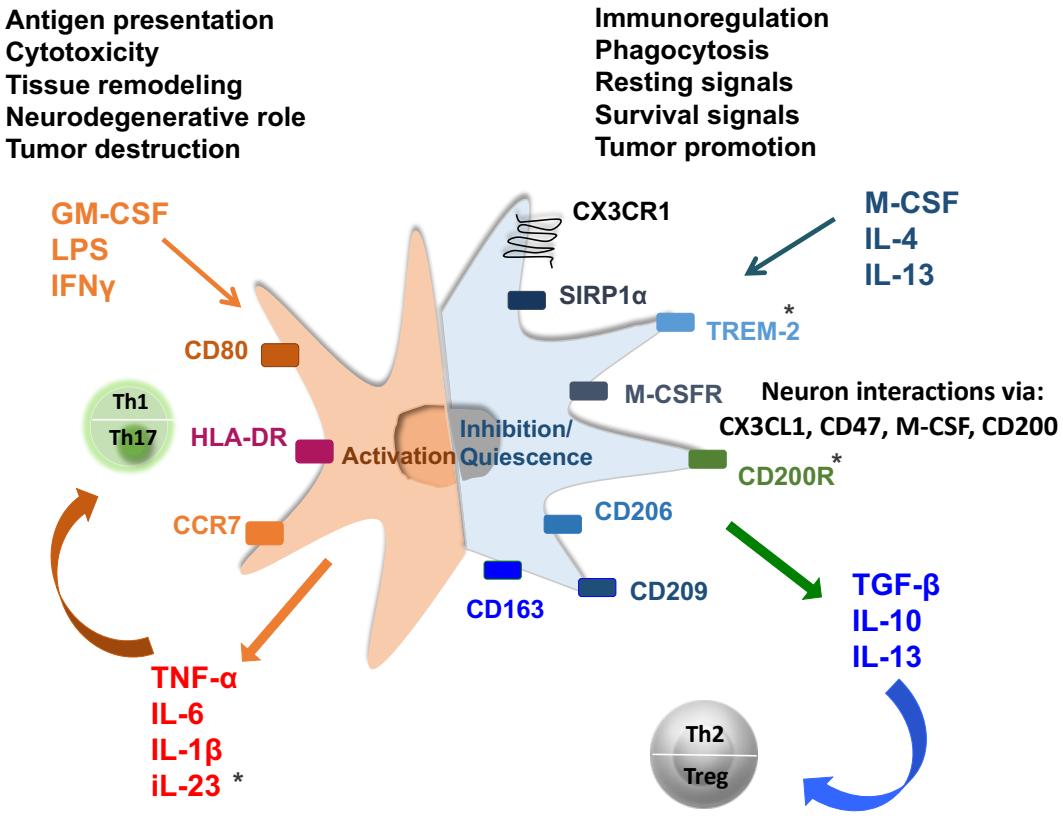

Figure1.2: Pro-inflammatory M1 and quiescent/anti-inflammatory M2 microglia regulated by the cytokine milieu. Both M1 and quiescent or M2 polarization profile could have beneficial role by either phagocytosing potential antigens, activating other immune cells or cleaning cell debris. * represents quiescence markers and soluble factors not yet validated in human microglia. Pro-inflammatory cell surface and soluble markers are up-regulated in response to pro-inflammatory stimuli (e.g.GM-CSF, LPS, IFN-g) known to induce Th1/Th17 cells. However, M-CSF alone or IL-4 & IL-13 could increase quiescent and M2 microglia cell surface and soluble makers and induce Th2 or Treg cells. In order for microglia to maintain a steady state, they interact with neurons interact through soluble or cell surface molecules (CX3CL1, CD47, M-CSFR & CD200). Pro-inflammatory M1 microglia are implicated in antigen presentation, which further activate immune cells such as Th1 or Th17, promote Cytotoxicity/tissue remodeling and could be implicated some neurodegenerative pathologies. Quiescent microglia are known to help maintaining resting state, induce phagocytosis and aide in cell survival. M2 microglia are known for their anti-inflammatory properties as they play a role in immuno-regulation and help tumor promotions

1.7. The inflamed MS CNS as a B cell fostering environment

The biology underlying CNS injury in progressive MS is thought to involve a combination of ongoing inflammation that is compartmentalized within the CNS and degeneration [207]. As previously noted, compartmentalized inflammation is characterized by the abnormal activation of glial cells such as astrocytes and microglia and is associated with loss of oligodendrocytes and neurons. However, the precise molecular mechanism(s) responsible for maintaining the chronic inflammation remain to be elucidated. B cells, and in particular the memory B cell subsets, are recognized to persist in the chronically inflamed MS CNS [172-174]. Clonally expanded B cells found in the CSF reportedly have the capacity to produce antibodies specific to CNS cells (including neurons and astrocytes) and can be shown to cause complement-mediated injury to such structures within the CNS [208]. In collaboration with the Lisak group, we used soluble factors derived from B cells of untreated RRMS patients or matching healthy controls in rodent oligodendrocyte and neuronal cultures. We have demonstrated that soluble factors of untreated MS patient B cells mediated apoptotic cell death of oligodendrocytes and neurons, while matching healthy control B cell-derived soluble factors did not impact their survival [209, 210]. Of note, this toxicity to CNS cells mediated by the MS patient B cells was not due to Ig and reflects an antibody-independent functional capacity of B cells. It is attractive to consider whether chronically persisting B cells in the inflamed CNS contribute both to propagation of compartmentalized inflammation and to injury locally. We consider that bi-directional interactions between B cells and glial cells mediated through release of secreted factors are responsible for why B cells are fostered, activated and able to contribute to propagating local inflammation and injury to brain cells within the underlying cortical tissue.

Preface, rationale, hypothesis and objectives - Chapter 2

The first chapter of my thesis introduced multiple sclerosis including current thinking about MS pathology and pathophysiologic mechanisms, identifying important conceptual gaps particularly related to the potential contribution of immune cells to progressive disease. With the implication of B cell responses as relevant to these processes, a fundamental question that remains to be answered is how B cells persist (i.e. are fostered) in the inflamed MS CNS and how immune:glial interactions may contribute to CNS-compartmentalized inflammation, cortical pathology and disease progression. In the next chapter, I will mainly concentrate on how human astrocytes support B cell persistence and activation, which may help propagation of local inflammation and contribute to subpial cortical pathology and disease progression. Most studies considering B cell functions in the CNS have been based on pathology investigations, which have the advantage of reflecting consequences of in vivo disease processes but, as a 'snapshot' in time, do not directly assess the process to identify potential cellular mechanisms that may be involved in this process. In the first part of this chapter, I used an *in vitro* culture system, whereby I co-cultured human B cells with human astrocytes under basal or pro-inflammatory conditions to assess the impact on survival and activation of B cells in response to astrocytes. In the second part of this chapter, I investigate the impact of astrocytes on survival and activation of B cells isolated from RRMS patients and whether requirement of cell to cell contact is necessary. My results suggest that B cell persistence in the CNS may be supported by soluble factors released by from astrocytes and that, when activated, the astrocytes-derived soluble factors can also enhance activation of MS relevant B cells. I further confirm that B cells exposed to these activated astrocytes could themselves mediate more efficient pro-inflammatory T cell activation.

Chapter 2. Human central nervous astrocytes support survival and activation of B cells: Implications for MS pathogenesis

Human central nervous system astrocytes support survival and activation of B cells: Implications for MS Pathogenesis

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Abstract

Background: The success of clinical trials of selective B cell depletion in patients with relapsing multiple sclerosis (MS) indicates B cells are important contributors to peripheral immune responses involved in the development of new relapses. Such B cell contribution to peripheral inflammation likely involves antibody-independent mechanisms. Of growing interest is the potential that B cells, within the MS central nervous system (CNS), may also contribute to the propagation of CNS-compartmentalized inflammation in progressive (non-relapsing) disease. B cells are known to persist in the inflamed MS CNS and are more recently described as concentrated in meningeal immune-cell aggregates, adjacent to the subpial cortical injury which has been associated with progressive disease. How B cells are fostered within the MS CNS and how they may contribute locally to the propagation of CNS-compartmentalized inflammation remains to be elucidated.

Methods: We considered whether activated human astrocytes might contribute to B-cell survival and function through soluble factors. B cells from healthy controls (HC) and untreated MS patients were exposed to primary human astrocytes that were either maintained under basal culture conditions (non-activated) or pre-activated with standard inflammatory signals. B cell exposure to astrocytes included direct co-culture, co-culture in transwells, or exposure to astrocyte-conditioned medium. Following the different exposures, B cell survival and expression of T cell co-stimulatory molecules were assessed by flow cytometry, as was the ability of differentially-exposed B cells to induce activation of allogeneic T cells.

Results: Secreted factors from both non-activated and activated human astrocytes robustly supported human B cell survival. Soluble products of pre-activated astrocytes also induced B-cell upregulation of antigen-presenting cell machinery, and these B cells, in turn, were more efficient

activators of T cells. Astrocyte soluble factors could support survival and activation of B cell subsets implicated in MS, including memory B cells from patients with both relapsing and progressive forms of disease.

Conclusions: Our findings point to a potential mechanism whereby activated astrocytes in the inflamed MS CNS not only promote a B-cell fostering environment, but also actively support the ability of B cells to contribute to the propagation of CNS-compartmentalized inflammation, now thought to play key roles in progressive disease.

Key words: multiple sclerosis, CNS-compartmentalized inflammation, human B cells, human astrocytes

Background

Studies of immune mechanisms that contribute to multiple sclerosis (MS) pathophysiology have traditionally highlighted processes of aberrant peripheral immune-cell activation and their subsequent trafficking into the central nervous system (CNS). This paradigm, commonly studied in animal models such as experimental autoimmune encephalomyelitis (EAE), continues to provide a useful framework for understanding as well as therapeutically targeting peripheral immune responses that underlie MS relapses. However, a major unmet clinical need in MS is represented by a relentless, progressive (non-relapsing) disease course, eventually experienced by most patients. The biology underlying such progression is less well understood but is now thought to reflect, at least in part, ongoing inflammation that is compartmentalized within the CNS. How such inflammation is maintained and how it is propagated within the CNS of patients is unknown.

Pathologic studies in recent years have highlighted the presence of immune cells (i.e., inflammation) within the meninges of patients with MS [61, 63, 64]. An important association has been identified between such meningeal inflammation and cortical injury, in particular, the subpial form of cortical pathology, which can be very extensive and is now thought to represent an important substrate of progressive disease [54, 211]. This subpial cortical pathology is characterized by oligodendrocyte injury, demyelination and astrogliosis, together with a graded degree of neuronal loss and microglial activation [61, 64]. The extent of neuronal loss and microglial activation in subpial lesions is reportedly greatest in superficial layer I of the cortex (immediately underlying the pia-meninges and cerebrospinal fluid (CSF)), with lesser abnormalities seen through the deeper cortical layers. An attractive hypothesis currently being

pursued in the field is that progressive subpial cortical injury may reflect the consequences of one or more soluble factors secreted by immune cells fostered in the meninges.

A spectrum of meningeal inflammation has been described in MS ranging from relatively scattered immune cells to considerably more organized immune-cell collections, some of which recapitulate features of tertiary lymphoid structures. While variable degrees of T cells and myeloid cells have been described across this spectrum, a commonly highlighted feature of meningeal inflammation in MS is the presence of B cells and plasma cells, with several studies describing meningeal immune-cell collections that are 'B-cell rich' [211] [64{Howell, 2011 #29, 212]. B cells of MS patients are known to be abnormally pro-inflammatory and their soluble products in vitro can be toxic to oligodendrocytes and neurons, fueling an interest in the potential contribution of B cells to CNS-compartmentalized inflammation and progressive CNS injury in MS [209, 210].

In this regard, the inflamed MS CNS appears to be a B cell fostering environment, as evidenced by the remarkable persistence and clonal uniformity of intrathecal immunoglobulin (Ig), and of serially-sampled B cells and plasma cells [213]. However, the mechanisms that may support B cell survival within the MS CNS and the implications for propagation of local disease processes have not been fully elucidated. Here, we assessed whether and how soluble factors released by human astrocytes may impact survival and activation of B cell responses that could, in turn, contribute to propagating CNS-compartmentalized inflammatory responses.

Results

Human astrocytes support B cell survival and increase their co-stimulatory molecule expression

While human B cells cultured alone survived poorly (as expected), survival of B cells co-cultured with human astrocytes was significantly enhanced (Figure 2.1a, representative donor; Figure 2.1b, summary, $n = 9$), whether the astrocytes were previously cultured under basal conditions ($p < 0.001$, $n = 9$), or pre-activated with IFNγ and IL-1β ($p < 0.001$, $n = 9$) as a proxy for astrocytes pre-activated by an inflammatory environment. While the enhancement of B cell survival mediated by exposure to astrocytes was similar whether the astrocytes had been pre-activated or not (Figure 2.1b), B cells exposed to pre-activated astrocytes also exhibited significant upregulation of the T cell co-stimulatory molecule CD86 (B7.2), as indicated by the percentage of B cells expressing CD86 (Figure 2.1c; $p < 0.001$, $n = 8$) and their CD86 mean fluorescence intensity (MFI) (Figure 2.1d; $p < 0.001$, $n = 8$).

The effects of astrocytes on B cells can be mediated by astrocyte-secreted products

To determine whether the effects of astrocytes on B cells are contact-dependent or can be mediated by astrocyte-soluble factors, we used two approaches: (i) co-culture of astrocytes and B cells in transwells, in which cells are not in direct contact but soluble factors can cross the 0.4-μm pore-size membrane bi-directionally; or (ii) addition of astrocyte-conditioned medium (ACM) to the B cell cultures for 3 days. As previously observed with the direct co-culture described above, incubation of B cells across a transwell from astrocytes also significantly enhanced B cell survival (Figure 2.2a; $p < 0.0001$, $n = 10$), whether B cells were cultured in transwell with stimulated ($p = 0.0002$) or unstimulated astrocytes ($p = 0.0003$). ACM from pre-stimulated astrocytes also significantly increased B cell survival (Figure 2.2b; $p = 0.0032$), while

ACM derived from unstimulated astrocytes did not (Figure 2.2b; $p > 0.99$). Compared to unstimulated astrocytes, stimulated astrocytes in transwell also significantly increased B cell surface expression of CD86 (Figure 2.2c; $p = 0.0007$, $n = 10$), which was also observed with astrocyte-conditioned medium. Compared to ACM from unstimulated astrocytes, ACM from stimulated astrocytes was able to significantly increase B cell expression of CD86 (Figure 2.2d; $p = 0.032$, $n = 5$). In a series of blocking experiments, neutralizing antibodies to either IL-6, IL-15, or BAFF (compared to the appropriate isotype control) did not abrogate the effects of astrocyte-soluble products on B cell responses (Supplementary Fig 2.2).

Secreted products of activated astrocytes enhance the ability of B cells to activate T cells

Based on the observations above, we predicted that B cells pre-exposed to stimulated astrocytes might exhibit an enhanced capacity to activate T cells. As shown in Figure 2.3 ($n = 10$), T cells cultured alone (negative control) did not proliferate. The addition of allogeneic B cells that were not pre-exposed to astrocytes induced an expected degree of allogeneic T cell proliferation (Figure 2.3a, summarized in Fig 2.3b). B cells that were pre-exposed in transwell to unstimulated astrocytes did not substantially enhance T cell proliferation; however, pre-exposure of B cells to stimulated astrocytes in transwell resulted in B cells that induced significantly greater T cell proliferative responses (Figure 2.3b) and T cell secretion of IFNγ (Supplementary Figure 2.3).

Astrocyte-secreted factors impact responses of MS-patient-derived B-cell subsets

To establish whether the ability of astrocytes to support B cell survival and activation is recapitulated with MS-patient-derived B cells, we isolated B cells from untreated patients with

secondary progressive MS (SPMS, Supplementary Table 2.1). Since the majority of B cells identified within the CNS of MS patients belong to the memory B cell pool [164, 173, 214], we further sorted the SPMS B cells into memory ($CD27^{+}IgD^{-/+}$), naive ($CD27^{-}$ IgD^{+}), and transitional ($CD24^{high}$ $CD38^{high}$) B cell subsets (Figure 2.4a) prior to exposing them to astrocyte-soluble factors. As shown in Figure 2.4b, survival of total B cells derived from SPMS patients tended to be enhanced following exposure to non-activated ($p = 0.043$) and particularly to pre-activated ($p < 0.0001$) astrocytes. With some variability, astrocyte-derived soluble products supported survival of B cell subsets of SPMS patients. Though stimulated astrocytes did not decrease memory B cell survival (compared to the memory B cells alone), they did not appear to enhance B cell survival to the same extent as the unstimulated astrocytes did, possibly reflecting a process analogous to activation-induced cell death described in memory T cells. Soluble products of pre-activated astrocytes also induced substantial upregulation of surface expression of the T cell co-stimulatory molecule CD86 by the total B cells ($p = 0.0038$) and all B cell subsets (memory: $p = 0.0039$; transitional: $p = 0.0045$; and naive: $p = 0.0004$) (Figure 2.4c).

