B CELL MODULATION OF T CELL RESPONSES IN MULTIPLE SCLEROSIS

APPROVED BY SUPERVISORY COMMITTEE

Nancy L. Monson, Ph.D.

Dorothy Yuan, Ph.D.

David R. Karp, M.D., Ph.D.

Laurie S. Davis, Ph.D.

Amy E. Lovett-Racke, Ph.D.

DEDICATION

This work is dedicated to all of the people in my life, both personal and professional, who have given me immense support in helping me accomplish my goals. I would like to begin by thanking Nancy, who served not only as my scientific mentor, but also became a trusted friend whom I could lean on in difficult personal times. Thank you Nancy for all of the encouragement, support, and joyfulness you gave me during my graduate research career; you have truly had a major impact on the scientist I have become.

I would also like to thank my friends and colleagues with whom I shared much time in lab. Thank you Mari Pili, Beth, Ann, and Petra for your friendship, and helpful scientific discussions throughout the years. A special thanks to Mari Pili for teaching me many of the scientific technical skills I put to use in the preparation of this dissertation, and for always making time in the lab enjoyable.

I would like to thank my dissertation committee members, Dorothy, David, Laurie, and Amy, as well as Todd, Nitin, and Olaf who helped push my project in the right direction and for your continued support in my research career.

I would not have made it to this point in my career without the love and support of my family. To my Mom and Dad: you have always allowed me to explore any and all of life's possibilities. I could not have accomplished all that I have without the sacrifices you made for me in supporting my life and education. You have taught me that anything is possible and I love you very much. Additionally I would like to thank my Grandparents and especially my grandmother Marjorie, who's dedication and commitment to my education both personally and financially, helped me immensely in making it to where I am today. I love you and appreciate all that you have done for me. To my brother Tom, thank you for always listening.

Finally I would like to thank my husband, Jonathan. You have truly made me understand what it is to sacrifice for someone you love. Your unwavering support through all of the ups and downs during my time in graduate school allowed me to accomplish this goal. You have taught me how to reach higher than I ever thought I could. I am grateful for all of your support and I love you very much,

B CELL MODULATION OF T CELL RESPONSES IN MULTIPLE SCLEROSIS

by

CHRISTOPHER TODD HARP

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2009

B CELL MODULATION OF T CELL RESPONSES IN MULTIPLE SCLEROSIS

CHRISTOPHER TODD HARP, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2009

NANCY L. MONSON, Ph.D.

Until recently, a definitive role for B cells in the pathogenesis of the autoimmune neurological disorder multiple sclerosis (MS), had not been widely accepted, and remains poorly understood. B cells have multiple functions in the immune system and can both positively and negatively modulate immune responses through the production of antibody, cytokine secretion, and/or antigen presentation. Several studies indirectly suggest that B cell-T cell cooperation may be paramount in MS disease pathogenesis, although this interaction has not been well studied in MS. Therefore, the focus of my thesis project was to test the hypothesis that B cells could be efficient neuro-antigen presenting cells in the

context of MS. My work has demonstrated that the cerebrospinal fluid (CSF) B cell population in MS shows characteristics of both auto-reactivity and antigen driven selection in a germinal center reaction.

These findings suggest that neuro-antigen driven selection had occurred in the periphery and prompted investigation of B cells as neuro-antigen presenting cells. Examination of CD40 ligand (CD40L) and interleukin-4 (IL-4) activated peripheral B cells demonstrated for the first time that B cells could efficiently elicit myelin basic protein (MBP) specific CD8+ and CD4+ T cell proliferation from resting T cells in vitro through a mechanism that was partially dependent on presentation through HLA-DR. Further inquiry into the antigen presentation capacity of specific subpopulations of resting B cells revealed that memory B cells from MS patients (but not healthy donors (HDs)) were significantly better neuro-antigen specific presenting cells than their naïve counter parts. This data indicated that a specific peripheral immune response had been generated in response to neuro-antigens in RRMS patients but not HDs. Taken together, these data provide a model where antigen experienced peripheral B cells from MS patients (but not HDs) provide important T cell support through antigen presentation and add to our understanding of the role of B cells in the pathogenesis of this autoimmune disease of the CNS.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Cameron E.M., Spencer S., Lazarini J., **Harp C.T.** Ward E.S., Burgoon M., Owens G.P., Racke M.K., Bennett J.L., Frohman E.M., Monson N.L. (2009). Potential of a unique antibody gene signature to predict conversion to clinically definite multiple sclerosis. *J. Neuroimmunol.* 213(1-2):123-30

Harp C.T., Lovett-Racke A., Racke M. Frohman E., Monson N. (2008). Impact of myelin specific antigen presenting B cells on T cell activation in multiple sclerosis. *Clin Immunol*. 128(3):382-91.

Harp C., Lee, J., Lambracht-Washington D., Frohman E., Racke M., Monson N.(2007). Cerebrospinal fluid B cells from multiple sclerosis patients that have not undergone clonal expansion are subject to normal germinal center selection. *J Neuroimmunol.*183(1-2):189-99

Ge H., Cha J.Y., Gopal H., **Harp C.**, Yu X., Repa J.J., Li C. (2005). Differential regulation and properties of angiopoietin-like proteins 3 and 4. *J Lipid Res*. 46(7):1484-90

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA – amino acid

AID – activation induced cytidine deaminase

ANA – antinuclear antibody

ANOVA – analysis of variance

APC – antigen presenting cell

ASC – antibody secreting cell

B-APC – CD40L/IL-4 activated B cell

B-CLL – B cell chronic lymphocytic leukemia

BBB – blood brain barrier

BCR – B cell receptor

B_{reg} – regulatory B cell

BSA – bovine serum albumin

BTAC – bi-specific tetrameric antibody complexes

CCR7 – C-C chemokine receptor 7

CD40L – CD40 ligand (CD154)

CDR – complementary determining region

CDR3 – complementary determining region 3

CFDA-SE – carboxyfluoro-diacetate succinimidyl ester

CFSE – carboxyfluorescein succinimidyl ester

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

CIS – clinically isolated syndrome

CLP – common lymphoid progenitor

CNPase – 2,3-cyclic nucleotide 3 phosphodiesterase

CNS – central nervous system

CpG ODN – CpG oligodeoxynucleotide

Cq – chloroquine

CSF – cerebrospinal fluid

CSR – class switching recombination

CXCL9 – C-X-C chemokine ligand 9

CXCR5 – C-X-C chemokine receptor 5

DC – dendritic cell

D_H – heavy chain diversity (D) segment

DM – direct magnet

DMSO – dimethyl-sulfoxide

EAE – experimental allergic encephalomyelitis

ELISA – enzyme linked immunosorbent assay

FACS – fluorescence activated cell sorting

FITC – fluorescein isothiocyanate

FR – framework region

g – gravity

GA – glatiramer acetate (Copaxone®)