Discussion

Our study provides insights into cellular mechanisms by which B cells of MS patients may be fostered within an inflamed CNS environment, as well as into the potential for such B cells to contribute to the propagation of CNS-compartmentalized inflammation. The concept that B cells and plasma cells may be supported over time within the CNS of MS patients and hence might participate in ongoing disease activity has gained traction over the years (reviewed in [101]). Serial studies of cerebrospinal fluid (CSF) of patients have indicated that the same Ig oligoclonal banding (OCB) pattern of individual patients tends to persist over many years [213], suggesting

that the same antibody-producing clones are maintained for extended periods. Follow-up studies quantifying B cell somatic hypermutation confirmed the long-term persistence and expansion of the same clones within the CSF of individual patients, and the same approach applied more recently to distinct tissue compartments within the CNS identified the same B cell and plasma-cell clones populating the CSF, parenchymal lesions as well as meningeal immune-cell collections of the same patients [161, 173, 215]. This persistence of B cells within the chronically inflamed MS CNS is remarkable given the presence of several mechanisms that appear to normally limit responses to inflammation within the CNS. These include inflammation-mediated upregulation of expression of immune regulatory molecules such as HLA-G, CD200/CD200R, and SLAM5 by parenchymal CNS cells that can, in turn, downregulate responses of T cells and both resident microglia and infiltrating myeloid cells [216, 217]. The potential significance of B cell persistence in the MS CNS, including within meningeal immune-cell collections, is highlighted by the growing recognition that B cells may contribute to disease-relevant immune responses through mechanisms that extend beyond antibody production (reviewed in [102]). In this regard, abnormalities in several antibody-independent functions of B cells have recently been described in patients with MS, including the demonstration of aberrant responses of MS B cells at the innate-adaptive interface, abnormal effector-cytokine profiles, over-expression of co-stimulatory molecules, and exaggerated activation of pro-inflammatory T cells and myeloid cells [105, 110, 155]. While these abnormalities were all discovered in studies of B cells isolated from the periphery of patients, it would seem quite plausible that B cells within the CNS of these patients, which were populated from the periphery, may actively contribute to propagation of local inflammation and injury. Indeed, B cells of MS patients (unlike B cells from matched healthy controls) were recently found to mediate cytotoxicity to

rodent oligodendrocytes as well as both human and rodent neurons in vitro through induction of apoptosis. This effect was mediated by B cell soluble products independent of antibody production [209, 210]. Shedding light on processes involved in supporting B cell persistence in the inflamed MS CNS and how such B cells, in turn, may contribute to local inflammation and injury is therefore of interest since these processes may represent attractive targets for novel therapies aimed at limiting or abrogating progressive disease.

Through a series of direct co-culture experiments, transwell experiments and use of human astrocyte-conditioned medium, we demonstrated that soluble factors form human astrocytes could substantially enhance survival of both healthy-control B cells and MS-patient-derived B cell subsets. Soluble products from astrocytes that were pre-exposed to inflammatory molecules (known to be present in the inflamed MS CNS) could also enhance B cell activation and their subsequent ability to more efficiently activate T cells. The observation that these effects were mediated through astrocyte-secreted factors and do not require direct astrocyte:B cell contact is noteworthy, since the pathology that appears most relevant to progressive MS is the subpial cortical pathology, which—while notable for demyelination, neuronal loss, microglial activation, and astrogliosis—exhibits very little, if any, infiltrating immune cells. Any role of immune cells, including B cells, is more likely to be mediated by immune cells within the meninges, which means that any interactions between glial cells and these immune cells would be predicated on actions of soluble molecules.

We noted that B cells exposed to astrocytes in transwell cultures exhibited increased survival whether the astrocytes were pre-activated or not. In contrast, when using astrocyte-conditioned medium (ACM), only ACM derived from pre-activated astrocytes could enhance B cell survival. This points to a bi-directional interaction between B cells and astrocytes: while ACM from non-

activated astrocytes is not sufficient to support B cell survival, the non-activated astrocytes that were exposed to B cells in transwell produced soluble factors that were able to support B cell survival. We also noted that while B cell survival was enhanced by supernatants of both unstimulated and pre-stimulated astrocytes, only the pre-activated astrocytes could also enhance co-stimulatory molecule expression by B cells. This suggests that the astrocyte-derived soluble factors responsible for enhancing B cell survival differ from those that increase B cell activation/co-stimulatory molecule expression and APC capacity.

Prior reports demonstrating that astrocytes in the MS brain express abnormally high levels of BAFF (a known survival factor for B cells and plasma cells) and that BAFF is overexpressed in the CSF of MS patients [218] provide indirect support for the hypothesis that glial cells in an inflammatory environment may contribute to a local B cell fostering environment. In EAE, soluble TACI-Ig (atacicept) which can bind BAFF-ameliorated disease [219] while in the treatment of patients with optic neuritis and MS, atacicept appeared to exacerbate disease [98, 220]. Our series of experiments directly assessed the impact of human astrocytes on human B cells. We opted to focus on primary human astrocytes (rather than astrocytic cell lines) and since isolating primary adult human astrocytes remains a challenge we utilized human fetal-derived astrocytes. This is important since while using these cells is the closest one can model in vitro human astrocyte:immune-cell interactions, there are likely to be relevant differences between fetal and adult astrocyte responses. Indeed, we have found that fetal human astrocytes do not secrete measurable levels of BAFF (data not shown) and BAFF neutralization did not abrogate the effects of the astrocyte supernatants on B cells (Supplementary Figure 2.2e, f), indicating human fetal astrocytes are able to support B cell survival (and activation) through a BAFF-independent mechanism. The astrocyte-mediated enhancement of B cell survival and activation

was also not impacted with neutralization of IL-6 or IL-15 in the astrocyte supernatants (Supplementary Figure 2.2a-d). Future experiments will be aimed at elucidating the distinct molecular mechanisms that underlie the ability of astrocytes to support B cell survival, and the ability of activated astrocytes to enhance B cell activation and subsequent T cell activation, which may prove particularly relevant to propagating CNS-compartmentalized inflammation and progressive MS.

Given our findings that astrocytes can support survival and activation of human B cells, and given prior work indicating that B cells of MS patients exhibit abnormal immune responses as well as the capacity to injure CNS cells, it was important to demonstrate that these astrocyte-mediated effects would also apply to MS-patient-derived B cells. We could document that B cells derived from untreated patients with progressive MS also exhibited enhanced survival and activation upon exposure to astrocyte-soluble products and, furthermore, that this was true for all major B cell subsets, including patient-derived memory B cells, which comprise the great majority of B cells found within the MS CNS [173, 214]. Since there is growing recognition that progressive MS disease mechanisms are likely initiated well before patients manifest with clinical disease progression, we were curious to see how B cells of MS patients earlier in their disease course would respond and found that survival and activation of B cells derived from untreated patients with relapsing remitting MS were also enhanced following the same astrocyte exposure (Supplementary Figure 2.4).

It is now appreciated that activated astrocytes may exhibit distinct functional response profiles that may depend on how they are activated (Supplementary Figure 2.1). While our in vitro activation of astrocytes (using brief exposure to pro-inflammatory cytokines) may be more likely to induce pro-inflammatory A1 astrocytes with neurotoxic potential [221], it is of interest to

consider how alternatively activated (A2) astrocytes that can exhibit neuroprotective properties [222, 223] may differently influence CNS-compartmentalized B cells, and whether therapies aimed at modulating astrocytes from A1 to A2 responses may in turn also limit the potential contribution of B cells to pro-inflammatory cascades and CNS injury within the CNS.

Material and Methods

Participants

Healthy controls (HC) were recruited among members of the McGill University community. Well-characterized patients with confirmed MS (Supplementary Table 1) were recruited at the MS Clinic of the Montreal Neurological Institute and Hospital (MNI/H). All participants provided informed consent as part of a protocol approved by the university's internal review board (IRB). All MS patients were untreated at the time of blood draw, had not received any immune-modulating treatments within at least 6 months prior to sampling, and had not been treated with steroids within at least 30 days prior to sampling.

B cell and B cell subset isolation

B cells were isolated from fresh antecubital venous blood, as previously described [110]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from 100 to 120 ml venous blood of untreated MS patients and healthy volunteers using standard density-gradient centrifugation on Ficoll-Paque (Pharmacia Biotech). Magnetic beads (MACS, Miltenyi Biotech) were used according to the manufacturer's instructions to isolate $CD19^+$ B cells by positive selection, and their purity was confirmed by flow cytometry (routinely > 98% pure). B cells were then washed and resuspended in serum-free X-Vivo 10 medium. For experiments with B-cell subsets, total B

cells were initially sorted from PBMC by CD19+ MACS-separation and then stained for CD20 (2H7), CD27 (M-T271), IgD (BV510), CD24 (ML5) and CD38 (HIT2), all from BD Bioscience. The total B cells were subsequently sorted (using a BD LSRFortessa, BD Bioscience) into transitional ($CD20^{+}CD24^{+}CD38^{+}$), naive ($CD20^{+}CD27^{-}IgD^{+}$), or memory ($CD20^{+}CD27^{+}IgD^{-/+}$) B-cell subsets with routine purity confirmation (typically > 93 %).

Astrocyte Isolation and Culture

Human fetal astrocytes (HFA) were isolated as previously described [224]. In brief, fetal CNS tissue age 17-22 weeks of gestation, obtained from the Albert Einstein College of Medicine Human Fetal Tissue Repository (Bronx, NY), was first dissociated using trypsin-EDTA (Invitrogen Life Technologies) and DNase I (Roche, Laval, QC), followed by mechanical dissociation. The cell suspension was then washed and seeded in complete Dulbecco's modified Eagle's medium (DMEM; containing 10% fetal calf serum (FCS), penicillin, streptomycin, L-glutamine, and glucose) into poly-L-lysine coated flasks, at a concentration of 3-5 x 10^6 cells/mL. To obtain near-pure astrocyte cultures, the HFA were further enriched in DMEM by at least three passages, starting two weeks post isolation. Astrocytes used in all experiments were between passages 3 and 5 and > 90% pure, as determined by glial fibrillary acidic protein (GFAP, clone EPG 724) immunostaining as previously described [225]. Purified astrocytes were then washed twice in phosphate-buffered saline (PBS) and plated in complete DMEM at a density of 0.1 x 10^6 cells/well in 300µL (48-well plates) or at 2.4 x 10^5 cells/well in 500µL (24-well plates). Upon reaching confluency (usually following 24 hours in culture), the astrocytes were again washed in PBS, and 300µL or 500µL serum-free X-Vivo 10 medium (Lonza, Walkersville, MD) supplemented with penicillin, streptomycin and L-glutamine was added to

each well. Astrocytes were either left unstimulated (Supplementary Figure 2.1) or activated with a combination of IFNγ (10ng/mL) and IL-1β (10ng/mL) as previously reported [180]. After 24 hours of incubation, the astrocytes were again washed thoroughly in PBS to minimize possible carry-over effects of activating cytokines, and fresh medium was added. Where indicated, astrocytes were either used in co-culture experiments with B cells (described below) or maintained in culture for an additional 24 hours, with astrocyte-conditioned medium (ACM) then collected and stored at -80°C until use.

B-cell:astrocyte Co-Cultures

For direct B-cell:astrocyte co-cultures, astrocytes were isolated as described above and plated at a density of 2.25 x 10^5 cells in 300µL per well in 48-well plates in DMEM supplemented with 10% FCS until they reached 80% confluence (2-3 days). DMEM was then removed and B cells, purified from the periphery of healthy donors, were directly co-cultured at a density of 3x10^5 cells in 300µL of X-Vivo 10. Survival responses were measured after 5 days of co-culture, while activation changes were assessed after 48 h of co-culture. For B-cell:astrocyte transwell co-cultures, astrocytes were isolated and plated at a density of 2.4 x 10^5 cells/well in 500µL in 24-well plates and were either left unstimulated, or activated, as described above. BD Falcon cell-culture inserts (0.4µm pore diameter) were then placed into the wells, and freshly-isolated B cells were added to the upper compartments at a density of 2 x 10^5 B cells in 200µL serum-free X-Vivo 10 medium for the indicated duration, at which time the B cells were collected from the upper well and survival and activation were measured. For experiments assessing the effects of astrocytes on B-cell subsets, the sorted B cell subsets were exposed in transwell to astrocytes, as described above, for 40 h. For experiments utilizing ACM, B cells were plated at a density of 1.5

x 10^5 cells in 150µL serum-free X-Vivo 10 medium per well in U-bottom 96-well plates, and 50µL of ACM (or control medium, as indicated) was added to each well (representing 25% of the final volume). For functional blocking experiments, neutralizing antibodies to human IL-6 (clone 6708) human IL-17 (clone 34559) and BAFF (clone 148725), as well as appropriate isotype controls (all R&D Systems), were incubated at a final concentration of 1µg/ml with ACM for at least 20 min prior to addition to the B cell cultures. At the end of all cultures, B cell survival and expression of the T cell co-stimulatory molecule CD86 were assessed by flow cytometry, as described below.

T cell Isolation and allogeneic stimulation

To determine whether astrocyte exposure can modulate the capacity of B cells to induce T-cell responses, B cells that were pre-exposed to astrocyte soluble factors in the transwell system described above were washed and co-cultured with T cells isolated from different healthy donors. Following PBMC separation, CD4+ T cells were isolated by positive selection using standard MACS separation (Miltenyi Biotec). T-cell purity was confirmed by flow cytometry (routinely > 98%) and the T cells were then washed, resuspended in serum-free X-Vivo 15 medium, and stained with CFSE (20µL CFSE 1% per mL). Allogeneic B cells that were previously cultured for 48 h under the different conditions described above were added to the freshly-isolated CD4+ T cells in 300µL serum-free X-Vivo 15 medium in 48-well plates at a B-cell:T-cell ratio of 1:4 (0.75 x 10^5 B cells per 0.3 x 10^6 T cells). T cells were also cultured alone, as a negative control, or with 2µg/mL phytohemagglutinin (PHA, Sigma), as a positive control for T-cell proliferation. Following 6 days in culture, the T cells were harvested and analyzed by flow cytometry as described below.

Flow cytometry for T cell and B cell responses

Depending on the assay, B cell survival was assessed following 2, 3 or 5 days in culture by co-staining for CD20 (anti-CD20, 2H7, BD Bioscience), 7AAD (BD Bioscience) and Annexin V (BD Bioscience), with forward- (FSC) and side-scatter (SSC) properties also considered. Surviving B cells we considered to be Annexin V^-/$7AAD^-$ $CD20^+$. B cell activation was assessed by quantifying upregulation of surface expression of the co-stimulatory molecule CD86 (anti-CD86, FUN-1, BD Bioscience). Following 2 days in culture, B cells were collected and washed using PBS containing 5% FCS, incubated with CD20 and CD86 antibodies for 20 minutes at 4°C, washed again and stained with Annexin V and 7AAD for 10 minutes at room temperature. T cell proliferation was quantified based on CFSE dilution following 6 days of culture as described above. All FACS acquisition was done using either FACSCalibur or LSRFortessa (BD Biosciences), and data were analyzed using FlowJo flow-cytometry10 analysis software (TreeStar, OR, USA).

Statistical analyses

GraphPad Prism (versions 7 and 8) was used for all statistical analyses. Student's unpaired *t* tests were used for statistical comparisons between two groups when the assumption of normal distribution was deemed appropriate. One-way ANOVA was used to compare across groups or conditions, and two-way ANOVA was used to compare several groups across different conditions.

Conclusions:

Our findings shed some light on human glial:immune interactions that may support survival of B cells within the inflamed CNS and their potential to propagate CNS-compartmentalized disease processes in MS. Future definition of molecular mechanisms underlying these interactions could contribute to novel therapeutic strategies aimed at targeting progressive disease, a major unmet need in the field of MS.