GC – germinal center

Gy – Gray

HBcAg – hepatitis B core antigen

HCPB – healthy control peripheral blood

HD – healthy donor

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

IFNβ – interferon-beta

IFNγ – interferon gamma

IgH – immunoglobulin heavy chain

IL-10 – interleukin-10

IL-12 – interleukin-12

IL-2 – interleukin-2

IL-4 – interleukin-4

IL-6 – interleukin-6

J_H – heavy chain joining (J) segment

LD – lymphocyte depletion

LDA – limiting dilution assay

LN – lymph node

LTα – lymphotoxin-alpha

MAG – myelin associated protein

MBP – myelin basic protein

MFI – mean fluorescence intensity

MHCII – major histocompatability complex, class II

mL – milliliter

MLR – mixed lymphocyte reaction

MOBP – myelin-associated oligodendrocytic basic protein

MOG – myelin oligodendrocyte glycoprotein

MS – multiple sclerosis

ng – nanogram

NO – nitric oxide

OIND – other inflammatory neurological disease

OND– other neurological disease

ONND – other non-inflammatory neurological disease

ORF – open reading frame

OSP – oligodendrocyte-specific glycoprotein

OVA – ovalbumin

PAMP – pathogen associated molecular pattern

PBBC – peripheral blood B cell

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PE – phycoerythrin

PLP – proteolipid protein

PP – primary progressive

PPMS – primary progressive multiple sclerosis

R:S ratio – replacement to silent mutation ratio

RAG – recombinase activating gene

RPM – rotations per minute

RR – relapsing remitting

RRMS – relapsing remitting multiple sclerosis

SA – streptavidin

SHM – somatic hypermutation

TCR – T cell receptor

TD – thymus dependent

T_{FH} – B helper CD4+ T follicular helper cell

TGFβ – transforming growth factor-beta

Th – $CD4^+$ T helper cell

TI – thymus independent

TLR – toll like receptor

TLR9 – toll like receptor 9

TNF – tumor necrosis factor

TNFα – tumor necrosis factor alpha

TP – time point

T_{reg} – regulatory T cell

TT – tetanus toxoid

UTSW – University of Texas Southwestern Medical Center

V_H – heavy chain variable (V) segment

VLA-4 – very late antigen 4

WBCE – white blood cell enriched

WVPB – whole venous peripheral blood

μL – microliter

μM – micromolar

CHAPTER ONE
Introduction

The fundamental purpose of the immune system is to constrain infection of the host by foreign species of microorganisms including viruses, bacteria, fungi, helminthes, and protozoa. A well functioning immune response requires the coordinated action of multiple types of immune cells that develop a highly targeted response resulting in the elimination or subversion of the invading pathogen while at the same time limiting damage to the host. Multiple exquisite mechanisms exist to prevent immune recognition of the host as foreign. These mechanisms include the elimination, reduction or inactivation of lymphocytes that recognize self, through central tolerance, as well as multiple peripheral tolerance mechanisms that prevent the activation of immune cells that may recognize the host if they escape central tolerance. Even though numerous mechanisms are built into the immune system to prevent recognition of the host as foreign, these mechanisms can fail leading to the development of immunity against the host, a condition known as autoimmunity.

Multiple sclerosis (MS) is the second most common neurological disease affecting young adults in the world behind traumatic injury (1). The etiology of MS remains unknown, however the consensus within the field is that relapsing remitting MS (RRMS), the most common subtype of MS, is chiefly an autoimmune disease. It is generally thought that during the course of RRMS, a variety of immune cells cooperate to mediate the destruction of the myelin sheath, a fatty substance produced by oligodendrocytes in the central nervous system (CNS) that acts as an insulator in promoting the propagation of electrical signals used by neurons to communicate with

each other. The destruction of the myelin sheath is thought to lead to a deficit in neuronal communication, and results in severe neurological symptoms observed during the course of RRMS.

There are several types of immune cells observed at the sites of demyelination in RRMS including macrophages, T cells, and B cells (2). The contribution of these cells types and their function in disease pathogenesis is an active area of research. Currently, it is thought that T cells and macrophages contribute most significantly to the pathogenesis of MS. The role of B cells has remained somewhat controversial because early studies in the mouse model of MS, experimental allergic encephalomyelitis (EAE), indicated that B cells were not necessary for disease initiation or exacerbation (3). Yet several observations in human MS indicate that humoral immunity (B cells and their antibody products) contributes to the pathogenesis of MS. For example, one of the hallmark diagnostic features in MS is the presence of oligoclonal bands in the cerebrospinal fluid (CSF, (4)). Several other studies indicate that B cells are clearly responding to antigens in the CNS as evidenced by: increased kappa (κ) to lambda (λ) light chain ratio in the CSF compared to blood (5-7), immunoglobulin (Ig) production in the CSF (4, 5, 8), and intrathecal B cell clonal expansion in the CSF (6, 9-13). Most recently, B cell depletion therapy was shown to be efficacious in restricting the development of CNS lesions and limiting disease progression in MS (14), demonstrating a necessary role for B cells in pathogenesis of MS. However, the particular role that B cells are playing in MS is still unknown.

B cells have several important functions in the immune system including antibody production, cytokine secretion, and antigen presentation to T cells, yet it

remains unclear which of these functions plays a central role in exacerbation of RRMS. Several studies suggest an important role for B cells in both the production of pathogenic auto-antibodies (14, 15) and modulation of T cell responses (16) in the pathogenesis of MS. Therefore, it became my hypothesis that a central role for B cells in the pathogenesis of MS was as neuro-antigen specific presenting cells. This hypothesis was novel since very few studies had assessed if antigen experienced B cells could serve as efficient APCs, much less in the context of neuro-antigens. The studies presented in this dissertation begin by documenting that B cells in the CSF of MS patients show characteristics of classical antigen driven selection indicative of peripheral germinal center reactions. This was important to document as some clonally related B cells isolated from the CSF of MS patients show atypical mutational characteristics that were suggestive of antigen driven proliferation but not selection in a germinal center reaction. These findings provided a license to assess the peripheral circulating B cell population in MS patients for the capacity to present neuro-antigen to autologous T cells as I hypothesized that at least a subset of the circulating B cell pool would contain neuro-antigen specific B cells selected in the context of a germinal center. I demonstrate here that primary peripheral pre-activated B cells from MS patients can effectively initiate neuro-antigen specific T cell activation and proliferation in vitro in a subset of both healthy donors (HD) and MS $CD4^+$ and $CD8^+$ T cells, a finding that for the first time provides direct evidence of the functionality of B cell antigen presentation in MS. Finally, I go on to demonstrate that memory B cells from MS patients are the most efficient neuro-antigen specific presenting cells, indicating that selection of neuro-antigen specific B cells has occurred in the periphery. These findings are important as they

provide a rationale for the function of B cells as antigen presenting cells in MS and favor a model where memory B cells function as a powerful stimuli for the reactivation of neuro-antigen specific T cells.

CHAPTER TWO
Review of the Literature

MULTIPLE SCLEROSIS

Incidence and Pathology

Multiple Sclerosis (MS) is a disease of the central nervous system that was first described by Charcot in 1868, and is the most prevalent neurological disorder diagnosed in young people with an incidence of approximately 0.1% in the general population (17). Sensory disturbances, unilateral optic neuritis, diplopia, paresthesias, peripheral limb weakness, clumsiness, gait ataxia, and incontinence are typical neurological-associated disabilities observed in patients with MS (18). Symptoms are thought to be the result of central nervous system (CNS) demyelination resulting in conduction block, however remyelination can occur, and neurological function can return even in the absence of remyelination (19). Although demyelination is the most prominent histological aberration observed in MS lesions, axonal damage and cell death do occur but are primarily seen in the progressive phase of the disease (20). Perivascular lesions are common, yet lesions that damage the cortical parenchyma are rare (18). Inflammatory cells are present in post-mortem CNS tissue samples from MS patients, specifically at perivascular lesion sites (18) consisting mainly of lymphocytes (B and T cells) and macrophages (21). In fact, Lucchinetti et al. have described a system of classifying lesion types into 4 categories based primarily on the types of cellular infiltrates occupying them (21).

Although MS lesions are heterogeneous in their pathologies and lymphocyte infiltrate composition, adoptive transfer of activated myelin basic protein antigen specific

$CD4^+$ T cells into recipient mice recapitulates many of the pathological findings present in MS (2), suggesting that $CD4^+$ T cells are central mediators of inflammation in this disease. This has lead to the hypothesis that potentially auto-reactive peripheral T cells escape tolerance mechanisms, become activated and traffic to the CNS where they mediate the development of MS (22). Lesion pathology is helpful in understanding the disease from post-mortem tissues, but is not helpful in determining the extent of CNS damage in a living patient.