Figure 2.1:

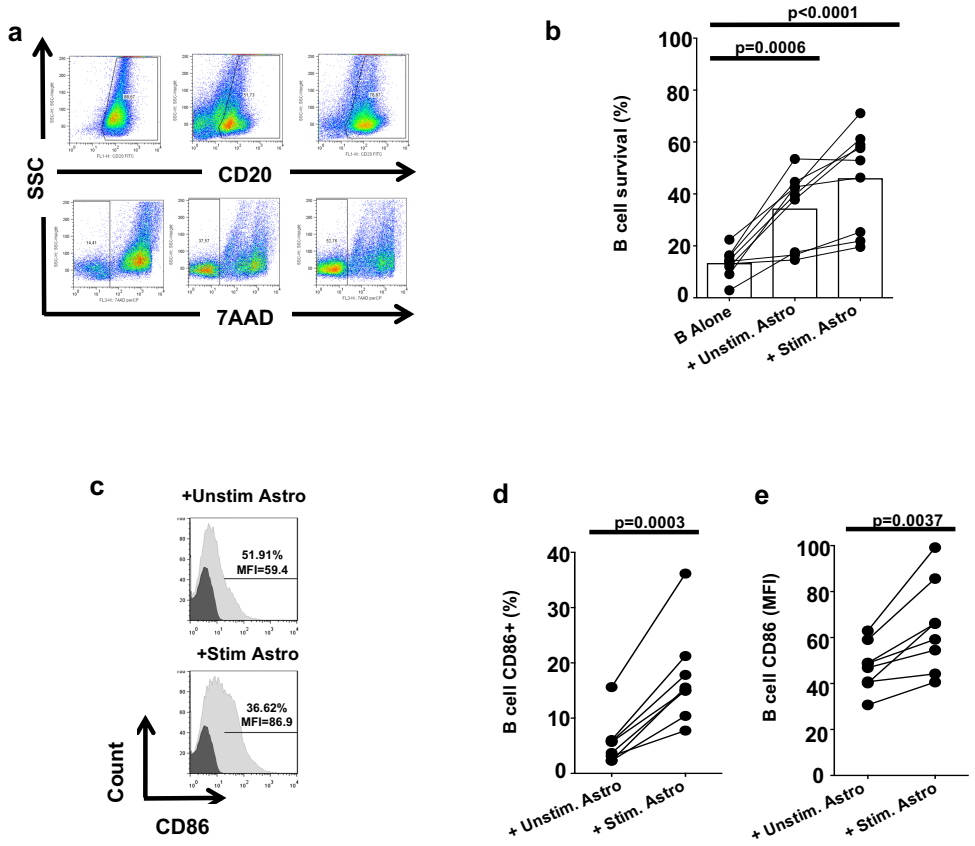


Figure 2.1: Human astrocyte co-culture increases B-cell survival and CD86 expression. Human astrocytes (Astro) were stimulated (Stim) with recombinant human IFNγ (10ng/mL) and IL-1β (10ng/mL) or received no stimulation (Unstim) for 24 hours. Astrocytes were then washed and co-cultured with human B cells in serum-free medium. B cells were also cultured without astrocytes in serum-free medium only (B alone). (a) Following 5 days of co-culture, we gated on CD20+ cells and assessed B-cell viability through 7AAD staining. (b) Percentage of viable B cells from 9 independent donors after 5 days of co-culture. (c) CD86 expression in B cells was assessed by flow cytometry after 2 days. Percentage of CD86-positive cells (d) and CD86 MFI (e) from 8 independent donors (data were analyzed with one way ANOVA (1b) and paired t-test (1c); *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 2.2:

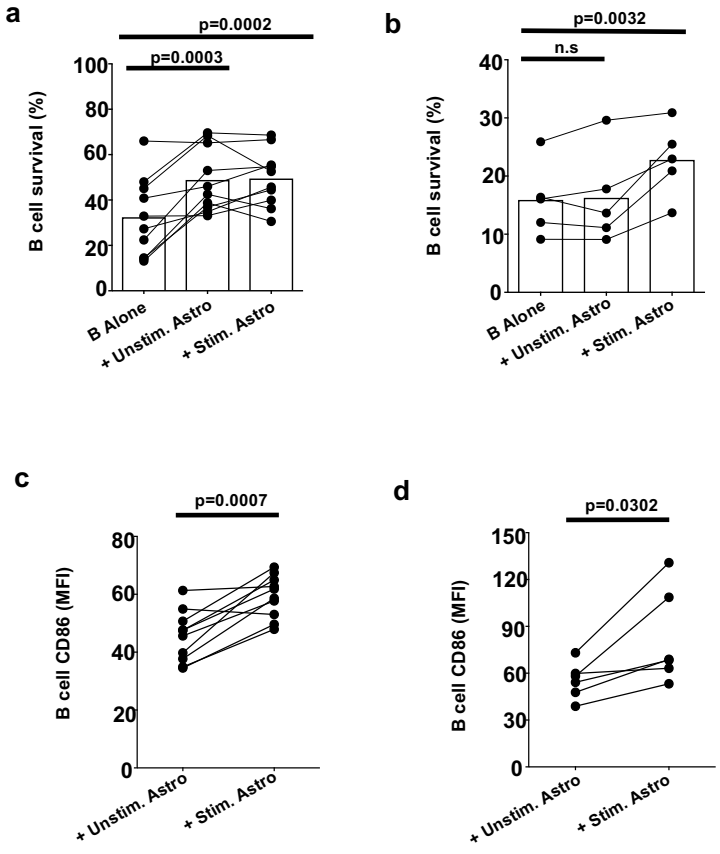


Figure 2.2: Soluble factors from human astrocytes increase B-cell survival and CD86 expression. (**a**, **c**) B cells were cultured in transwells in serum-free medium, either alone (B alone) or with either stimulated (Stim) or unstimulated (Unstim) human astrocytes (Astro). (**b**, **d**) B cells were cultured with 25% serum-free medium, which had not been conditioned by astrocytes (B alone), or with 25% astrocyte-conditioned medium (ACM) from stimulated (Stim) or unstimulated (Unstim) astrocytes. B-cell viability (**a**, **b**) was assessed after 2 and 3 days of co-culture using 7AAD and Annexin V staining. Data are shown from 9 independent experiments using different healthy-control donors. B-cell CD86 expression (**c**, **d**; MFI) was determined by flow cytometry following 2 days in culture. Data are shown for 5 independent experiments (one way ANOVA (2**a**, **b**), paired t-test (2**c**, **d**); n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 2.3:

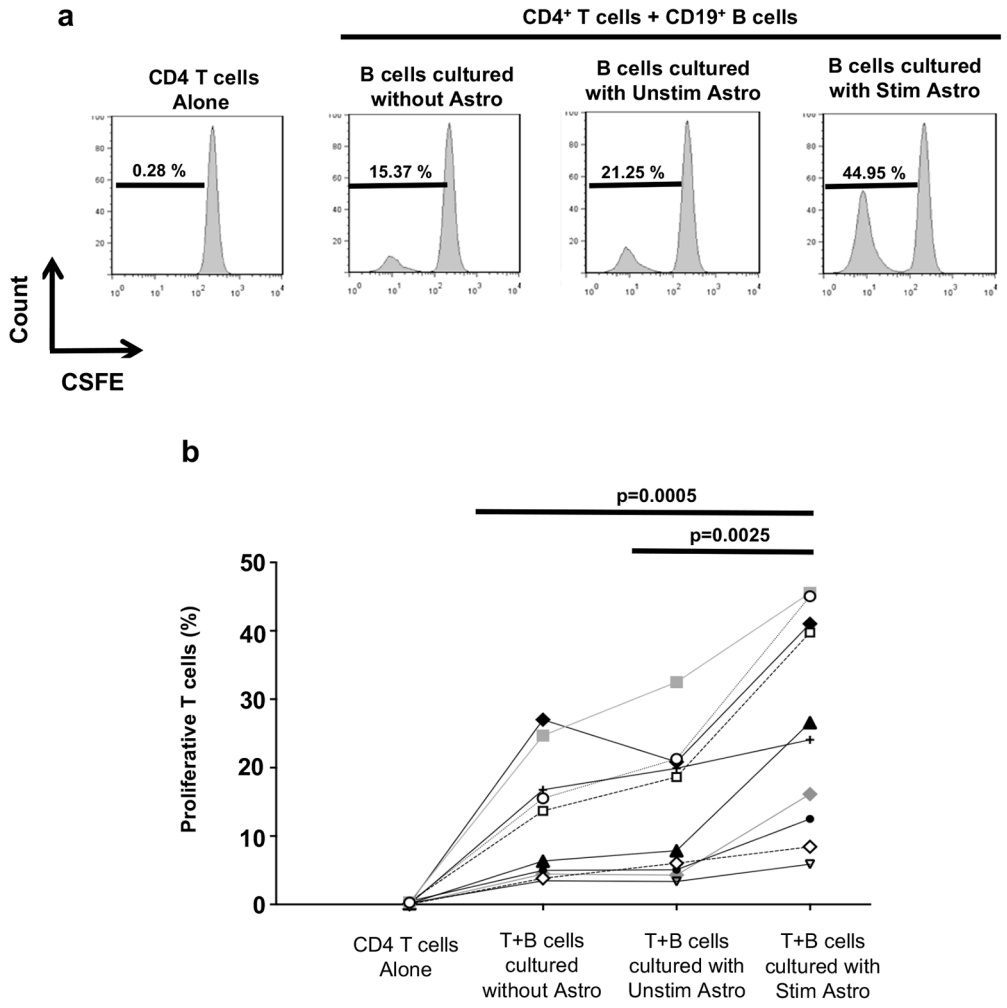


Figure 2.3: Astrocyte-exposed B cells enhance CD4+ T-cell responses.
Human B cells were cultured in transwell as described above, either alone or with stimulated or unstimulated astrocytes. Following 2 days in culture, B cells were harvested, thoroughly washed and co-cultured with human T cells from allogeneic donors for 6 days at a B-cell:T-cell ratio of 1:4. T-cell proliferation was assessed by flow cytometry using CFSE staining. (**a**) Representative data from a single donor, and (**b**) each line/symbol represents data from n=10 independent experiments using different donors. (paired t-test; **: $p < 0.01$; ***: $p < 0.001$).

Figure 2.4:

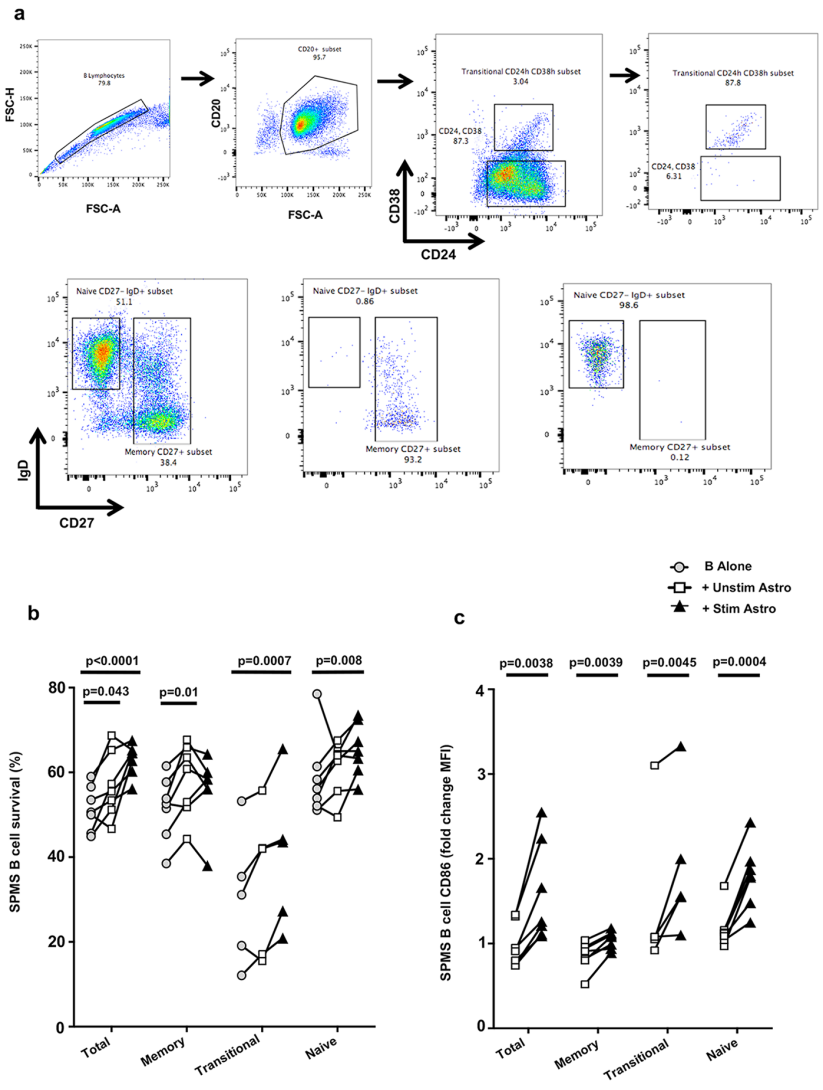
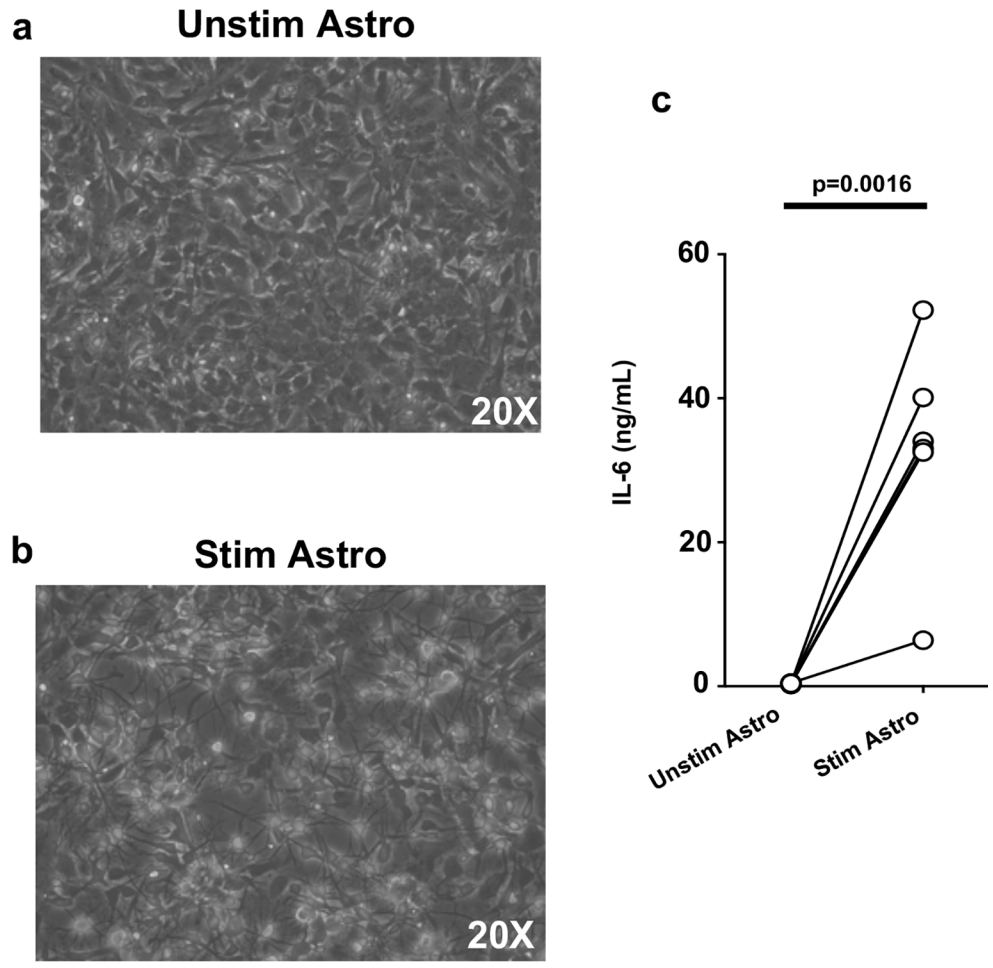


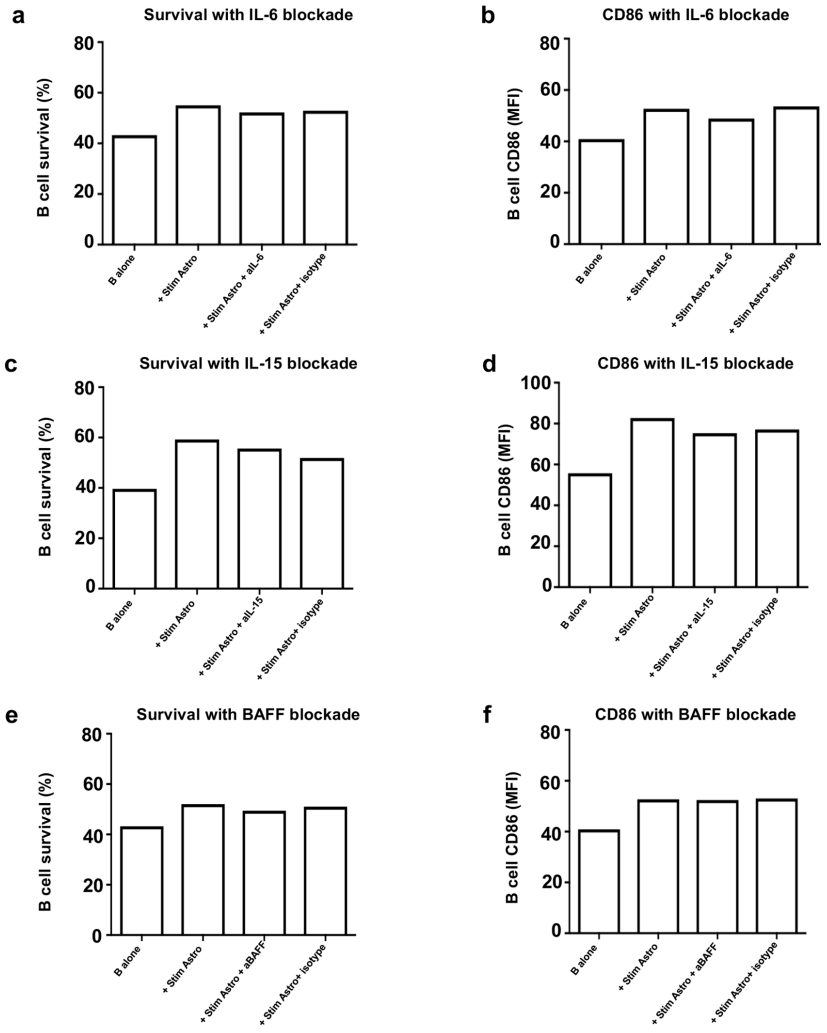
Figure 2.4: Astrocyte-secreted factors impact responses of MS-relevant B-cell subsets. (**a**) B cells derived from untreated SPMS patients were sorted into total CD20+ B cells, naive $CD20^{+}$ $CD27^{-}$ IgD^{+} B cells, transitional $CD20^{+}$ $CD24^{high}$, CD38 ^{high} and memory $CD20^{+}$ $CD27^{+}$ $IgD^{-/+}$ B cells and were then cultured either alone or with stimulated or unstimulated astrocytes in transwell, in serum-free medium X-Vivo 10 (n=7). (**b**) B-cell viability was assessed after 40 hours of transwell co-culture using 7AAD and Annexin V staining; (**c**) CD86 MFI was determined by flow cytometry following 40 hours in transwell co-culture. (two way ANOVA test and paired T test; n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Supplementary Figure 2.1



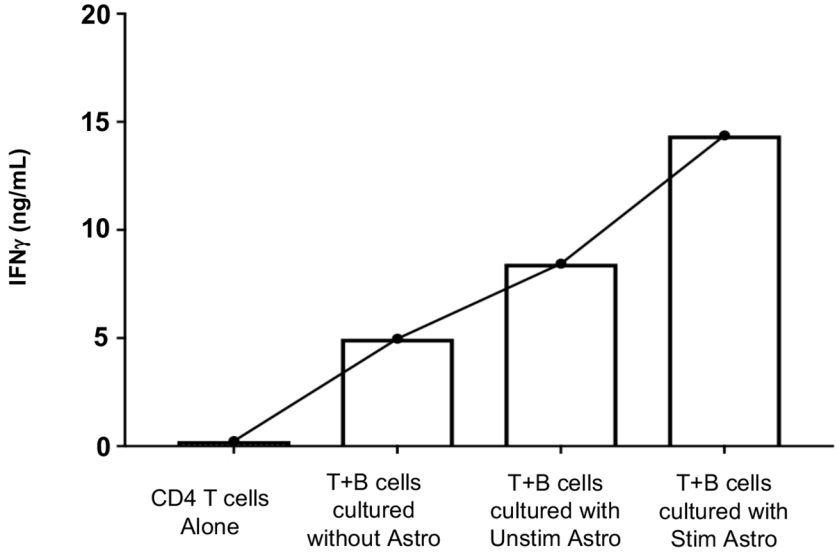
Supplementary Figure 2.1: Confirming activation of human astrocytes.
Astrocytes were cultured for 24h and were either left unstimulated or were stimulated with IFNγ (10ng/ml) and IL-1β (10ng/ml). After 24h, the astrocytes were washed thoroughly and fresh medium was added. After an additional 24h in culture, at which time cultures were imaged and supernatants were collected for subsequent measurement of astrocyte-secreted IL-6 by ELISA. Compared to unstimulated astrocytes (**a**), stimulated astrocytes exhibited activated morphology (**b**) and significantly-enhanced production of IL-6 (c; $p = 0.0016$; paired t-test).

Supplementary Figure 2.2:



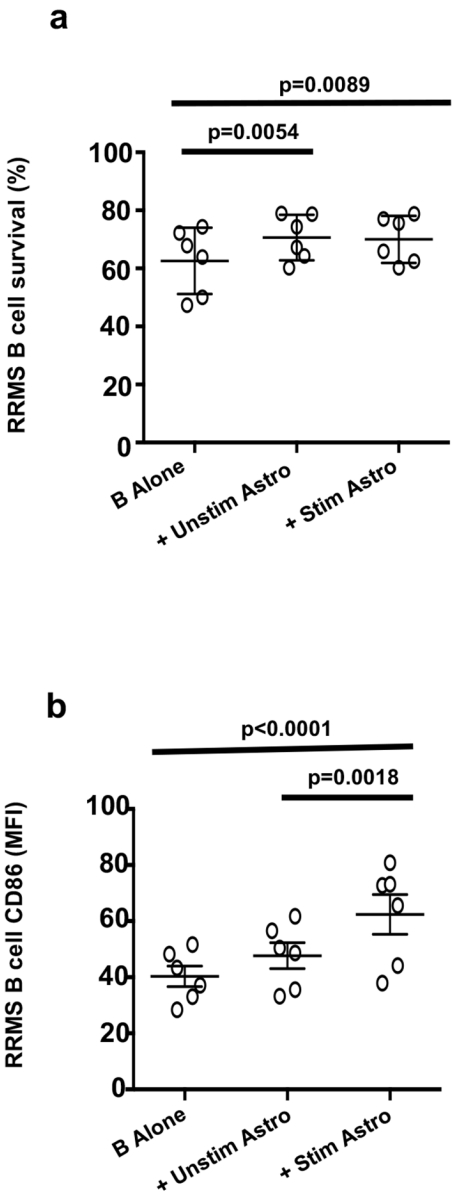
Supplementary Figure 2.2: Effects of astrocytes cytokine neutralization on B cell survival and activation. B cells from HC were either cultured alone, or with stimulated astrocyte conditioned-medium (ACM), or with ACM pre-treated with neutralizing antibodies to IL-6 (**a**, **b**; anti-IL6: aIL-6), IL-15 (**c**, **d**; anti-IL-15: aIL-15) or BAFF (**e**, **f**; anti-BAFF: aBAFF); or pre-treated with corresponding isotype control antibodies. After 2 days of culture B cell viability was assessed using ANNEXIN V and 7AAD staining, and CD86 expression was measured by flow cytometry (representative experiment).

Supplementary Figure 2.3:



Supplementary Figure 2.3: IFNγ production by proliferative T-cells. Human B cells were cultured in transwell as described previously, either alone or with stimulated or unstimulated astrocytes. Following 2 days in culture, B cells were harvested, thoroughly washed and co-cultured with human T cells from allogeneic donors at a B-cell:T-cell ratio of 1:4. Conditioned media of B-cell:T-cell co-culure was collected and IFNγ was measured using ELISA (representative experiment).

Supplementary Figure 2.4:



Supplementary Figure 2.4: Astrocyte-secreted factors also support survival and activation of relapsing remitting MS (RRMS) B cells. B cells derived from patients with RRMS were cultured in transwell either with unstimulated human astrocytes or with astrocytes that had previously been stimulated as described above. (**a**) B-cell viability was assessed after 48 hours of transwell co-culture using 7AAD and Annexin V staining; (**b**) CD86 MFI was determined by flow cytometry following 48 hours of transwell co-culture. Data were analyzed using one way ANOVA test (n = 6 independent experiments; n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Supplementary Table 2.1:

MS Patients	Age	Sex	Diagnosis	Treatment Status	Steroids within 30 days	Disease duration
RRMS 1	23	F	RRMS	Untreated	No	5
RRMS 2	24	M	RRMS	Untreated	No	1
RRMS 3	43	F	RRMS	Untreated	No	11
RRMS 4	37	M	RRMS	Untreated	No	1
RRMS 5	50	F	RRMS	Untreated	No	10
RRMS 6	43	F	RRMS	Untreated	No	1
SPMS 1	56	F	SPMS	Untreated	No	14
SPMS 2	72	F	SPMS	Untreated	No	12
SPMS 3	55	F	SPMS	Untreated	No	24
SPMS 4	72	M	SPMS	Untreated	No	5
SPMS 5	71	M	SPMS	Untreated	No	14
SPMS 6	60	F	SPMS	Untreated	No	13
SPMS 7	68	F	SPMS	Untreated	No	12

Preface, rationale, hypothesis and objectives - Chapter 3

My previous chapter focused on how human astrocytes may support the survival and activation of B cells as part of immune:glial interactions mediating CNS-compartmentalized inflammation and progressive disease. Additionally, I have been part of collaborations whereby we demonstrated that soluble factors secreted by B cells from untreated MS patients were cytotoxic to both oligodendrocytes and neurons, a toxicity not seen with soluble factors derived from healthy control B cells studies in parallel. This current chapter will concentrate on another type of immune:glial interaction and a potential feature of CNS-compartmentalized inflammation, namely on the cross-talk between MS relevant B cell subsets and microglia/macrophage, and how such interactions may contribute to subpial cortical injury and disease progression. While most studies considering the disease-relevance of the balance between pro-inflammatory B cell (Beff) and anti-inflammatory regulatory B cell (Breg) responses have considered their contributions in the periphery of patients to relapse-related biology, we believe CNS-compartmentalized pro-inflammatory B cells in MS patients may also contribute to propagation of CNS injury through aberrant activation of myeloid cells (both resident microglia and potentially infiltrating macrophage).

In the first part of this chapter, I will present my findings using the simplistic M1/M2 paradigm, to establish that differentially polarized human microglia have the capacity to influence B-cell survival and activation profiles. In the second part of this chapter, I investigate the impact of MS-relevant B cell subsets (Beff & Breg) on the phenotype, on cytokine and phagocytic responses of human microglia and monocytes-derived macrophages. My results suggest that microglial activation present as part of the subpial cortical injury and associated with

progressive MS, may be partially due to the bi-directional interactions between B cells and microglia and participating in CNS-compartmentalized inflammation.

Future investigations based on my studies will help the identification of molecular mechanisms behind such cellular interactions, and have the capacity to provide new insights on therapeutic strategies for progressive MS.

Chapter 3. Cross-talk between MS relevant B cell subsets and myeloid cells: potential contribution to the CNS-compartmentalized inflammation and disease progression in MS

Cross-talk between multiple sclerosis relevant B cell subsets and microglia/macrophage: potential contribution to the CNS-compartmentalized inflammation and disease progression in multiple sclerosis

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Abstract:

Background: Patients with multiple sclerosis (MS) have been shown to harbor abnormally increased proportions of pro-inflammatory memory effector B cells (Beff), producing high levels of IL-6, TNFα and GM-CSF compared to matching healthy controls. These abnormalities are thought to contribute to the peripheral cascades of cellular immune interactions (involving B cells, T cells and myeloid cells) that underlie disease relapses. In the central nervous system (CNS) of MS patients, memory B cells appear to be fostered within different compartments including meningeal immune cell aggregate. The latter are found adjacent to subpial cortical injury, involving demyelination, neuronal loss, and a gradient of microglia/macrophage activation which is higher at the superficial layers of the cortex. This subpial cortical injury is thought to be a pathologic substrate of (non-relapsing) progressive MS. Given our prior implication that B cell:myeloid cell interactions may contribute in the periphery to relapse biology, here we considered whether and how B cell:myeloid cell (including distinct MS-relevant B cell subsets:microglia) interactions may propagate CNS-compartmentalized inflammation and contribute to injury and disease progression

Methods: We carried out a series of *in vitro* experiments, using human-derived microglia and monocyte-derived macrophage as well as human (including MS-patient)-derived B cells to investigate bi-directional interactions between the myeloid cells and MS-relevant B cell subsets. First, we used soluble factors (supernatants) derived from polarized conditions of human microglia (M1, M2a & M2c) to assess their potential to impact B-cell survival and activation using flow cytometry. Next, we exposed microglia and macrophage to supernatants of distinct B-cell subsets including pro-inflammatory (Beff) B cells implicated in MS and regulatory (Breg) B

cells and assessed changes in the myeloid cell phenotype as well as functional responses (cytokine profiles by ELISA and phagocytic function by flow cytometry).

Results: First, we demonstrated that M1 supernatants enhanced B-cell activation while M2c supernatants were cytotoxic to MS relevant B-cell subsets. In turn, pro-inflammatory MS-implicated Beff B cells substantially increased secretion of pro-inflammatory cytokines (IL-12, IL-6, TNFα) by both microglia and macrophage, an effect that was GM-CSF dependent for macrophage. Beff supernatants also significantly down-regulated IL-10 production by the myeloid cells, while IL-10 derived from Breg induced myeloid cell IL-10 production. Finally, supernatants derived from both Beff and Breg B cells influenced microglia and macrophage phenotype (CD80 & TREM-2) and differentially impacted their capacity to phagocytose human myelin.

Conclusion: Our findings indicate a potential bi-directional interaction between disease-relevant human B cell subsets and both resident CNS microglia and infiltrating myeloid cells, which may influence the propagation of MS-CNS compartmentalized inflammation associated with disease progression.

Key words: Multiple sclerosis, CNS-compartmentalized inflammation, Human microglia, Human macrophage, Human B cells.

Introduction

Multiple Sclerosis (MS) is debilitating autoimmune disease involving injury in the central nervous system (CNS). Eventually, most patients experience disease progression which may include both physical and cognitive impairments, yet the pathological mechanisms underlying such progression remain unknown. Aberrant peripheral immune activation and subsequent trafficking to the CNS was traditionally thought to mainly involve abnormal T cell responses in both the periphery and the CNS. However, clinical trials based on the selective depletion of CD20 expressing cells, indicate an important contribution of B cells to the development of new MS relapses [91, 94, 95, 104, 106, 226]. The ability of B cells to contribute to peripheral cellular immune cascades involved in relapses appears to be independent of their capacity to produce antibodies, as evidenced by the lack of change in the abnormal cerebrospinal fluid profile of antibodies in MS patients following the highly effective aCD20 treatment [226, 227]. The ability of B-cell targeting therapies to impact relapses in MS is now thought to reflect an abnormal pro-inflammatory propensity of B cells in MS patients. Indeed, our group and others have previously demonstrated that B cells derived from MS patients harbor abnormally higher proportions of pro-inflammatory effector B cells (Beff), producing elevated levels of IL-6, TNFα and GM-CSF and that these B cells are able to perpetuate pro-inflammatory myeloid and T cell responses, while IL-10 expressing (and putatively regulatory) B cells (Breg)) that can down-regulate myeloid cell responses appeared diminished in patients [105, 129, 155, 227].

Unlike relapsing remitting MS which is thought to involve the above peripheral immune cell interactions and waves of immune cells trafficking into the CNS, the biology underlying (non-relapsing) disease progression is thought to involve CNS-compartmentalized inflammation and degeneration [207]. Compartmentalized inflammation in MS is in part reflected by the

appearance of activated microglia within the CNS tissue as well as the presence of immune cell collections in the meninges of patients, often described as B-cell rich [36, 60, 61, 161]. Pathological studies highlight the potential association between meningeal inflammation and abnormal subpial cortical pathology, which is now thought to be an important contributor to progressive disease [54, 64, 211]. Adjacent to the B cell-enriched meningeal immune cell collections, subpial cortical injury includes a gradient of neuronal and oligodendrocyte loss, astrogliosis as well as what appears to be a 'surface-in' gradient of microglial activation [61, 64]. The latter is reflected by presence of the greatest extent of microglial activation in layer I of the cortex immediately underlying the pia, and a lesser degree of pathology in deeper cortical layers [65].

As part of the MS inflammatory CNS microenvironment, both resident microglia and infiltrating macrophage have been found to acquire distinct activation phenotypes and functional properties manifesting with pro-inflammatory cytokine production, and changes in myelin debris phagocytosis [192, 203, 205, 228]. Nonetheless, the mechanisms contributing to the chronic myeloid cell activation in MS (both the graded subpial activation of microglia, as well as the peri-vascular changes in both resident microglia and infiltrating macrophage) have not been fully elucidated. Of considerable interest is the possibility that meningeal immune cells (and potentially B cells that commonly constitute such structures) contribute to the sub-pial cortical changes through release of soluble factors. In this context, our *in vitro* studies have previously shown that soluble factors of MS-derived B cells can be cytotoxic to both oligodendrocytes and neurons [209, 210]. We have also shown that activated human astrocytes can promote a B cell fostering environment through soluble factors [177] emphasizing the potential role for glial

cell:B cell interactions in promoting CNS-compartmentalized inflammation, and potentially progressive CNS injury, in MS. Here, we consider whether activated CNS-myeloid cells can promote B cell activation and whether, in turn, MS-implicated B cells have the potential to induce changes to the MS CNS myeloid cells, including both infiltrating monocyte-derived macrophages and resident microglia. Our overarching hypothesis is that bidirectional cross-talk between disease implicated B cells and microglia/macrophage mediated by release of soluble factors, contributes to the propagation of CNS compartmentalized inflammation and injury in MS.

Material and Methods

Participants

Healthy control donors were recruited at the Montréal Neurological Institute, or at the University of Pennsylvania (Supplementary table 3.1). Well characterized MS patients (with confirmed relapsing remitting, secondary progressive or primary progressive MS [9] were recruited at the MS clinic of the Montreal Neurological institute (MNI), and the MS division, Department of Neurology, at the University of Pennsylvania; all patients had no prior history of any immune-suppressive drugs, had no exposure to immune-modulating treatments for at least 6 month, and no steroid exposure for at least 30 days prior the blood draws. All subjects provided informed consent using protocols approved by the Montréal Neurological institute, McGill University and the University of Pennsylvania institutional ethics review boards.

Human B cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll (GE Healthcare) using our well-established standardized protocols [155], and B cells were purified from PBMC by positive selection using CD19 beads (Miltenyi Biotec) according to the manufacturer's protocol. B cell purity was regularly verified using flow cytometry, with typical purities of > 98 %. B cells were freshly plated in U-bottom 96 well-plates at a density of $2x10^5$ cells/well, in a total volume of 200 ul of serum free x-vivo culture media (Gibeco, Life Technologies). B cell survival and activation profile was assessed following 2 days in culture, after exposure to conditioned media derived from microglia by flow cytometry. We considered live B cells to be Annexin $V^-/7AAD^-$ $CD20^+$. To generate effector B cell supernatants (Beff sups), an activation cocktail of CD40L (1 mg/ml, Enzo Life Sciences), IgM BCR cross-linking antibody (10 mg/ml, Jackson ImmunoResearch) and IL-4 (20 ng/ml, R&D System) was used. To generate regulatory B cell supernatants (Breg sups), B cells were activated with CpG DNA (1 mM; ODN2006, InvivoGen). All B cells were washed after 12 hours of culture and serum free media was replaced. Supernatants of the non-activated (Nil B sups), effector B cell sups (Beff sups) and regulatory B cell sups (Breg sups) were collected after a total of 3 days of culture. Subsequently, B cell supernatants were added at a 1:1 ratio to non-polarized microglia and macrophage cultures.