Diagnosis and Subtypes of MS

Patients who present with symptoms of MS, who have not had a history of MS or other neurological disorders, undergo several diagnostic assessments to determine if MS might be suspected. CSF, which is a clear colorless fluid that surrounds the subarachnoid spaces surrounding the brain and spinal chord (23), is often sampled by lumbar puncture to aid in the diagnosis of clinically definite of MS (23). For example, a mononuclear cell count of 0 to 5 cells per μL is normal in adult human CSF, but a higher mononuclear cell count (typically greater than 10 cells per μL) is considered abnormal (23). The presence of oligoclonal Ig and lymphocyte pleocytosis in the CSF of MS patients are commonly observed, although these features are not unique to MS (18). Gadolinium (Gd) enhanced magnetic resonance imaging (MRI) of the brain and spinal cord additionally detect blood brain barrier (BBB) disruption which may be one of the earliest detectible events that occurs in MS (19). The Gd contrasting agent (Gd-diethylenetriamine penta-acetic acid) is unable to diffuse across the intact BBB readily; therefore, Gd enhances areas of BBB permeability on MRI. Lesions are detected on MRI

based on water displacement of lipid, which changes the contrast between areas of demyelination that appear hyper-intense compared to healthy tissue. Therefore, active and inactive lesions can be differentiated based on Gd enhancement. However, CNS pathology detected by MRI can occur in the absence of clinical symptoms and vice versa (19). It should also be noted that neurologic dysfunction must be separated in space and time in order for formal diagnosis of clinically definite MS (CDMS) to be confirmed (18).

80-85% of patients initially diagnosed with MS have a relapsing remitting (RRMS) course (17, 18), characterized by exacerbations of neurological dysfunction that occur episodically, and are reversible (17). Approximately 10-20% of RRMS patients show a benign course with little to no progression of disability (17), but the majority of RRMS patients subsequently develop secondary progressive MS (SPMS), characterized by progressive disability in the absence of superimposed episodic relapses and remissions (24). The remaining 15% of patients with MS are diagnosed with primarily progressive MS (PPMS). Normal appearing white matter in addition to inflammatory lesions in the CNS of PPMS patients show pathological neuroaxonal degeneration, and lesions are characterized by more prominent cortical demyelination in comparison to RRMS lesions (25). First line immunomodulatory therapies currently approved in therapeutic intervention of RRMS and SPMS are ineffective in PPMS, raising questions about whether PPMS is primarily an autoimmune disease (25).

MS is a complex genetic disorder with an environmental component

Multiple genetic and environmental factors are thought to contribute MS disease risk. RRMS has a clear gender bias with a female:male ratio of 2:1 (18), while PPMS is more commonly diagnosed in men (25). Monozygotic twins have at least a 300 fold increase risk of developing MS if present in the opposite twin as compared to the general population (26) even though MS concordance in monozygotic twins is uncommon (27). (28). A clear environmental effect has also been documented as MS incidence increases dramatically above the 35th parallel in the United States (US) (29). Recently, a major advancement in the field was made when IL-2 receptor alpha, and interlekukin-7 receptor alpha were identified as new risk alleles (and HLA-DRB*1501 reaffirmed as a risk allele) in the development of MS in a genome wide study (28). These provide further credence to the theory that MS is as an autoimmune disease. The types of immune cells involved and their interactions between immune cells and the CNS are the subjects of much investigation.

FUNCTION OF T CELLS IN MS AND EAE

T cells are the most well studied immune cell in the pathogenesis of MS and are clearly involved in the disease process. Experimental autoimmune encephalomyelitis (EAE), is an animal model of MS that has been used extensively to understand the pathogenesis of MS especially as it relates to T cells. For example, EAE can be induced by adoptive transfer of activated neuro-antigen specific $CD4^+$ T cells, demonstrating that neuro-antigen specific $CD4^+$ T cells are necessary and sufficient for the development of EAE (30).

A dominant view in the field postulates that during the initiation of MS, the peripheral lymphoid system is primed by antigens draining from the CNS into cervical lymphatics (31). $CD4^+$ T cells activated in the periphery are then thought to traffic to the CNS where they recognize specific antigen in the context of MHCII in areas of blood brain barrier (BBB) breach and subsequently promote a local inflammatory response (18, 32). It is hypothesized that $CD4^+$ T cells mediate the destruction of myelin in part through activation of macrophages and microglia, as well as activation of $CD8^+$ cytotoxic T lymphocytes (33). Activated macrophages and microglia may also secrete nitric oxide (NO) and matrix-metalloproteinases, which can contribute to the neuronal and oligodendrocyte cytotoxicity observed in MS lesions (34). Humoral immune components also contribute to lesion pathology, but are discussed in more detail in a later section.

T cell response to neuro-antigens in MS

Because specific demyelination observed in MS typically can occur in the absence of axonal damage or loss (35), the immune system is implicitly assumed to be recognizing and reacting against myelin or oligodendrocyte associated proteins as opposed to neuronal derived proteins. Furthermore, because many symptoms of MS can be recapitulated in EAE simply by immunizing animals with a single whole neuro-antigen or peptide in adjuvant (30), it is thought that T cell responses against epitopes within these proteins dominate the immune response in MS. There are several neuro-antigens that have been studied in the context of MS and EAE including, Myelin-associated Oligodendrocytic Basic Protein (MOBP), Oligodendrocyte-Specific Glycoprotein (OSP), Myelin Associated Protein (MAG), 2,3-Cyclic Nucleotide 3

Phosphodiesterase (CNPase), Alpha-B Crystalin, but the most well studied neuro-antigens in MS and EAE are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) (33). The role of T cell responses against neuro-antigen epitopes in MS remains in contention since the frequency of neuro-antigen specific T cells in healthy donors (HD) and MS patients are similar (2, 36). These estimates were calculated experimentally by determining the frequency of neuro-antigen reactive T cells using limiting dilution analysis (LDA) with autologous irradiated PBMCs as APCs, and calculated assuming a Poisson distribution (2, 36). However, neuro-antigen reactive $CD4^+$ T helper cells from MS patients tend to secrete a Th1 profile in response to restimulation, in contrast to HDs, whose neuro-antigen reactive $CD4^+$ T cells tend to secrete a Th2 profile more often in response to restimulation (37). In addition, neuro-antigen reactive $CD4^+$ T cells from MS patients are less dependent on costimulation than those from HDs (36, 38, 39), suggesting that they are most likely memory in phenotype (40).

Myelin Basic Protein

MBP is an 18.5 Kd protein (41) and constitutes 30% of the protein component in myelin (the major non-protein component being lipid) (42). MBP is not expressed on the surface of myelin, but is instead embedded along with lipid in the cytosolic portion of the myelin sheath, making it inaccessible to immune cells when myelin is intact (43). Free MBP is detected in the CSF of MS patients and patients with other neurological diseases (44). Adoptive transfer of MBP specific T cells results in a chronic relapsing form of EAE with demyelination (45). Purified MBP, recombinant MBP and MBP

peptides can initiate EAE through active immunization in various mouse strains and animal models (30). Viral and bacterial peptides presented in MHCII that mimic MBP in structure can activate MBP 85-99 specific T cells, which is the immunodominant MBP epitope in humans (46).

Glatiramer Acetate

Glatiramer acetate (GA) is a US Federal Drug Administration (FDA) approved immunomodulatory therapy for the treatment of MS. GA is a random copolymer of 4 amino acids (alanine, lysine, glutamate, and tyrosine) in a specific ratio of 4.2:3.4:1.4:1 respectively, with an average molecular weight of 7.5 kilo-Daltons (Kd), within a range of 40-90 amino acids in length (47). It was originally designed as an synthetic MBP mimic for use in the induction of EAE (48). GA is though to modulate the immune system in many ways, such as induction of APC promotion of Th2 responses, cross reactivity with MBP associated peptides as an altered peptide ligand and deviation of immune responses, also known as "bystander suppression" (47).

Myelin Oligodendrocyte Glycoprotein

MOG is a small transmembrane 28 Kd protein that constitutes 0.01-0.05% of the myelin sheath (49). MOG is expressed on the outermost lamellae of the myelin sheath (50) and therefore can be a target of demyelinating antibodies in intact myelin in animal models (51). Both whole recombinant MOG and MOG peptides can be used to actively initiate EAE (30, 52). Although several studies have demonstrated increased MOG

specific PBMC proliferative responses in MS as compared to HDs (53-55), others have not (56).