Monocyte-derived macrophage isolation and culture

Monocytes were purified from PBMC using positive selection with CD14 beads (Miltenyi Biotec) according to the manufacturer's protocol, and their purity was routinely confirmed by

flow cytometry (consistently > 98 %). Freshly purified monocytes were then suspended at a density of $5x10^5$ cells/mL and plated in either flat bottom 12-well plates (1 mL/well) or 96 well-plates (200 uL/well). Cells were differentiated into macrophage for a period of 5 days in culture, using Roswell Park Memorial Institute (RPMI) media containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and glutamine. Where noted, the monocytes were polarized into either pro-inflammatory M1 macrophage using Granulocytes macrophage colony-stimulating factor (GM-CSF) (5 ng/mL, Peprotech), IFNγ (20 ng/mL, Gibeco Lifetechnologies) and lipopolysaccharide LPS (100 ng/ml, sigma), M2a using macrophage colony-stimulating factor M-CSF (25 ng/Ml Peprotech), IL-4 (20 ng/mL, Gibeco Lifetechnologies), IL-13 (20 ng/mL, Gibeco Lifetrechnologies), or M2c macrophages using the same polarization cocktail used to induce M2a in addition to TGFβ (20 ng/mL, Miltenyi) and IL-10 (10 ng/mL, Peprotech), or left unpolarized treated with M-CSF (25 ng/mL, Peprotech), as described by [205]. Unpolarized, M1, M2a and M2c macrophages were carefully washed three times with warm PBS and fresh x-vivo media was added for an additional 24 hours - 30 hours to collect conditioned media that was subsequently added to B cell cultures. For phenotype characterization, macrophage were either polarized *in vitro* (as described above) and their phenotype was assessed after 5 days of culture, or kept at basal condition (without polarization) for 3 days, and exposed to B cell supernatants for 2 days and phenotype was measured.

Human brain-derived microglia isolation and culture

Human adult microglia were isolated from temporal lobe brain tissue obtained from patients undergoing brain surgery for intractable epilepsy, following the Canadian institutes of Health

Research (CIHR) guidelines, and using previously described protocol [229]. All tissue used was outside of the suspected focal epileptiform site. Human adult Microglia were cultured in Minimum Essential Media (MEM) supplemented with 5% FBS, and 1% penicillin/streptomycin and glutamine and cultured in either 12-well plates (1 mL/well) or 96 well-plates (200 uL/well). Where noted, the microglia were polarized into M1and M2a and M2c phenotype for 5 days, as previously published [205]. Briefly, microglia were polarized into either pro-inflammatory M1 microglia using GM-CSF (5 ng/mL, Peprotech), IFNg (20 ng/mL, Gibeco Lifetechnologies) and lipopolysaccharide LPS (100 ng/ml, sigma); M2a microglia using macrophage colony-stimulating factor M-CSF (25 ng/Ml Peprotech), IL-4 (20 ng/mL, Gibeco Lifetechnologies), IL-13 (20 ng/mL, Gibeco Lifetrechnologies), or M2c microglia using the same polarization cocktail used to induce M2a in addition to TGFβ (20 ng/mL, Miltenyi) and IL-10 (10 ng/mL, Peprotech), or left unpolarized without any treatment. The microglia were then washed three times with PBS, and replenished with fresh x-vivo serum-free media for another 24 hours to 30 hours before collecting the microglia conditioned media, subsequently added to B cell cultures. Phenotype characterization of microglia was assessed similarly to macrophage, as described above.

Flow cytometry

Following 2 days in culture, B cells exposed to microglia conditioned media were collected and washed with PBS containing 5% FCS, and incubated with surface antibody staining: CD20 (anti-CD20, 2H7, BD Bioscience), CD69 (anti-CD69, L78 BD Bioscience), CD86 (anti-CD86, FUN-1, BD Bioscience) and CD95 (anti-CD95, DX2, BD Bioscience) for 20 min at 4°C. B cells were then washed twice and stained with Annexin V (BD Bioscience) and 7AAD (BD Bioscience) for 10 min at room temperature using Annexin V buffer (BD Bioscience). To phenotype human

microglia and macrophage under different differentiation conditions or in response to supernatants derived from functionally distinct B cell subtypes, we used Annexin V (BD Bioscience), 7AAD (BD Bioscience), CD11 (3.9, BD Bioscience), CD80 (2D10, Biolegend), CD115 (9-4D2-1E4), CD172 (15-1729-42), CD200R (OX-108, Biolegend), HLA-DR (G46-6, BD Bioscience), CD274 (MIH1, BD Bioscience), MerTK (125518; R&D System), TREM-2 (Kindly gifted by Dr. Laura Piccio), SIRP1α (SE5A5, Biolegend). Briefly microglia were detached using dissociation buffer (Thermofisher), washed with PBS containing 5% FCS, and incubated with cell surface staining cocktail antibody, including CD11, CD80, CD115, CD200R, HLA-DR, CD274, CCR7, MerTK, TREM-2 and SIRP1α for 20 min at 4°C. Microglia were washed and stained with Annexin V and 7AAD for 10 min at room temperature using Annexin V buffer (BD Bioscience).

Enzyme-linked immunosorbent assay

After exposure of myeloid cells to different B cell supernatants (non-activated 'B sup', effector B cells 'Beff' and regulatory B cells 'Breg'), microglia and macrophage supernatants were then collected and used to quantify the cytokines (IL-12, TNFα, IL-6 and IL-10) with OptEIA ELISA Kit (BD Bioscience) according to the manufacturer's protocol. Briefly, ELISA plates were coated with capture antibody for at least 12 hours ahead. Non-specific binding sites were blocked for 1 hour with blocking buffer (10% fetal calf serum and phosphate saline buffer (PBS)), and samples were added to the plate and incubated for 2 hours at room temperatures. Detection antibody was added for 1 hour at room temperature. The plates were developed by trimethylborn (BD Biosciences), and the reaction was stopped by 0.005 M H_2SO_4 and read by a Bio-Rad

microplate reader (Model 5550, Bio-Rad). Plates were washed with ELISA washing buffer (0.05 % Tween 20 and PBS) between each step above.

Cytokine blocking assays

For functional blocking experiments, we added neutralizing antibodies to GM-CSF (anti-GM-CSF, 3209, R&D System) and matching isotype controls (monoclonal mouse IgG_1, R&D System) at a concentration of 1µg/mL, for 30 minutes at room temperature. Anti- GM-CSF was used to neutralize GM-CSF within pro-inflammatory (Beff) supernatants, Macrophages were exposed to B sup, Beff or Breg supernatants, with the neutralizing antibodies or matching controls were added for 2 days, and macrophages were then activated using LPS (100 ng/ml, sigma) for the last day of culture, prior to measurement of myeloid cytokine production (IL-6, TNFα and IL-10) using ELISA as described above.

Myelin purification, labeling and phagocytosis assay

Human myelin was isolated from white matter obtained from post-mortem brain tissue, using percoll gradient separation as previously described [230]. Briefly, white matter tissue was homogenized in 0.32 M sucrose, 1mM MgCl, 10 mM Tris-HCL (pH=7.5) and 0.1 mM PMSF. The homogenate was then layered with 1 M and 0.32 M sucrose containing 20 mM Tris- HCL (pH=7.5) and 0,1 mM PMSF and centrifuged for 100 000g over night at 4 degrees. Crude myelin membrane and astrogliosome layers were carefully collected and diluted 1:1 in ice cold ultrapure H2O (containing 20 mM Tris pH 7.5 + 0.1 mM PMSF). To concentrate myelin membranes,

crude myelin membranes were pelleted at 100,000g for 1 hour and Hyposomatically shocked by homogenization in 20 vol. of ice cold ultrapure H2O (containing 20 mM Tris pH 7.5 + 0.1 mM PMSF). Shocked membranes are subjected to serial centrifugations (40,000g for 20 minutes, 100,000g for 3h) and resuspended in 0.32M sucrose at a final concentration of 1ug/mL for subsequent use in phagocytosis experiments. Myelin was confirmed to be endotoxin-free using the *Limulus ambebocyte* lysate test (Sigma Aldrich) and western-blot experiments confirmed presence of myelin proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) within the isolated myelin preparation.

Phagocytosis assay

To assess the capacity of myeloid cells to ingest myelin, we first incubated myelin with a pH-sensitive dye (pH-Rodamine; Invitrogen) for 1 hour in PBS (pH=8) and incubated microglia and macrophage pre-exposed to B cell supernatants or differentially polarized to dyed myelin for 1 hour at 37°C at a final concentration of 20 μg/mL. Following 1 hour incubation of myeloid cells to dyed myelin we used epifluorescence microscopy (Leica Microsystems, Wetzlar, Germany) to visualize myelin ingestion. To quantify phagocytosis by microglia and macrophage, cells were washed and detached after 1 hour exposure to dyed myelin, and internalized myelin was quantified using flow cytometry (FACS Fortessa, BD Bioscience).

Results:

Soluble factors derived from human M2c polarized microglia appear to be cytotoxic to B cells

To evaluate whether microglia across a range of polarization conditions may modulate B cell responses through soluble products, we generated conditioned media from microglia that were either cultured under basal conditions or polarized under traditional M1, M2a or M2c conditions, then exposed B cells *in vitro* to the different microglia conditioned media. Using flow cytometry, and Annexin V/7AAD staining, we subsequently quantified B cell survival for up to 4 days. Conditioned media of microglia cultured under basal conditions or induced into pro-inflammatory M1-like microglia or M2a-like microglia, did not impact the survival of total ($CD20^+$), memory ($CD27^+$) or naïve ($CD27^-$) human B cells, compared to the survival of B cells cultured with no exposure (Figure 3.1 b, c, d, *n=7*). However, conditioned media derived from M2c-like microglia induced significant B cell death which was mediated via both apoptosis and necrosis to total $CD20^+$ B cell subsets (Figure 3.1 b; $p<0.0001$, $n=7$), which involved both memory $CD27^+$ B cells (Figure 3.1 c; $p=0.0006$; $n=7$) and naïve $CD27^-$ B cells (Figure 3.1 d; $p<0.0001$; $n=7$), with perhaps a preferential cell death directed towards naïve B cells. Supplementary Figure 3.1 provides an example of a kinetic experiment, further supporting the potential toxic effect that M2c-differentiated human microglia may exert on human B cells.

Soluble factors derived from human pro-inflammatory M1 polarized microglia induce B cell activation

To investigate whether a pre-existing pro-inflammatory CNS milieu influenced by microglia might impact the activation profile of B cells, we pre-exposed human B cells to conditioned media derived from differentially polarized (M1, M2a & M2c) microglia and using flow cytometry subsequently assessed the B cell phenotypic activation profile. We observed that B cells exposed to M1 pre-activated microglia soluble products, but not M2a or M2c soluble products, exhibited a substantial increase of the surface co-stimulatory molecule CD86 (Figure 3.2 a; $p<0.0001, n=9$) and the activation markers CD95 (Figure 3.2 b; $p<0.0001, n=8$) and CD69 (data not shown), while not impacting B cell expression of HLA-DR (Figure 3.2 c; $p>0.99, n=7$).

Soluble factors derived from Beff defines the pro-inflammatory profile of microglia and macrophage, in a GM-CSF dependent manner for macrophage

To assess the possible functional consequences of exposing myeloid cells to supernatants derived from functionally distinct B cells, we transiently exposed the myeloid cells to conditioned media from the Beff or Breg, or from non-activated B cells for 2 days, then activated the myeloid cells with LPS for the last day in culture, to subsequently assess their cytokine expression profile. Pre-exposure of microglia to Beff supernatants significantly increased the capacity of microglia to secrete the pro-inflammatory cytokines IL-12 (Figure 3.3 a; $p=0.03, n=5$), IL-6 (Figure 3.3 b; $p=0.01, n=5$), and TNFα (Figure 3.3 c; $p=0.001, n=5$), while Beff supernatants significantly

down-regulated their IL-10 production (Figure 3.3 d; p=0.002, n=6). Similarly, Beff supernatants significantly enhanced the capacity of monocyte-derived macrophages to secrete IL-12 (Figure 3.3 e; *p=0.0004, n=6*), IL-6 (Figure 3.3 f; *p=0.01, n=4*) and TNFα (Figure 3.3 g; *p=0.002, n=3*). While Beff significantly down-regulating IL-10 production (Figure 3.3 h; *p=0.002, n=5*).

To address the molecular mechanisms underlying these effects of B cell supernatants on myeloid cell responses, and given the limiting number of available human-derived microglia, we carried out a series of experiments focusing on monocyte-derived macrophage, using neutralizing antibodies to selected B cell cytokines. We found that the observed pro-inflammatory effect of Beff B cells on macrophage cytokine responses was GM-CSF-dependent, as the selective blocking of GM-CSF within Beff supernatants (compared to control antibody) abrogated the subsequent myeloid cell IL-6 production (Figure 3.3 f; *p=0.01, n=3*), TNFα production (Figure 3.3 g; *p=0.005, n=3*) as well as IL-12 production (data not shown).

Soluble products of Beff and Breg B cell subsets differentially modulate the balance between quiescence and activation markers of human microglia and macrophage

The contribution of myeloid cells (both CNS-resident microglia and infiltrating monocyte-derived macrophage) to CNS compartmentalized inflammation in MS is thought in part to be influenced by the balance between their surface expression of activation-associated molecules (CD80; HLA-DR) and quiescence-associated molecules (TREM-2, MerTK, M-CSFR, SIRP1a, CD200R). We wished to evaluate the potential for functionally distinct (Beff & Breg) B cell subsets to impact the balance between myeloid cell activation-associated and quiescence-associated molecules. To establish the read-out, we first assessed how differential in vitro (M1 or

M2a) polarization of myeloid cells would impact the expression of these molecules. We could readily show that, as expected, M1-polarization induced myeloid surface expression of CD80 in both Microglia (Figure 3.4 a, b; *p=0.2, n= 6*), and monocyte derived-macrophages (Figure 3.4 h, I; *p<0.0001, n=12*). M1-polarization also induced HLA-DR expression by macrophage (Figure 3.4 j; *p<0.001, n=8*) but not microglia. The same M1-polarization resulted in decreased surface expression of the quiescence molecule M-CSFR by both microglia (Figure 3.4 g; *p=0.003, n= 3*) and macrophage (Figur 3.4 o; p*<0.001, n=6*), as well as decreased surface expression of the quiescence molecule MerTK by both microglia (Figure 3.4 d, e; *p=0.008, n=3*) and macrophage (Figure 3.4 m; p=0.02, n=6). In contrast, in vitro M2a polarization of the same myeloid cells induced substantial increases in the expression of TREM-2 on both microglia (Figure 3.4 d, e; *p=0.01, n=5*) and monocyte derived-macrophages (Figure 3.4 k, I; *p=0.02, n=8*), with the M2a-polarized macrophage also exhibiting induced levels of expression of the quiescence molecules SIRP1α (Figure 3.4 p; *p=0.03, n=4*) and CD200R (Figure 3.4 q; *p=0.02, n=4*).

Using this readout system we could now assess how conditioned media derived from Beff and Breg B cell subsets may impact the balance between activation and quiescence molecule expression by human microglia and macrophages. While distinct B cell supernatants did not substantially affect microglia survival (Supplementary Figure 3.2, a, *n=4*), or macrophage survival (Supplementary Figure 3.2, b, *n=4*), as shown in Figure 3.5, Beff derived conditioned media increased CD80 expression on both microglia (Figure 3.5 a, b; *p=0.007, n=4*) and monocyte-derived macrophages (Figure 3.5 g, h; *p=0.02, n=6*) without significantly changing their expression of HLA-DR (Figure 3.5 c, i; *n=4-9*). The Beff soluble products tended to also decrease TREM-2 expression by microglia, although no significant difference. In contrast, Breg derived conditioned media substantially up-regulated TREM-2 surface expression by both the

microglia (Figure 3.5 d, e; *p=0.03, n*=5) and monocyte-derived macrophages (Figure 3.5 j, k; *p=0.03, n=5*). Overall, these findings indicate that secreted products of functionally distinct B cell subsets can differentially modulate the balance between quiescence and activation markers of microglia and macrophage. Specifically, they suggest that pro-inflammatory B cells (as implicated in MS) may induce, through their secreted products, an activated, pro-inflammatory state of both CNS-resident and potentially infiltrating myeloid cells. Therapies able to shift CNS-fostered B cells towards a more regulatory (Breg) profile, may be expected to limit activation and possibly enhance quiescence profiles of the myeloid cells. We next wished to assess whether and how the Beff and Breg may modulate functional response profiles of CNS microglia and macrophage.

Soluble factors derived from Beff and Breg differentially modulate myelin phagocytosis function of microglia and macrophage

To assess the potential impact of B cell soluble product exposure on the capacity of CNS resident microglia and infiltrating macrophage to phagocytose human myelin, we pre-exposed purified microglia and macrophage to conditioned media derived from Beff and Breg, and used flow cytometry of fluorescently-tagged myelin to quantify subsequent myelin ingestion by the myeloid cells.. Fluorescent microscopy indicated that microglia exposed to Beff or Breg supernatants appeared to differentially impact phagocytosis of human-derived myelin, compared to the basal phagocytosis level. Moreover, quantification of phagocytosis using flow cytometry indicated that M1-polarized microglia demonstrated limited phagocytic capacities compared to microglia cultured under basal conditions, while supernatants derived from Breg substantially

increased phagocytic functions of microglia (Figure 3.6 c, d; p=0.01, n=4) and macrophage (Figure 3.6 e; p=0.03 , n=6). Beff supernatants might have slightly decreased myelin phagocytosis compared to basal phagocytosis level, we did not detect any statistical significance for either microglia (Figure 3.6 b, d; ns, n=4) or macrophage (Figure 3.6 e; ns, n=6). Similar observations were generated using Beff and Breg supernatants purified from the periphery of MS patient (Supplementary Figure 3.3).

Discussion

While traditionally MS was viewed as a T cell mediated disease, the established efficacy of anti-CD20 B cell depleting therapy [91, 93, 94], emphasizes an important contribution of peripheral B cell responses to the aberrant cascades of immune cell activation involved in relapse biology. In this regard, MS patients are found to harbor increased frequencies and abnormal responses of pro-inflammatory memory effector (Beff) [105, 110, 122, 126, 129, 155, 231]. Of interest is whether memory B cells of MS patients, known to be chronically fostered in the inflamed MS CNS including within meningeal immune-cell collections [36, 122, 161]; reviewed in [101,103], may also propagate the CNS-compartmentalized inflammation and injury, and particularly the subpial cortical injury subjacent to the meninges that is now thought to importantly contribute to progressive MS pathology. This subpial cortical injury is notable for demyelination, neuronal loss and microglial activation, yet a paucity of immune cells, such that any meaningful interaction between meningeal immune cells and CNS cells would need to be mediated by secreted products. We have previously reported that secreted factors derived from B cells of untreated MS patients (independently of B-cell derived antibodies) can induced cell

death of both oligodendrocytes and neurons via apoptosis [209, 210] and that activated human astrocytes can support both the survival and activation state of B cells [177].

Our current study examines how soluble products of functionally distinct B cells (including MS-implicated Beff B cells) may interact with both resident CNS microglia, as well as infiltrating monocyte-derived macrophage. Our findings point to potentially disease-relevant bi-directional cross-talk between functionally distinct B cells and CNS myeloid cells (both resident CNS microglia, and infiltrating macrophage), wherein differentially activated myeloid cells may influence survival and immune responses of the B cells while, in turn, functionally distinct B cells can differentially impact the state of activation and disease-relevant functional responses of both human CNS resident microglia and macrophage. Elucidating such interactions may point to novel therapeutic targets aimed at interrupting immune:glial interactions that propagate injury, and/or promoting interactions that may generate anti-inflammatory/acquiescing responses. For example, we observed that soluble products of M1-polarized 'pro-inflammatory' microglia were able to induce activation of B cells while soluble factors derived from anti-inflammatory M2c microglia induced B cell death. The MS field is in active pursuit of therapies that access the CNS to modulate myeloid cells away from pro-inflammatory responses, and our result suggest that such modulation may also serve to counter the B cell fostering environment with the potential to limit cascades of B cell:glial interaction that unchecked would continue to propagate CNS-compartmentalized inflammation.

We further report that soluble products released by MS-implicated Beff enhance pro-inflammatory responses of both microglia and macrophage, including the induction of myeloid cell expression of the T-cell costimulatory molecule CD80 and secretion of the pro-inflammatory cytokines IL-6, IL-12 and TNFα; all of which have been proposed as disease severity

biomarkers, as they were reportedly increased in CSF and within meningeal tissue of patients presenting with more aggressive forms of progressive MS [232]. While Beff derived conditioned media suppressed myeloid cell secretion of IL-10. This pro-inflammatory modulation of the myeloid cells is partly driven by Beff B-cell secreted GM-CSF. Since IL-12 and IL-6 are known to respectively contribute to Th1 and Th17 T cell differentiation as reviewed by [233], providing a potential mechanism by which B cells fostered in the inflamed CNS could indirectly contribute to pro-inflammatory T cell responses (possibly within the meningeal immune cell collections) as part of ongoing propagation of CNS-compartmentalized inflammation. This would add to prior observations that soluble products of activated human astrocytes can support both survival and activation of B cells which, in turn, promoted more efficient T cell activation [177].

In contrast, we found that soluble products of Breg B cells can drive both CNS microglia and macrophage to increase their expression of TREM-2, a molecule implicated in myeloid cell quiescence rather than pro-inflammatory activation. TREM-2 has been reported to be highly expressed by foamy myeloid cells in actively demyelinating MS lesions, while levels of its soluble form (sTREM-2) were increased in CSF of MS patients [234]. Moreover, natalizumab treatment decreased levels of sTREM-2 in CSF of patients to levels comparable to healthy controls [235], which suggests that future targeting therapies might also have the capacity to lower CNS inflammation in MS through TREM-2 modulation. The Breg soluble products also substantially increased myelin phagocytosis of both microglia and macrophage (thought to be important for myelin debris take-up), while this myeloid cell myelin phagocytosis tended to decrease following exposure to Beff soluble products. These results are in keeping with prior reports that M2-polarization of myeloid cells increases their phagocytosis [203], while M1-

polarization of the same myeloid cells decreases their phagocytosis, as also observed in the present study.

Conclusion

Our findings that human microglia can influence the activation profile of B cells and that, in turn, distinctly activated B cells can differently influence the state of underlying microglia and macrophage, provide novel insights into bi-directional immune cell:glial interactions that may contribute to propagating CNS-compartmentalized inflammation and injury underlying disease progression. Our study is limited to *in vitro* experiments, hence complementary pathological studies that would demonstrate the presence of pro-inflammatory GM-CSF^{+}IL-6^{+}TNFα^{+} B cells in association with meningeal inflammation would re-inforce our findings. An important next step would be to define whether B cell:glial interactions can take place *in vivo*. A general limitation in our field represent the lack of a good animal model for progressive MS. Ultimately, how important such cascades of immune:glial interactions may be to progressive disease mechanisms will likely require successful biological-proof-of principle in patients.

Figure 3.1:

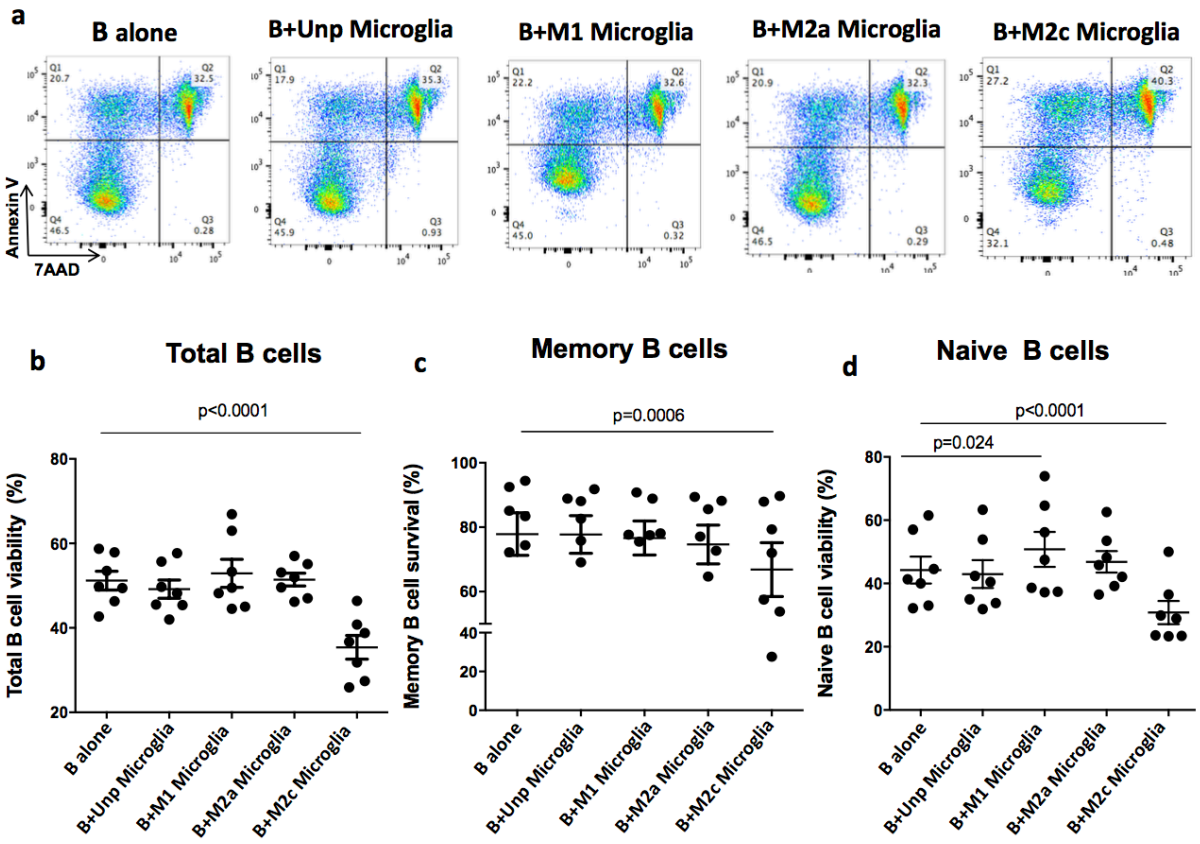


Figure 3.1: Soluble factors derived from human M2c polarized microglia are cytotoxic to MS-relevant B cells subsets. (a to **d)** Human microglia were either left under basal culture conditions (unp), activated with GM-CSF, IFNγ and LPS (M1), with M-CSF, IL-4, IL-13 (M2a) or with M-CSF, IL-4, IL-10, IL-13 and TGFβ (M2c). Microglia were then washed and left in culture for 2 days before collection of conditioned media. B cell were cultured either cultured with serum-free media alone or with 25% microglia conditioned media for up to 4 days. **(a)** Following 2 days of culture, we gated on $CD20^+$ B cells and measured B cell viability gating on Annexin V^- and $7AAD^-$ viable B cells. **(b)** Conditioned media derived from M2c microglia induced B cell death via apoptosis and necrosis on $CD20^+$ total B cells. **(c)** Conditioned media derived from M2c microglia induced B cell death via apoptosis and necrosis on $CD27^+$ memory B cells. (**d**) Conditioned media derived from M2c microglia induced B cell death via apoptosis and necrosis on $CD27^-$ naïve B cells, while M1-conditioned media may have marginally supported $CD27^-$naïve B cell survival (though this may reflect false discovery given multiple comparisons, and limited, heterogeneous effect). Percentage of B cell viability represents n=8 healthy control independent individuals; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 3.2:

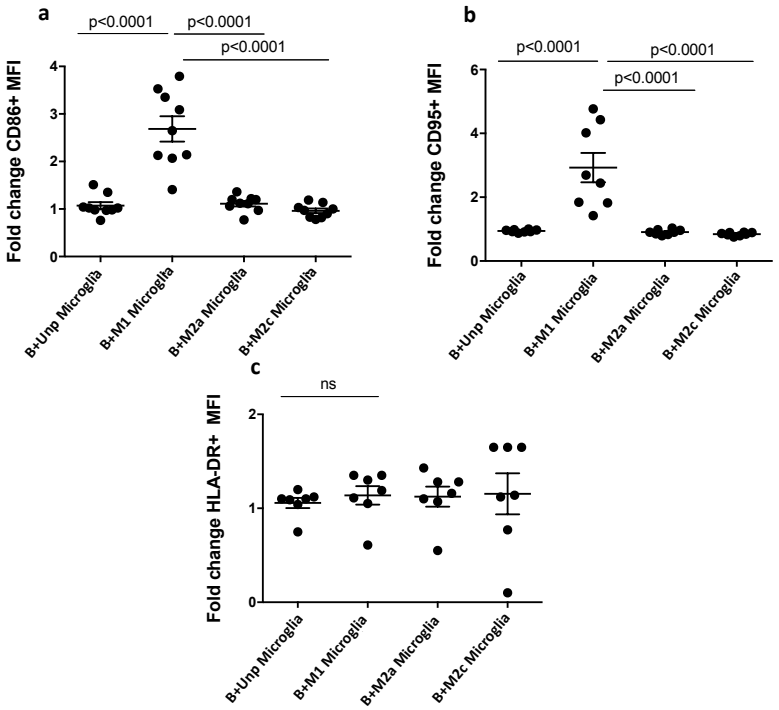
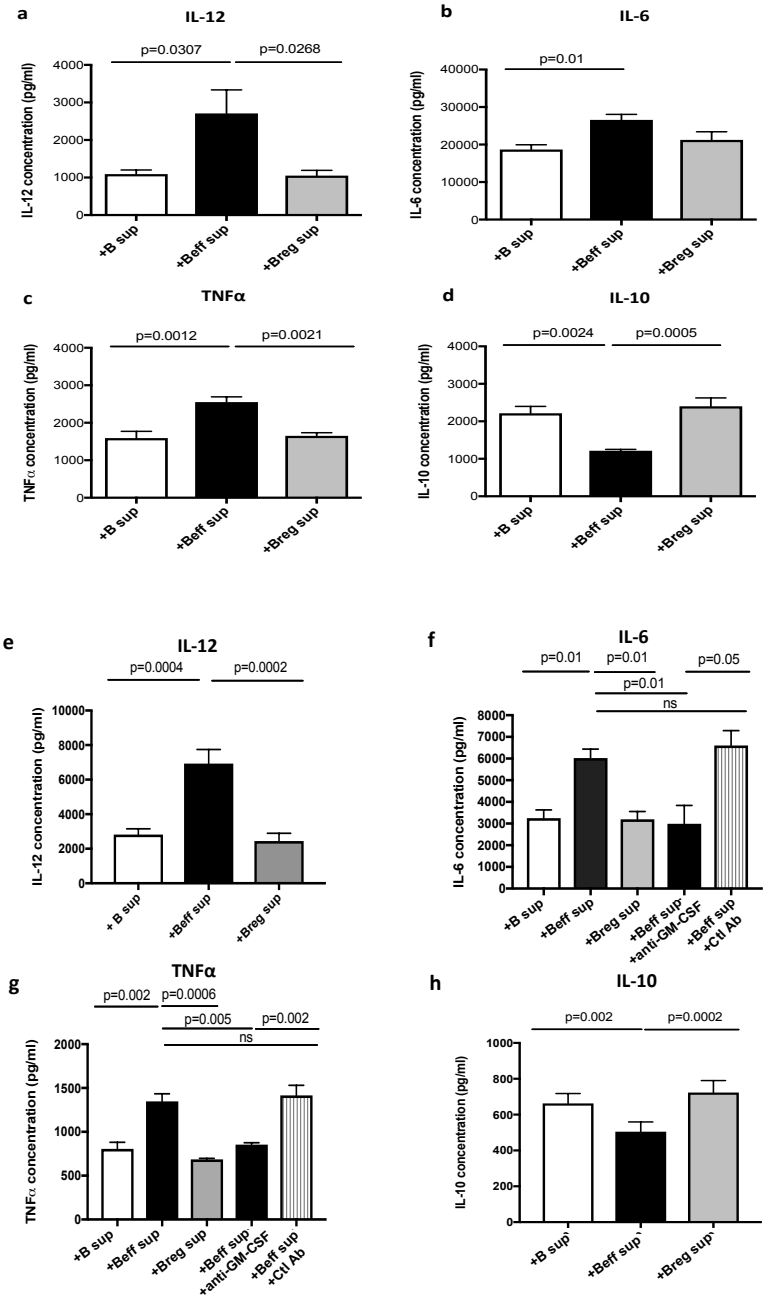


Figure 3.2: Soluble factors derived from human M1 polarized microglia increase B cell activation. (a to **c)** B cells were either cultured in serum-free media alone or with 25% conditioned media derived from M1, M2a or M2c polarized microglia, and activation profiles of $CD20^+$ total B cells were measured using flow cytometry, after 48 hours. (**a)** Microglia M1 conditioned media increased CD86 co-stimulatory molecule expression on the surface on live $CD20^+$ B cells (Annexin V^- $7AAD^-$) (Fold change MFI). (**b)** Microglia M1 conditioned media increased CD95 activation marker on surface of live $CD20^+$ Annexin V^- $7AAD^-$ B cells (Fold change MFI, compared to basal level). (**c)** HLA-DR activation marker measured on surface of live $CD20^+$ Annexin V^- $7AAD^-$ B cells (Fold change MFI, compared to basal level). Each symbol represents data from n=7 independent experiments; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 3.3