CD4+ T helper cell differentiation in MS

The types of signals that a naïve $CD4^+$ T cell receives through interactions with APC are of paramount importance to determining the type of effector T helper (Th) cell or T regulatory (T_{reg}) cell that it will become. Th1 cells are believed to be important in orchestrating cellular immunity to intracellular pathogens (57), while Th2 cells are thought to be important for immunity against helminth infection (58). The Th1/Th2 paradigm has recently been challenged, as new Th subsets such as Th17, T_{reg}, follicular B helper T cells (T_{FH})and Th9 have been identified, however many of the features defining this paradigm are still applicable. Naïve $CD4^+$ T cells can differentiate into divergent Th cell subsets simply by the addition of polarizing cytokines and obligate signaling through the T cell receptor. For example, human naïve T cells can be polarized into Th1 cells through CD3 stimulation in the presence of IL-12 (59). Conversely, Th2 cells can be polarized in the presence of IL-4 (58) and secrete the prototypic Th2 cytokines IL-4, IL-5, and IL-13. Although IL-10 is sometimes considered a Th2 related cytokine, it not mutually exclusive with Th2 polarization, as some IFN-γ producing cells also secrete IL-10 (60). Currently, T_{regs} are associated with IL-10 and TGFβ secretion, and Th17 cells (which secrete IL-17 IL-21 and IL-22), can be primed with different combinations of IL-6, IL-21, TGFβ, and IL-23 (61).

The type of interaction a T cell has with resident APCs is important to study in the context of autoimmune disease. Th1 cells are thought to mediate MS, as adoptive

transfer of neuro-antigen specific T cells polarized in Th1 promoting conditions cause severe EAE, while neuro-antigen specific T cells polarized in Th2 promoting conditions do not cause EAE unless transferred into RAG deficient or αβ T cell deficient mice (62). This finding is one reason that immune deviation of Th1 cells to a Th2 phenotype has become an important treatment goal. Indeed, multiple rounds of restimulation can cause the permanent differentiation of effector Th cells into Th1 or Th2 cells through epigenetic regulation of these genes (63), and Copaxone, a drug commonly used in the treatment of MS, is thought to induce immune deviation of the immune system towards Th2 (64), as well as $CD8^+$ T_{reg} cell enrichment (65) in MS patients. B cells are capable of secreting IL-2, IL-6, IFN-γ, and IL-12 and therefore could potentially help polarize naïve CD4+ T towards Th1 or Th17 differentiation.

THE ROLE OF B LYMPHOCYTES IN MS

B Cell Development

B cells are a differentiated subset of hematopoietic cells that have many specialized functions in the immune system. B cells develop from lymphoid precursors in the bone marrow called common lymphoid progenitor cells (CLP), which are derived from $CD34^+$ hematopoietic stem cells, in several discrete steps to ensure the productive rearrangement and membrane expression of membrane immunoglobulin as well as the prevention of autoreactive B cell precursors from entering the mature peripheral B cell repertoire.

Signals derived from the bone marrow stroma potentiate the development of some CLPs into Pre B cells, the earliest detectable stage of B cell development. At this

point in B cell differentiation, the B cell begins the process of developing a productive immunoglobulin heavy chain (IgH). This occurs through a process known as V-D-J recombination, and is the first significant event in the generation of B cell specificity for antigen. The human genome contains at least 44 Variable heavy chain antibody segments (V_H) with an open reading frame (ORF, (66)), 25 Diversity heavy chain antibody segments (D_H) (67), and 6 Junction heavy chain antibody segments (J_H) (66). These segments are located on chromosome 14 (68). The V segments show a certain amount of homology to one another, and most likely appeared in the genome due to gene duplication events (69). These V genes can therefore be classified together according to sequence homology into discrete family groups (V_H1, V_H2, V_H3, V_H4, V_H5, V_H6, and V_H7). The same is true of the J segments (J_H1, J_H2, J_H3, J_H4, J_H5, J_H6). The genetic recombination event, known as VDJ recombination, takes place in discrete steps, and is mediated by the recombination activating genes (RAG1 and RAG2). RAG, along with other adaptor molecules, recognize specific non-coding intervening sequences between the segments and mediate the excision of intervening sequences to form a transcriptionally functional heavy chain gene segment that is translated along with constant regions to form the heavy chain of the antibody. The heavy chain is expressed on the cell surface along with the surrogate light chain (made up of lamda5 and VpreB) as well as the signaling adaptor molecules Ig-alpha and Ig-beta (CD79a and CD79b) as the Pre-B cell receptor. Upon expressing the Pre-B cell receptor, the Pre-B cell is now considered a pro-B cell, the stage at which a B cell undergoes positive selection, and is so called because the Pre-B-cell receptor must be properly assembled on the cell surface to initiate down stream signaling by recognition of self-antigens in the bone marrow. If the

Pro-B cell properly initiates signaling through the pre-B cell receptor, the Pro-B cells undergoes several (30-60) rounds of division and enters the late Pro-B cell stage.

At this time, rearrangement of the light chain takes place through V_L-J_L rearrangement. Human B cells express either kappa or lambda light chains. Kappa light chain genes are preferentially rearranged first. If an non-productive or autoreactive rearrangement is formed in both kappa chains, rearrangement of the lambda light chain is allowed (70). Once a productive light chain is formed, it is expressed on the cell surface along with the previously rearranged functional heavy chain, and CD79, as a B cell receptor (BCR). The cell is now considered an immature B cell. The immature B cell undergoes negative selection to self-antigen at this point. If the BCR expressed by the immature B cell recognizes self-antigen in the bone marrow with sufficient affinity to induce downstream signal transduction, the cell will either apoptose, become anergic, or undergo subsequent rounds of light chain rearrangement utilizing upstream V_L segments and downstream J_L segments in a process known as light chain receptor editing. If strong signal is not obtained through the BCR, then the B cell exits the bone marrow and becomes a naïve B cell. IgM and IgD are expressed through means of alternative splicing mechanisms on naïve B cells. Naïve B cells typically express CCR7 and CD62L, so that they can home to secondary lymphoid organs such as the spleen or lymph node.

Thymus independent (TI) naïve B cell activation

Naïve B cells can be activated through thymus-dependent (TD) or thymus-independent activation (TI). TI activation does not require cellular contact with a T cell. TI B cell activation can occur in at least two different ways, TI-I: by recognition of

pathogen associated molecular patterns (PAMPs) through innate receptors such as toll like receptors (TLRs) or TI-II: recognition of repeated antigenic epitopes that cause B cell receptor aggregation and crosslinking. This is in contrast to thymus-dependent activation of naïve B-lymphocytes, which require cell contact with T cells to acquire the necessary signals for activation and differentiation.

Thymus dependent (TD) B cell activation and germinal center reaction

Naïve B cells that recognize whole protein epitopes through the antigen specific BCR, internalize antigen, then process and display peptide products from cognate antigen on MHC II molecules for subsequent interaction with T cells (Figure 2-02). Unlike the majority of other professional APCs, B cells express HLA-DO, which is thought to inhibit the action of HLA-DM. In the absence of HLA-DO, HLA-DM catalyzes the removal of CLIP from MHCII molecules and replaces them with proteolytically degraded antigens (71). HLA-DO is active at high pH, but is in inhibited by low pH environments, thus serving to focus the loading of MHCII molecules with BCR internalized antigens that have matured into low pH compartments (72). TD antigens are unable to stimulate activation of a B cell because of the inability of this type of antigen to crosslink the BCR. T cell recognition of cognate antigen presented by a B cell on MHCII, results in the expression of CD40L. CD40L a member of the TNF super-family of proteins and is an important cell surface receptor in promoting the activation of APCs. CD40L interacts with constitutively expressed CD40 on the surface of the B cell to induce profound downstream signaling events that result in B cell activation, expression of costimuatory molecules such as CD80/CD86, release of cytokines such IL-6, TNF-alpha (TNFα) and

lymphotoxin, increased survival and increased adhesion molecule expression such as CD58. The production of cytokines and costimulatory molecules, and adhesion molecules acts to further increase the activity of the T cell, which in turn provides a feed forward loop of B and T cell cooperation in activation. Naïve B cells activated in this way undergo affinity maturation, clonal expansion, and differentiation into antibody secreting cells or memory B cells (Figure 2-03).