<u>Figure 3.3:</u> Soluble factors derived from pro-inflammatory B effectors cells (Beff) induce a pro-inflammatory myeloid cell responses. (**a** to **d**) Human microglia and (**e** to **h**) macrophages were generated by M-CSF treatment of freshly isolated $CD14^+$ peripheral monocytes. Macrophage and Microglia were then exposed to B cell supernatants derived from non-activated B cells (B sup) or effector B cell supernatants (Beff) or regulatory B cell supernatants (Breg) for 24 hours, then activated with LPS for additional 24 hours; supernatants were subsequently collected and IL-12, IL-6, TNFα and IL-10 secretion was measured using ELISA. (**a** to **c**) Supernatants derived from Beff increase IL-12, IL-6 and TNFα production by microglia; while (**d**) Beff supernatants down-regulate IL-10 production by microglia and macrophage. Beff enhanced (**e**) IL-12 production by macrophage and (**f** and **g**) IL-6 and TNFα production by macrophage in a GM-CSF dependent manner. Each column represents median data from n=6 (microglia) and n=3-6 (macrophage) independent experiments; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 3.4:

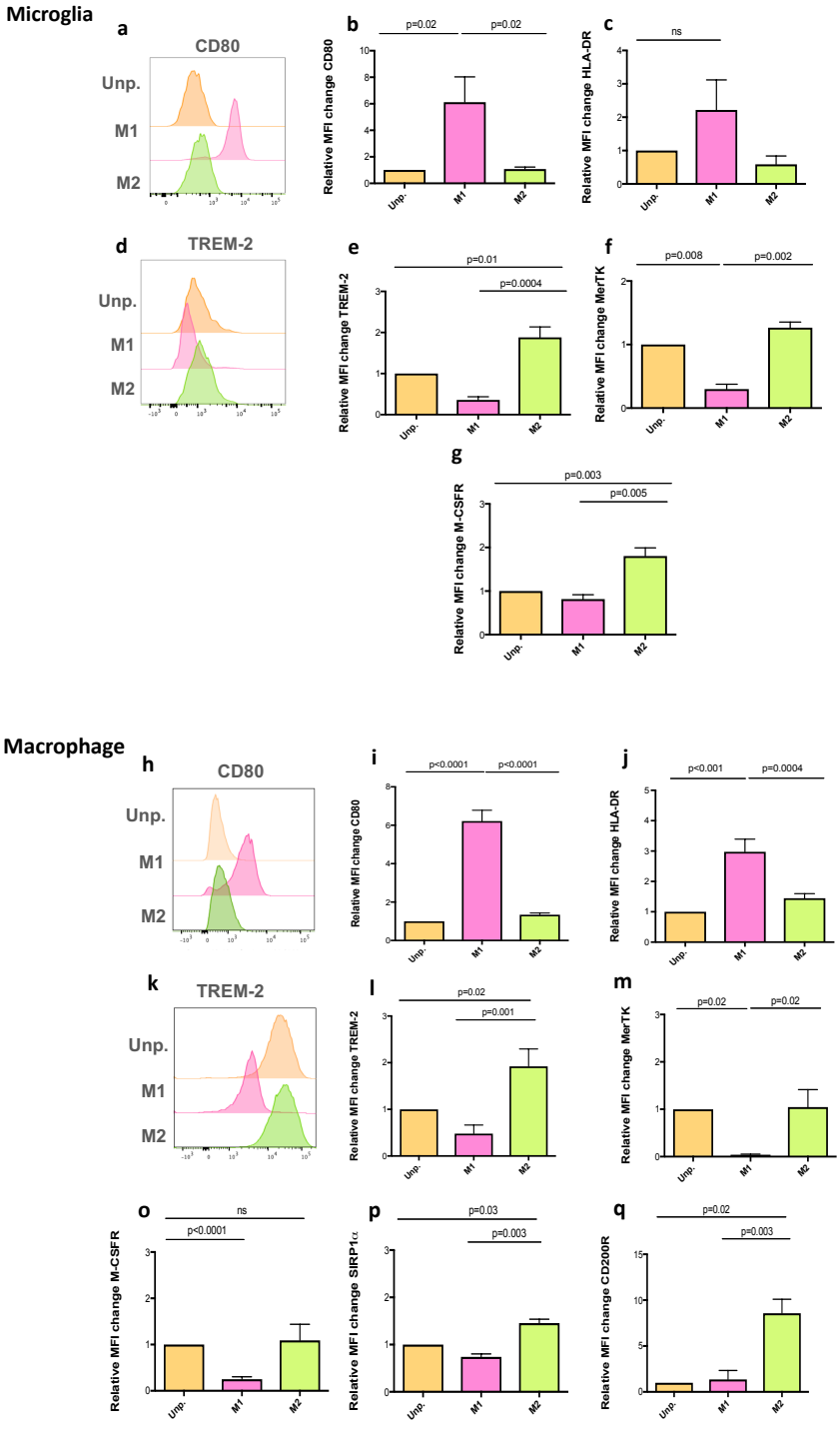


Figure 3.4: Soluble factors derived from MS-relevant B cell subsets (Beff & Breg) differentially impact phenotype of microglia and macrophage. (**a** to **f**) Human microglia were purified from epileptic surgery tissue, and (**g** to **i**) macrophages were generated by M-CSF treatment of freshly isolated $CD14^{+}$ peripheral monocytes. Macrophage and Microglia were then exposed to B cell supernatants derived from effector B cell supernatants (Beff) or regulatory B cell supernatants (Breg) for 48 hours and cell surface marker changes was assessed by flow cytometry. Flow cytometry histogram representation of increase of CD80 expression by (**a**) microglia and (**g**) macrophage in response to Beff supernatants. Summary data of CD80 increase in response to Beff supernatants by both (**b**) Microglia and (**h**) macrophage, (**c** and **i**) but not HLA-DR. Flow cytometry histogram representation of TREM-2 expression decrease by (**d**) microglia and (**j**) macrophage; summary data of TREM-2 up-regulation in response to Breg supernatants by both (**e**) Microglia and (**k**) macrophage. (**i**) Macrophage down-regulate MerTk expression in response to Beff supernatants. Each column represents median data from n=4 (microglia) and n=3-5 (macrophage) independent experiments; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 3.5:

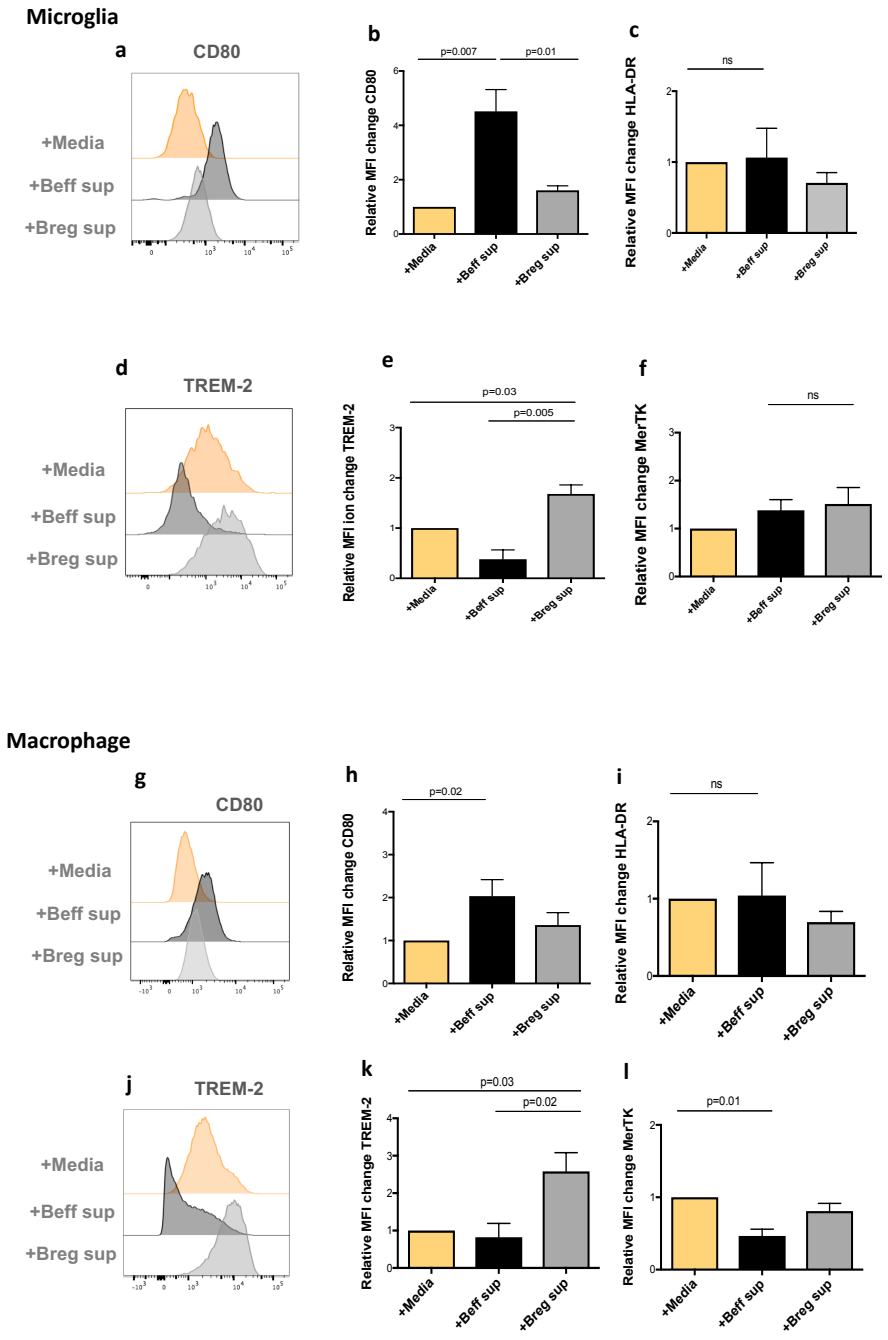


Figure 3.5: Soluble factors derived from MS-relevant B cell subsets (Beff & Breg) differentially impact phenotype of microglia and macrophage. (**a** to **f**) Human microglia were purified from epileptic surgery tissue, and (**g** to **i**) macrophages were generated by M-CSF treatment of freshly isolated $CD14^+$ peripheral monocytes. Macrophage and Microglia were then exposed to B cell supernatants derived from effector B cell supernatants (Beff) or regulatory B cell supernatants (Breg) for 48 hours and cell surface marker changes was assessed by flow cytometry. Flow cytometry histogram representation of increase of CD80 expression by (**a)** microglia and **(g)** macrophage in response to Beff supernatants. Summary data of CD80 increase in response to Beff supernatants by both **(b)** Microglia and **(h)** macrophage, (**c** and **i**) but not HLA-DR. Flow cytometry histogram representation of TREM-2 expression decrease by (**d)** microglia and **(j)** macrophage; summary data of TREM-2 up-regulation in response to Breg supernatants by both **(e)** Microglia and **(k)** macrophage. (**i)** Macrophage down-regulate MerTk expression in response to Beff supernatants. Each column represents median data from n=4 (microglia) and n=3-5 (macrophage) independent experiments; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 3.6:

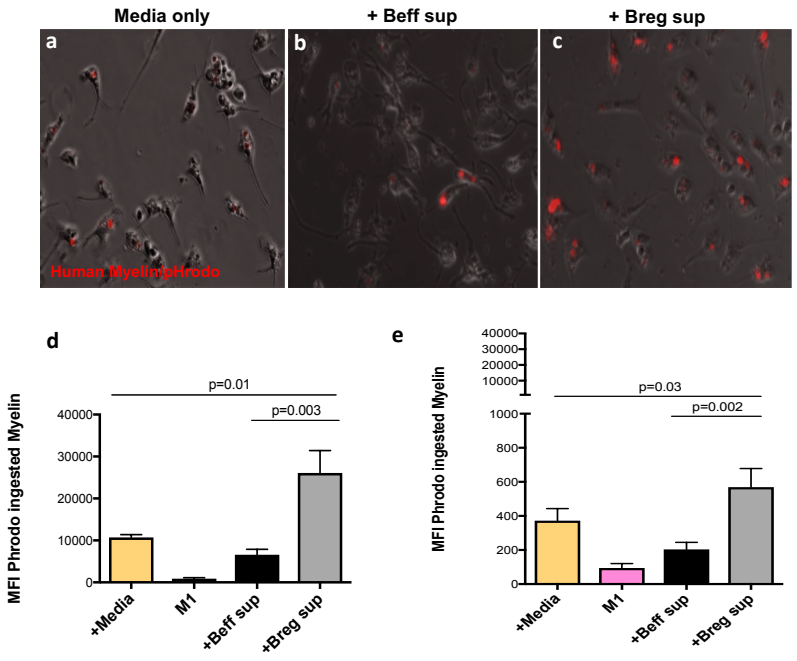
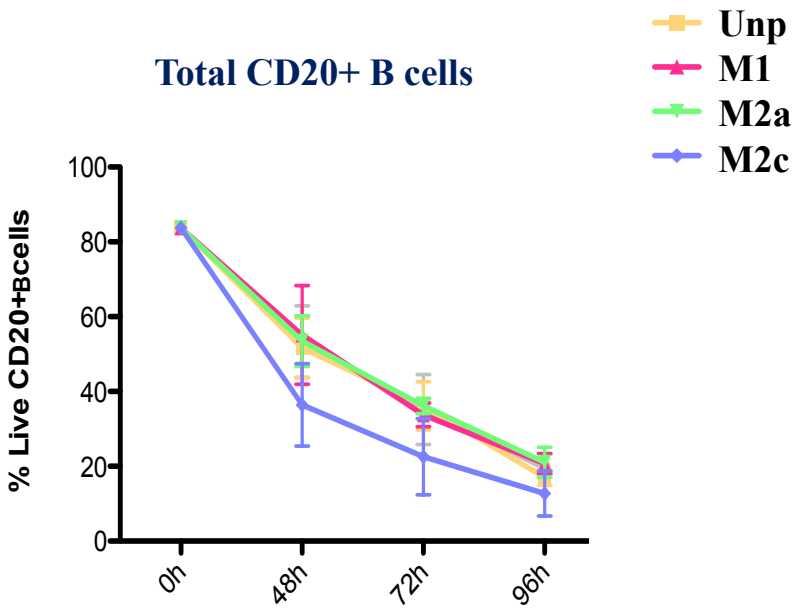


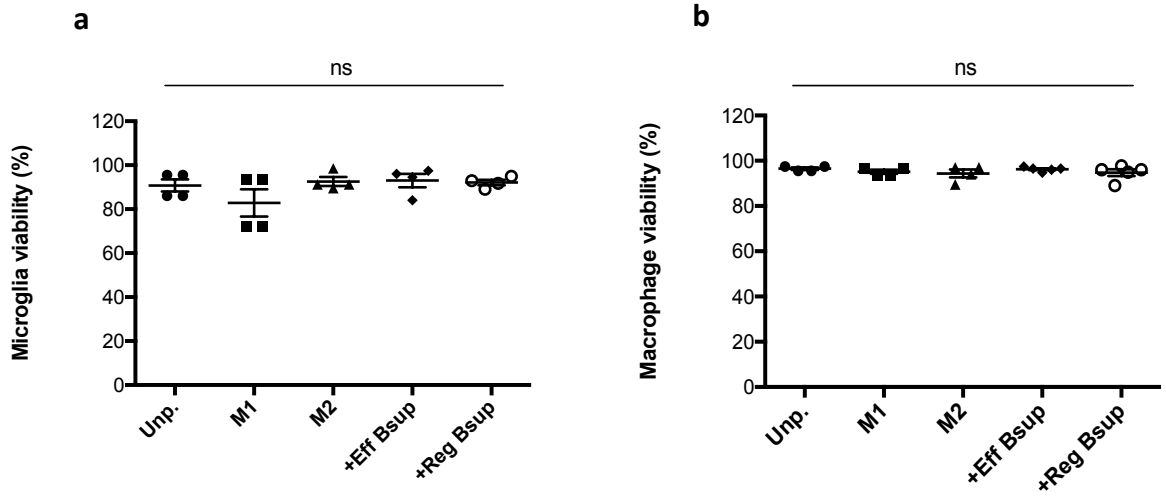
Figure 3.6: Differential ability of soluble factors derived from distinct B cell subsets (Beff & Breg) to impact microglia and macrophage phagocytic function. (**a** to **c**) Human microglia and (**e**) macrophages were generated by M-CSF treatment of freshly isolated $CD14^+$ peripheral monocytes. Macrophage and Microglia were then exposed to B cell supernatants derived from effector B cell supernatants (Beff) or regulatory B cell supernatants (Breg) for 2 days, and during the last hour of culture, pH-rodomycin stained human myelin was added to microglia or macrophage in culture. Myelin ingestion by microglia previously exposed to Beff or Breg: immunofluorescence images captured by fluorescence microscopy X20. (**a**) basal level of phagocytosis by microglia, (**b**) Beff supernatants down-regulate microglia phagocytic functions, while (**c**) Breg supernatants enhance the capacity of microglia to uptake myelin. (**d**) Flow cytometry quantification of myelin ingestion by microglia and **(e)** macrophage. Each column represents median data from n=4 (microglia) and n=6 (macrophage) independent experiments; data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Supplementary Figure 3.1:



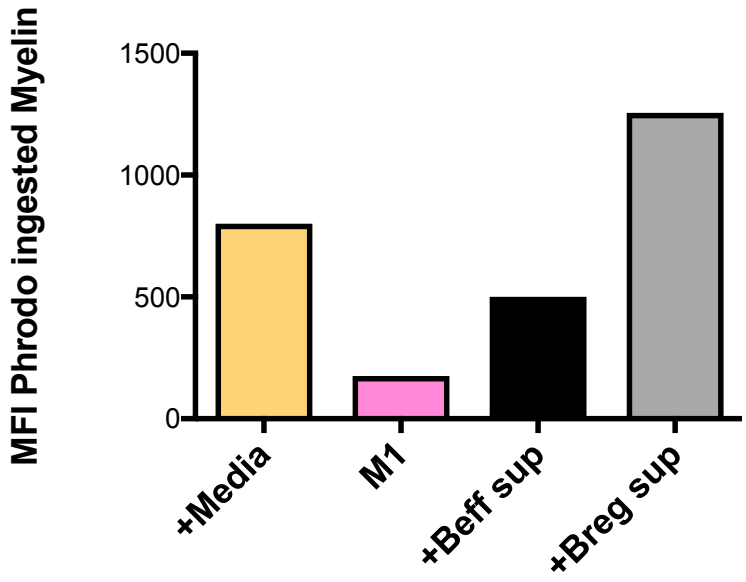
Supplementary Figure 3.1: Kinetics of total CD20$^+$ B cell death in response of M2c microglia conditioned media, over 4 days in culture. Human microglia were either left under basal culture conditions (unp), activated with GM-CSF, IFNγ and LPS (M1) or M-CSF, IL-4, IL-13 (M2a) / IL-4, IL-10, IL-13 and TGFβ (M2c). Microglia were then washed and left in culture for 2 days before collection of conditioned media. B cell were cultured either cultured with serum-free media alone or with 25% microglia conditioned media for up to 4 days. We measured B cell viability on fresh B cells, or after 2 days, 3 days or 4 days in culture, by gating on CD20$^+$ Annexin V$^-$ 7AAD$^-$ viable B cells. Conditioned media derived from M2c microglia mediate B cell death via apoptosis and necrosis at day 2, day 3 and day 4. Graphics represent a median data of n=2.

Supplementary Figure 3.2:



Supplementary Figure 3.2: B cell supernatants do not impact microglia and macrophage survival. (**a**) Human microglia were purified from epileptic surgery tissue, (**b**) and macrophages ere generated by M-CSF treatment of freshly isolated $CD14^+$ peripheral monocytes. Microglia and macrophages were then exposed to B cell supernatants derived from non-activated B cells (B sup) or effector B cell supernatants (Beff), or regulatory B cell supernatants (Breg), for 2 days the microglia and macrophage survival was assessed using 7AAD and ANNEXIN V staining. **(a)** B cell supernatants (Beff & Breg) did not impact the survival of microglia. **(b)** B cell supernatants (Beff & Breg) did not impact the survival of macrophage. Each data point represents data from n=4 (microglia) and n=5 (macrophage) independent experiments; data data were analyzed with one-way ANOVA: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Supplementary Figure 3.3:



Supplementary Figure 3.3: SPMS B cells soluble factors derived from Beff and Breg differentially modulate myelin phagocytosis function of microglia and macrophage. Human microglia were purified from epileptic surgery tissue. Microglia were then exposed to MS B cell supernatants derived from effector B cell supernatants (Beff) or regulatory B cell supernatants (Breg) for 2 days, and during the last hour of culture, pH-rodomycin dyed myelin was added to microglia in culture. Each column is a representative data of one donor.

Supplementary Table 3.1:

	Age	Sex
Healthy Control 1	**28**	**F**
Healthy Control 2	**62**	**F**
Healthy Control 3	**54**	**F**
Healthy Control 3	**24**	**M**
Healthy Control 4	**25**	**M**
Healthy Control 5	**32**	**F**
Healthy Control 6	**58**	**F**
Healthy Control 7	**27**	**M**
Healthy Control 8	**26**	**F**
Healthy Control 9	**42**	**F**
Healthy Control 10	**57**	**F**
Healthy Control 11	**30**	**F**
Healthy Control 12	**52**	**F**
Healthy Control 13	**31**	**F**
Healthy Control 14	**50**	**F**
Healthy Control 15	**25**	**M**
Healthy Control 16	**29**	**M**
Healthy Control 17	**30**	**M**
Healthy Control 18	**30**	**F**
Healthy Control 19	**26**	**F**
Healthy Control 20	**36**	**F**

Chapter 4: Discussion, conclusion and future directions

4.1. General discussion

The overarching goal of my PhD thesis was to investigate the cross-talk between MS relevant B cell subsets and glial cells, and how these interactions may play a role during disease progression by propagating CNS-compartmentalized inflammation and contributing to injury. My findings provide novel insights into how particular B cell subsets may persist, become activated, and contribute to local pro-inflammatory activation of T cells as well as underlying tissue resident microglia and infiltrating macrophage. My results emphasize how the balance between pro-inflammatory (Beff) and anti-inflammatory (Breg) B cell subsets may shape CNS-compartmentalized inflammation, via a variety of relevant microglial responses. My thesis reinforces our appreciation of how biologically complex MS disease progression is, with the potential to involve cascades of cellular interactions between immune and brain cells. An important question is whether targeting B cells within the CNS of patients by either modulating them into an anti-inflammatory direction, or depleting them (which aCD20 therapies only marginally accomplish in the CNS) may break B cell:glial cell cascades, thereby limiting the process of disease progression more effectively than achieved to date with aCD20.

4.1.1. Glial cells support B cell persistence and activation

Previous findings indicated that B cells abnormally persist in the CNS of patients, and can be found in the CSF, parenchymal lesions and also within the meningeal immune cell collections that are now thought particularly relevant to sub-pial cortical pathology [161, 173, 215]. Increased levels of BAFF, IL-6, IL-10 IL-15 and CXL12 are reported in the CSF of MS patients [232], some of which could be secreted by activated astrocytes and microglia. It is thought that such glial-derived factors may contribute to a favorable environment supporting B

cells persistence, which maybe particularly relevant to maintenance of B cells within meningeal immune cell collections, in turn propagating compartmentalized inflammation. The subpial cortical injury appears to be an important contributor to progressive MS and any involvement of meningeal immune cells would require communication through soluble molecule/s whether the interactions are unidirectional or bi-directional (as opposed to direct cell-to-cell contact). My *in vitro* data clearly indicate that soluble factors secreted by glial cells can support B cell survival (including memory B cells known to be enriched in the in MS CNS) and activation in a contact independent manner. In this context, differentially activated microglia can have quite different effects on B cells ranging from activation (by secreted factors from M1 microglia) to death (secreted factors from M2c-microglia). This observation may have therapeutic implications since ongoing efforts to develop agents that access the CNS and modulate microglial responses may have consequences on the survival and activation of CNS-compartmentalized B cells. Our observations also indicate that the astrocyte soluble factors responsible for supporting B cell survival, differ from those that enhance B cell activation/co-stimulatory molecule expression. My initial attempts to identify the molecules responsible for mediating these effects by blocking several of these cited molecules (alone or in combination) did not produce definitive results. Hence, our lab is currently applying proteomic analysis of astrocyte and microglia conditioned media, with the hope to reveal the glial-drived molecules that influence these different aspects of B cell biology. To increase our chances to identify culprit molecules, we are also analyzing the proteomic profile of CSF obtained from post-mortem MS cases, previously stratified according to disease severity.

4.1.2. MS-relevant B cell subsets modulate underlying glial tissue responses

Our group and others have shown that untreated MS patients harbor a higher proportion of pro-inflammatory memory (Beff) B cells that produce abnormally increased levels of pro-inflammatory cytokines (IL-6$^+$, LTα^+ , TNFα^+ & GM-CSF$^+$), and lower proportions of naïve anti-inflammatory (Breg) B cells (producing IL-10$^+$) [105, 110, 155], compared to matching controls. These abnormalities are now firmly implicated in the contribution of peripheral B cells to new disease relapses in MS. In the context of CNS-compartmentalized inflammation, of further interest is how MS-implicated B cells may participate in propagating inflammation and injury to the CNS. In collaboration with Dr. Lisak's group we have shown that soluble factors derived from MS B cells (but not B cells from matched controls) were cytotoxic to neurons and oligodendrocytes *in vitro* [209, 210], an effect that may be mediated by exosomes secreted by the B cells (unpublished data). I could further show that the same supernatants I generated from differentially activated Beff or Breg B cells could differentially impact myeloid cell (including microglia) responses. Specifically, secreted products of Beff B cells (enriched in MS patients) could enhance both microglia and macrophage pro-inflammatory cytokine (IL-6, IL-12 & TNF) responses. I have been able to implicate GM-CSF from the Beff as a pro-inflammatory modulator of the macrophage and further investigation is currently taking place to demonstrate whether the same is true for microglia. I also found that soluble factors derived from the distinct Beff and Breg B cells differentially impacted the state of quiescence versus activation, as well as the myelin phagocytic capacity, of both microglia and macrophage (a function thought to be important for clearance of myelin debris around lesions). Our observations are in line with the recent report that increased levels of IL-6, IL-12 and TNF in MS CSF are associated with increased meningeal inflammation, a greater extent of subpial cortical injury and more severe

disease progression [232]. Overall, my findings offer new insights into the potential importance of cross-talk between B cells and glial cells, and on how the balance between pro-inflammatory Beff and anti-inflammatory Breg subsets can modulate glial cell responses, by either propagating CNS-compartmentalized inflammation or inducing quiescent glial cell responses. Our study reinforce the view that compartmentalized immune responses (and particular immune:brain interactions) may importantly contribute to the propagation of CNS. To better understand the molecular mechanism involved in such interactions it could be of particular interest to investigate the down-stream signaling of myeloid cells in response to B cell supernatants. For this purpose, I have already generated RNA material that would be used for RNAseq profiling, which is an ongoing project in the Bar-Or lab. Further investigation to identify more soluble factors responsible of functional changes on microglia and macrophage is also needed.

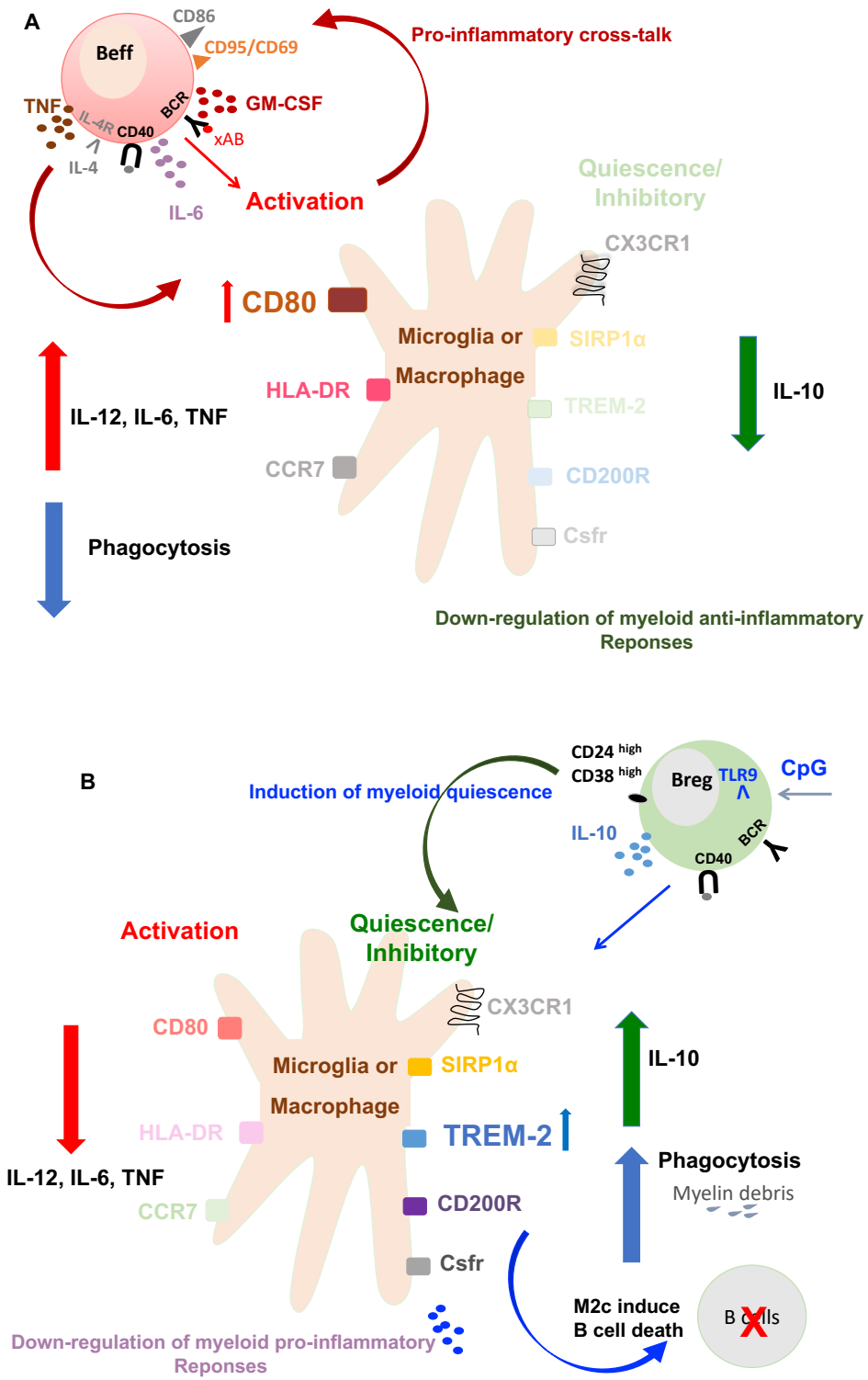
4.2. Conclusion and future directions

My overall PhD findings reveal novel cellular mechanisms by which CNS-compartmentalized inflammation links with underlying pathologic changes seen as part of the subpial cortical injury which is now thought to importantly contribute to progressive MS. More precisely, I have shown that the cross-talk between MS-relevant B cell subsets and glial cells (astrocytes, microglia) and infiltrating macrophage may sustain ongoing cascades of inflammation and CNS injury. Our discoveries attribute novel roles and potential for therapeutic targeting of pro-inflammatory (Beff) and anti-inflammatory (Breg) B cell subsets as modulation of the balance between them (away from Beff and towards Breg) would be expected to result in less pro-inflammatory glial responses. I consider, my thesis is the first work that aimed at

implicating functionally distinct cytokine-defined B cells within the target organ of MS. While my project is limited to *in vitro* observations I have used human sources of both CNS-derived glial cells and immune cells (including, for the latter, from MS patients) and have provided a 'proof-of-principle' that bi-directional interactions between CNS-resident glial cells (microglia/astrocytes) and immune cells (B cells) may occur and in a way that could potentially propagate local inflammation and subpial injury. Identification of the molecular mechanisms underlying such immune:glial cell interactions, and perhaps a better characterization of distinct B cell subsets according to their surface markers and transcriptional factors may help us dissect pathway and identify attractive novel therapeutic targets aimed at limiting progressive MS, which is a major unmet clinical need.

Below is a model summarizing the proposed cross-talk between Beff, Breg subsets and microglia and macrophage, via supernatants. (a) Supernatants from Beff enhances CD80 expression by microglia and macrophage their IL-12, IL-6 and TNF production, while down-regulating IL-10 production and phagocytic function; while M1-derived soluble factors increase B cell activation (Higher CD86, CD95 & CD69 expression) (b) Supernatants from Breg enhance TREM-2 expression, and induce IL-10 secretion and phagocytic responses of microglia and macrophage; while unkown factors derived from M2c induce B cell death.

Figure 4.1. Cross-talk between MS implicated B cell subsets (Beff & Breg) and microglia and macrophage responses : A model for CNS-compartmentalized inflammation



4.3. Original contributions and research highlights – by Hanane Touil, PhD candidate

Neuroimmunology, multiple sclerosis and progressive disease

1- Identification of novel cellular mechanism involving bi-directional interactions between human B cells and glial cells, as a proposed cellular mechanism mediating ongoing CNS-compartmentalized inflammation, cortical injury and disease progression.

2- Demonstration of a preferential MS and HC B cell persistence in response to soluble factors derived from human astrocytes at basal and inflammatory conditions.

3- Demonstration that MS-relevant B cell subsets: $CD27^{+}$memory, $CD24^{high}CD38^{high}$ transitional and $CD27^{-/+}IgD^{+}$ naïve B cells are more prone to persist in response to soluble factors derived from astrocytes.

4- Demonstration of the ability of M2c-derived soluble factors to induce B cell death via apoptosis and necrosis.

5- Demonstration that soluble factors derived from activated human astrocytes and microglia substantially enhance B cell expression of the co-stimulatory molecule CD86 molecule, and activation markers CD95 and CD69.

6- Demonstration of enhanced T cell activation upon co-culture with B cells previously exposed to soluble factors derived from activated astrocytes and M1 microglia.

7- Demonstration that soluble factors derived from pro-inflammatory Beff and anti-inflammatory Breg cells did not impact survival of microglia and macrophage.

8- Demonstration that soluble factors derived from pro-inflammatory Beff cells enhanced CD80 and IL-12, IL-6 and TNF production by human microglia and macrophage, and that this is mediated via GM-CSF (for macrophage).

9- Demonstration that soluble factors derived from pro-inflammatory Beff down-regulate IL-10 production by microglia and macrophage.

10- Demonstration that soluble factors derived from pro-inflammatory Beff and anti-inflammatory Breg reciprocally regulate microglia and macrophage myelin phagocytosis.

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