Development of B cell memory

Memory B cells differ from naïve B cells in many phenotypic and functional ways that may influence subsequent interactions with T cells and modulate T cell responses through antigen presentation. Specifically, memory B cells have qualitatively higher affinity for antigen due to advantageous mutations gained and selected for during the germinal center reaction. In humans, the vast majority of memory B cells express the TNF family molecule CD27 (73). The function of CD27 on B cells has not been fully elucidated, however ligation of CD27 with CD70 in the presence of IL-10 (74, 75) allows for the development of plasma cells. Interestingly the ligand for CD27 (CD70) is only present on activated T cells but not resting T cells (76). Memory B cells, and particularly $CD80^+$ memory B cells increase with age suggesting that $CD80^+$ B cells are not activated (77). Memory B cells are less dependent on T cell help during activation and differentiation given the high capacity of $CD27^+$ memory B cells in differentiating into ASCs in vitro following TLR9 stimulation with CpG (78). In mice, naïve and memory IgG^+ cells are activated to proliferate in response to bystander (TLR or CD40L) activation signals *in vitro*, but specific antigen is required to induce proliferation and

differentiation into antibody secreting cells *in vivo* (79). Additionally, IgG^+ memory B cells did not re-enter or express markers typical of GC reactions indicating that specific antigen induces memory IgG^+ B cells to differentiate directly into antibody secreting cells in mice in vivo (79). This is in contrast to human data in which pathogen-specific memory B cells can be activated and differentiate into antibody secreting cells in response to a *different* pathogen (80). Responsiveness to differentiation into ASC is a major functional difference between naïve and memory B cells; furthermore memory and naïve B cells express differentially regulated chemokines and integrins that allow for differential homing to sites of inflammation. Normal human B cells from the PB migrate more efficiently than T cells across human brain derived endothelial cells, and migration is dependent on VLA-4, LFA1/ICAM, MCP-1, IL-8, and TIMP-1 (81). Importantly the majority of tonsilar VLA-4 expressing B cells are memory and not GC or naïve (82). Memory B cells can rapidly respond to secondary challenge by differentiating into plasmablasts outside of a B cell follicle (83). CXCL9 and CXCL10 are both ligands for CXCR3 and are upregulated in MS CSF compared to normal controls and CXCL10 correlates with leukocyte counts in the CSF (84). CXCR3 is present on a subset of memory B cells but not naïve B cells and is most often found on memory B cells expressing IgG1 in comparison to other isotype expressing cells found in the blood and expression on B cells is induced by IFNγ (85).

Typically, both naïve and memory B cells in the resting state express CXCR4 and are responsive to the chemokine CXCL12, but lose this responsiveness after exposure to CD40L activation (86). In contrast, only a subset of memory B cells, but not naïve B cells express, the chemokine receptor CXCR3 (85). A portion of memory B

cells, but not naïve B cells, express CD80 and another portion is $CD25^+$ (77, 87). $CD80^+$ $CD27^+$ B cells proliferate more rapidly and secrete more Ig than naïve or CD80- memory B cells (77). Both $CD27^+CD80^+$ and $CD27^+$CD80- B cells induced T cell proliferation in response to glatiramer acetate, but $CD27^+CD80^+$ memory B cells induce greater T cell proliferation in response to GA (77).

B-T CELL INTERACTIONS IN THE IMMUNE SYSTEM AND MS

On a per cell basis, DCs more efficiently induce antigen specific T cell proliferation to tetanus toxoid expressed on the cell surface of bacteria (88). Therefore it is generally assumed that in all cases, DCs are far superior APCs when compared to B cells. However, in those cases where the majority of B cells are antigen -specific, B cells can present antigen much more efficiently than dendritic cells or macrophages. For example, in the case of hepatitis B core antigen (HBcAg), a large precursor frequency of naïve B cells that recognize HBcAg can stimulate IL-2 production by T cells in the presence of minute concentrations of HBcAg, but non-B cells can not (89). HBcAg presentation could be inhibited by using anti-mouse Ig Fab fragments or anti-HBcAg antibodies that competed for epitopes recognized by the endogenous B cell receptor pool (89). It is unknown if the precursor frequency of neuro-antigen specific naïve B cells in the context of MS is higher than in healthy donors (HDs). Although B cell antigen presentation is clearly important in these cases where there is a large precursor frequency against the immunizing foreign antigen, other models reveal no need for B cells in priming T cell responses through antigen presentation.

B cell role in the induction of T cell tolerance

Regulatory B cells (B_{regs}) that secrete IL-10 and TGFβ are considered as important as T_{regs} in the maintenance of peripheral immune tolerance and the suppression of detrimental levels of immune activation (90). It is important to understand whether B cells act in a regulatory capacity in MS as they could potentially be targeted for immunotherapy. In addition, the naïve-like phenotype of many B cells shown to be important in immune tolerance, suggests that most naïve B cells may not be effective antigen presenting cells in the promotion of pathogenic T cell responses in MS.

For example, studies involving B cell deficient animals in the induction of EAE demonstrated that B cells were not required for the development of EAE when peptide antigen was used in active induction (3). In fact, B cell deficient mice (91), or mice that have been depleted of B cells prior to active immunization (92) tend to have a more severe course of EAE. The increase in EAE severity is mediated by elimination of an IL-10 producing subset of B cells (92) and has led to the conclusion that B cells are important mediators of immune regulation and the promotion of tolerance.

Further evidence of this tolerance promotion was demonstrated when B cells from BCR transgenic mice (specific for antigen) exposed to intranasal antigen in vivo induced T cell tolerance as measured by the inability of splenic and bronchial lymph node T cells to proliferate in response to Ag *in vitro* following priming (93). Along the same lines, pre-activated/memory T cells, but not naïve T cells, proliferate in response to MBP presentation by resting B cells, however subsequent restimulation of these activated/memory T cells after encounter with resting B cells presenting antigen, resulted in a diminished T cell response (94). However the response of T cells to activated or

memory B cells in this study was not addressed. Unlike CD40 sufficient B cells, resting CD40 deficient B cells cultured with naïve T cells do not induce tolerance (95). Antigen non-specific mouse B cells loaded with peptide (no processing necessary) and incubated with TCR transgenic T cells form immunologically mature synapses induced regulatory T cells in contrast to activated DCs which induce effector T cells (96). However, the conclusion that naïve B cells antigen presentation in general always results in Treg generation is flawed because these B cells did not process antigen and were not activated by recognition of antigen.

Clearly B cells in many contexts can induce T cell tolerance or suppression, but there are many instances where B cells are necessary in the development and promotion of effector T cell responses both in vitro and in vivo.

B cell role in the induction and maintenance of cellular immunity

Perhaps the most relevant, and elegant demonstration that B cells have a role in the promotion of effector T cell responses in RRMS comes from the seminal demonstration that B cell depletion therapy with Rituximab significantly decreased the total number of Gd^+ lesions, decreased the number of new Gd^+ lesions, decreased the volume of T2 lesions and decreased annualized rate of relapse without effecting the mean IgM, IgG, and IgA levels (14). Similarly, depletion of B cells 7 days after active EAE induction in mice resulted in a significant decrease in CNS pathology, decrease in neuro-antigen specific T cell reactivation in vitro, and decrease in clinical EAE score (92). As a group, Rituximab treated RRMS patients show a decrease in both CSF B cells as well as

CSF T cells indicating that B cells (either peripheral or CSF) have a significant role in maintaining T cell infiltration, activation, or presence in the CNS of RRMS patients.

Although these data provide striking evidence that B cells are involved in some way in the promotion of disease relevant T cell responses in MS and EAE, these studies did not address the specific function of B cells in regulating these responses (Figure 2-1). Much evidence has accumulated that would suggest that the important function for B cells in initiating and maintaining relevant $CD4^+$ T cell responses is through antigen presentation. For example, circulating $CD80^+$ B cells are increased during active relapse phases of MS, compared to patients in remission or controls, suggesting that this population may be involved in antigen presentation during the active phase of the disease (97). Prolonged CD40L stimulation enhances B cell antigen presentation through a mechanism dependent on CD80 upregulation (98) indicating that CD80 is an important molecule in enhancement of antigen presentation. Similarly, an increased B cell to monocyte ratio in the CSF is correlated with more rapid disease course in retrospective studies (99). B cells may play important roles in antigen presentation in other human systems as well. For example, allograft rejection is normally T cell mediated, but infiltration of $CD20^+$ B cells has also been associated with renal allograft injection (100), suggesting a role for B cell mediation of T cell rejection.

In animal models, the case for B cell antigen presentation in the promotion of effective T cell responses is much stronger than documented in humans. For example, B cell knockout mice show impaired $CD4^+$ T cell proliferation to a variety of protein antigens but not peptide as DCs preferentially take up peptide antigen and antigen specific B cells preferentially take up whole protein antigens (101). In contrast, SCID

mice engrafted with T cells and immunized with whole protein antigen exhibited no difference in the number of IL-2 producing T cells as compared to SCID mice engrafted with B and T cells (102). Additionally, no difference in T cell proliferation to whole protein antigens between B cell deficient (μMT) and sufficient animals was observed in an additional model (103). Nevertheless, the importance of B cells in the development of T cell memory has been documented by several different groups. For example, adoptive transfer of B cell sufficient bone marrow into mixed bone marrow chimeras (with B cell deficiency) before whole antigen priming, restored T cell proliferation, that was not observed in B cell deficient mixed bone marrow chimeras (104). In another model, following primary infection with lymphocytic choriomeningitis virus (LCMV), LCMV specific $CD4^+$ ($tetramer^+$) are similar in B cell sufficient and deficient mice, but after 154 days, virus specific memory $CD4^+$ T cells are undetectable in B cell deficient mice compared to B cell sufficient mice and $mIgM^+$ mice (105). Similarly, B cells can reconstitute the development of $CD4^+$ memory T cells to KLH adjuvant at similar levels observed in wild type mice (106). In another similar model, $CD8^+$ T cells underwent a larger contraction in number, in B cell deficient mice infected with *L. monocytogenes* compared to B cell sufficient mice (107). A model of T cell tolerance induction is maintained in B cell deficient mice suggesting that B cells are not necessary for the induction of tolerance (103). Similarly, mixed bone marrow chimeras with MHCII deficient B cells failed to clear *pneumocystis carinii* lung infection, and did not develop a sufficient number of memory $CD4^+$ T cells following infection (108). In addition, T cells from *pneumocystis carinii* infected JHD, did not expand, secrete sufficient levels of pro-inflammatory cytokines or infiltrate the lungs in when transferred into SCID mice

infected *pneumocystis carinii* in comparison to transferred T cells from wild type animals (108). In one model however, B cell cytokine secretion rather than antigen presentation provides optimal support for T cell responses (109).

Given the accumulating evidence that B cells can act as both immunoregulatory B_{regs} and as immunostimulatory B effectors, it is the focus of this dissertation to determine the subtypes of B cells that can promote neuro-antigen specific T cell activation through antigen presentation.

Summary

T cells are central mediators of inflammation in MS, and accumulating evidence suggests that T and B cell responses against neuro-antigens are pathogenic and have shown disparities between MS and HDs. The primary target of current immunomodulatory therapies in MS is interference with pathogenic T cells. However, recent evidence suggests a role for B cells in the pathogenesis of MS. B cells have pleotropic functions in the immune system including antibody production, cytokine secretion, and antigen presentation, but the function of B cells that is central to the pathogenesis of MS remains unknown. Therefore the studies presented in this dissertation aim to formally assess the neuro-antigen presenting capacity of B cell subsets in MS.

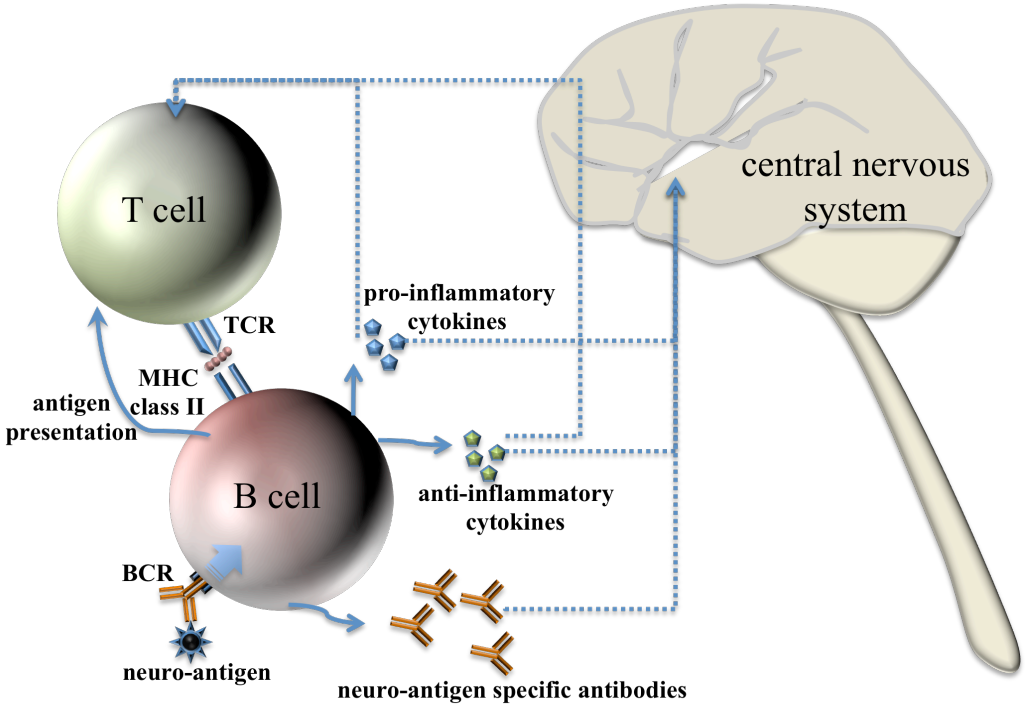


FIGURE 2-01. Potential functions of B cells during the course of MS. The pleotropic functions of B cells can modulate the immune system during the disease course of MS in fundamentally different capacities. The most well studied function of a B cells in MS is the production of auto-antibody. However because a clinically beneficial effect on MS is observed rapidly after treatment with Rituximab, the primary pathogenic role of B cells during the course of MS may likely be through relevant neuro-antigen presentation to $CD4^+$ T cells, and or production of inflammatory cytokines,

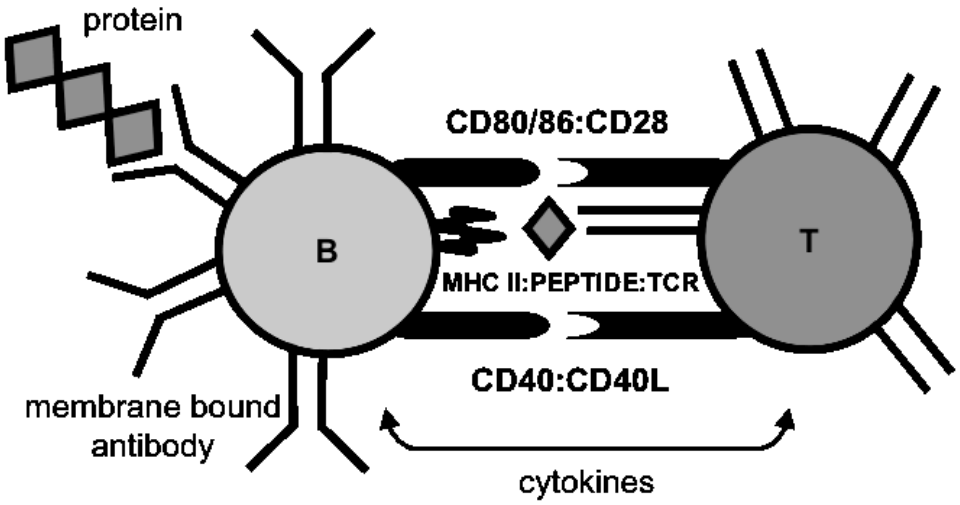


FIGURE 2-02. B-Cell-T-cell interactions following processing and presentation of BCR specific protein. B cells are capable of concentrating minute levels of protein antigen via recognition by antigen specific BCR. Presentation of epitopes derived from protein antigen on MHCII molecule results in cognate recognition by antigen specific T cell through T cell receptor (TCR). Recognition of MHCII:peptide by TCR and costimulation via CD28 interaction with CD80/CD86 results in T cell activation and upregulation of CD40L. CD40L in turn promotes B cell activation, proliferation, and survival of B cell via interaction with constitutively expressed CD40. Feed forward loop of mutual activation is established that is modified by secreted cytokines effecting both autocrine and paracrine activation.

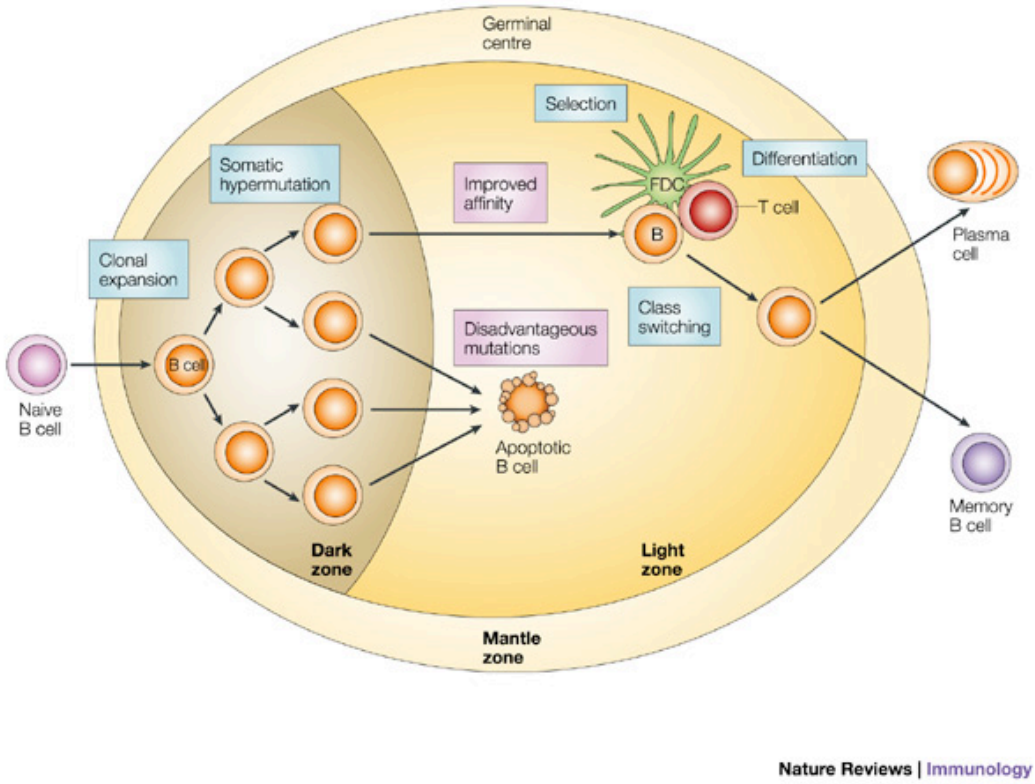


FIGURE 2-03. The germinal center reaction. Naïve B cells activated by antigen specific T cell, differentiate into centroblasts that inhabit the dark zone of a germinal center. Centroblasts are rapidly proliferating B cells undergoing clonal expansion and somatic hypermutation (SHM) of V genes in both heavy and light chains. SHM is mediated by activation induced cytidine deaminase (AID), which targets specific motifs and sequences present in V genes. After several rounds of proliferation and SHM, centrocytes migrate into the light zone, and down regulate genes involved in proliferation. Centrocytes are dependent on additional signals from antigen specific T cells for survival, and therefore centrocytes that have improved affinity for antigen as a result of SHM will receive correct T cell signals for survival. Centrocytes that have accrued disadvantageous mutations that prevent efficient recognition of cognate antigen will die by apoptosis because of lack of additional T cell help. Cytokine profile secreted by T cell in light zone mediates class switching recombination (CSR) of centrocytes. Centrocytes receiving T cell help can differentiate into long-lived plasma cells or memory B cells. Figure reproduced with permission from Macmillan Publishers Ltd: Nature Reviews Immunology, license 2292350932683, originally published in reference (110). Copyright Nature Publishing Group 2002.

CHAPTER THREE
Methodology

3.1.0 Human Samples

3.1.1 Patient Donors

Patients with Multiple Sclerosis (MS) were recruited at the University of Texas Southwestern (UTSW) Medical Center at Dallas in accordance with the UTSW Institutional Review Board. MS patients recruited for studies involving CSF B cell characteristics (Chapter 4) are listed in Table 3-01. Patients recruited for studies involving CSF B cell antibody gene characteristics (Chapter 4) that served as controls for our MS patient population were classified here as patients with other neurological disease (OND). Three of these OND patients had other non-inflammatory neurological diseases unrelated to MS: OND142, OND758 and OND766. Two of the OND patients had inflammatory symptoms not related to MS, and were categorized as other inflammatory neurological disease (OIND) (OND341 and OND658). And finally two OND patients were considered to have clinically isolated syndrome at the time of sampling: CIS132 and CIS681, although CIS132 converted to clinically definite MS 18 months after sampling. Of the 10 MS patients (Table 3-01) and 7 OND patients recruited for studies involving the analysis of B cells antibody gene characteristics from CSF (Chapter 4), 8 MS and 3 OND patients had sufficient CSF B cells to generate antibody gene databases that could be further analyzed for mutation characteristics. As an additional control, we compared the mutational characteristics of CSF B cells from MS and OND patients to a healthy control peripheral blood (HCPB) database consisting of 232 heavy chain rearrangements and 133 kappa chain rearrangements from peripheral blood B cells of two healthy donors.

This HCPB antibody database has been used extensively for comparison purposes in other studies (111-113).

For studies involving the functional analysis of peripheral blood B cells (Chapters 5 and 6), patients that had been diagnosed with clinically definite relapsing remitting MS (RRMS), or had monosymptomatic multiple sclerosis meeting the controlled high-risk subjects Avonex multiple sclerosis prevention study (CHAMPS) criteria (114) under went leukapheresis at three separate time points (TP). Patient characteristics are listed in table 3-02. TP 1 sampling occurred at month zero of the study when patients were treatment naive. Studies described in Chapter 6 utilized cryopreserved samples from leukapheresis TP 1 exclusively, at which point RRMS patients had not used corticosteroids for 60 days prior to leukapheresis and were treatment naïve to disease modifying immuno-modulatory therapies including interferons, monoclonal antibodies, glatiramer acetate, or methotrexate. TP 2 and TP 3 occurred at month 6 and month 12 of the study. Studies described in Chapter 5, utilized cryopreserved samples from leukapheresis TP 2 and 3, at which point patients were undergoing immunomodulatory therapy regimens that included either Interferon-β1a (Avonex), or Interferon-β1a plus mycophenolate mofetil (Cellcept). All RRMS patients had at least one exacerbation in the preceding two years but not within 60 days prior to TP 1 leukapheresis sampling. None of the RRMS patients included in this study had symptoms suggestive of primary progressive, secondary progressive, or progressive relapsing MS. Three additional MS patients samples were used for studies involving activated B cell antigen presentation (Chapter 5, Table 3-02). These patient samples were used in the examination of CD40L/IL-4 induced B cell expansion but not as activated

APCs in B-T culture experiments. These MS patients consisted of 2 females and 1 male between the ages of 35 and 53 years old. All 3 of these patients had secondary progressive MS.

3.1.2 Healthy Donors

White blood cell filter bags derived from approximately 500 mL of individual whole blood donation were purchased from Carter Blood Care (Bedford, TX, USA) for use as healthy controls for studies involving ex vivo B cell antigen presentation (Chapter 6). Blood donations were evaluated by Carter Blood Care and found to be negative for the presence of Hepatitis B surface antigen, Hepatitis C antibody, Hepatitis B core antibody, Human Immunodeficiency Virus (HIV)-1 antibody, HIV-2 antibody, HIV-O antibody, Human T-lymphotorophic Virus type I and II antibodies, West Nile Virus, and Syphilis. Additional healthy control subject leukaphereses were purchased from Lonza (Walkersville, MD, USA) and used in studies involving activated B cell antigen presentation (chapter 5). Lonza leukapheresis consisted of mononuclear cell enriched peripheral blood obtained from automated apheresis from single donors screened for general health and found to test negative for HIV-1, hepatitis B core, and hepatitis C antibodies. Leukapheresis samples from Lonza used in these studies included 3 females and 2 males between the ages of 33 and 64.

3.2.0 Preparation of cell populations from human samples

3.2.1 Isolation of peripheral blood mononuclear cells (PBMC) from leukapheresis

White blood cell filter bags (Carter Blood Care; Bedford, TX, USA), leukapheresis bags (Lonza; Walkersville, MD, USA) or leukapheresis bags obtained from UTSW facilities were ascetically sprayed with 70% ethanol after which a small cut was made a the top of the bag using sterile scissors to create an opening for pouring out white blood cell enriched blood (WBCE). WBCE blood was poured into 50 mL conical tube(s); typically a volume of approximately 60 mL was recovered from each white blood cell filter bag. WBCE blood was diluted 1:1 with phosphate buffered saline (PBS), and gently overlayed onto Ficoll Plus (GE Healthcare Life Sciences, Uppsala, Sweden) to create a discontinuous gradient, at a ratio of 2:1; diluted WBCE blood to Ficoll Plus respectively, in a 50 mL conical tube. 50 mL conical tubes were then centrifuged at 1800 rotations per minute (RPM) (650 x gravity (g)) for 25 min at 22°C with no brake. Buffy coat (layer of white mononuclear cells on top of Ficoll Plus) was removed by pipetting and placed into new 50 mL conical tube. The pooled PBMCs were then diluted 1:2 with PBS and centrifuged for 10 minutes at 1800 RPM (650 x g) at 22°C. Supernatant was then discarded by pouring off, or removal by vacuum pipette. The cell pellet was then resuspended by vortexing gently, and brought up to a volume of 50 mL with PBS, followed by centrifugation for 10 minutes at 1000 RPM (200 x g) at 25°C to remove platelets. Supernatant was again discarded, and the PBMC pellet resuspended by gentle vortexing. The PBMC pellet was brought up to a volume of 50 mL, and an aliquot of 10 μL of the cell suspension was removed and placed into a microcentrifuge tube. 90 μL of a 0.04% Trypan Blue (Sigma, St. Louis, MO, USA) solution was mixed with 10 μL aliquot of PBMC suspension by pipetting to create a 10X dilution. 10 μL of this Trypan Blue:PBMC suspension mixture was placed into the chamber of an improved Neubauer

hemocytometer (Hausser Scientific, Horsham, PA, USA) and placed under a light microscope using 100X magnification. PBMC were counted in 4 of the large grids of the counting matrix and the total number of cells were calculated using the formula ((number of cells counted)/(number of large squares counted)) x (dilution factor) x (volume of cell suspension in mL) x 10^4, which provides a cell count in cells/mL. The PBMC suspension in the 50 mL conical tube was then centrifuged at 1000 RPM (200 x g) for 10 minutes at 25°C to pellet PBMCs. The supernatant was discarded and the PBMC pellet resuspended to a concentration of 5-10x10^7 PBMC/mL in ice-cold Human Freezing Media (Table 3-03) by pipetting. 1 mL aliquots of the PBMC suspension were added to cryovials (Wheaton, Millville, NJ, USA), placed into "Mr. Frosty" Cryo1°C Freezing Containers (Nalgene, Rochester, NY, USA), and stored overnight in -80°C freezer. Cryovials containing frozen PBMCs were removed from -80°C freezer and placed into liquid nitrogen for long-term storage.

3.2.2 Thawing of PBMCs from cryostorage

Cryopreserved PBMC samples frozen in cryovials and stored at less than -130°C in liquid nitrogen (LN) vapor freezer were removed from storage and immediately placed in 37°C water bath for rapid thawing. If many samples were removed from LN storage at one time, cryovials were placed on ice and transported to water bath to prevent slow thawing. Once in water bath, cryovials containing samples were swirled by hand to increase the rate of thawing and to distribute heat evenly to the sample. Prior to complete thawing of the samples, cryovials were removed from water bath and sprayed with 70% ethanol to prevent contamination. Once thawed, samples were transferred with 2 mL

pipette (BD Falcon, Franklin Lakes, NJ, USA) drop by drop into ice cold human media (Table 3-03) at ratio of 1:10, sample:media in either a 15 mL or 50 mL conical tube (BD Falcon, Franklin Lakes, NJ, USA) depending on the final volume of the dilution. Conical tubes containing samples were then centrifuged at 1200 RPM (300 x g) for 7 minutes at 4°C. Supernatant was removed by vacuum aspiration leaving a small amount of media. The cell pellet was resuspended in a small volume of the remaining media by vortexing. The volume of the cell suspension was then brought up to 10 mL with ice cold BSA Buffer (Table 3-03), and centrifuged at 1200 RPM (300 x g) for 7 minutes at 4°C. The supernatant was removed by vacuum aspiration leaving a small amount of buffer for pellet resuspension. The cell pellet was resuspended in small amount of remaining buffer by vortexing, and brought up to 10 mL with ice cold BSA Buffer. Cells were then counted as described in section 3.2.1, and samples stored on ice until used in downstream applications.

3.2.3 Isolation of purified T cells from PBMCs

T cells were typically isolated from PBMC by magnetic negative selection using the Human T Lymphocyte Enrichment Set-DM (BD Biosciences, San Jose, CA, USA), as illustrated in Figure 3-01. This set contains anti-human antigen monoclonal antibodies to CD11b/Mac-1 (clone ICRF44), CD16 (clone 3G8), CD19 (clone HIB19), CD36 (clone CB38), CD41a (clone HIP8), CD56 (clone B159), CD235a (clone GA-R2) each conjugated to biotin; herein referred to as “enrichment cocktail.” Streptavidin particles plus-DM are included in the T lymphocyte enrichment set and contain streptavidin

conjugated to magnetic nanoparticles and are herein referred to as "SA-particles". PBMCs from single donors were resuspended in BSA Buffer (Table 3-03) at a concentration of $1x10^7$ cells /mL to which 50 μL per $1x10^7$ cells of enrichment cocktail was added and incubated in a stationary rack at 4°C for 15 minutes. Cell suspension was then brought up to a 10-fold excess volume with BSA Buffer and centrifuged at 1200 RPM (~300 x g) for 7 minutes at 4°C. As much supernatant was removed as possible without disturbing the cell pellet by vacuum aspiration. The cell pellet was resuspended in 50 μL per $1x10^7$ cells of SA-particles by pipetting up and down, transferred to a 12 x 75 mm round-bottom polystyrene tube (BD Falcon, Franklin Lakes, NJ, USA) and allowed to incubate in a stationary rack at 4°C for 30 minutes. BSA Buffer was then used to dilute the cell suspension to a concentration of $5x10^7$ cells/mL. The tube was placed into a BD IMAGnet™ and incubated at room temperature for 8 minutes. Using a 2 mL serological pipette (BD Falcon, Franklin Lakes, NJ, USA), the negative fraction (T cell enriched fraction; free-floating in the tube) was removed from the tube using a sterile pipette and placed in new sterile 12 x 75 mm round-bottom polystyrene tube. The original tube still in the IMAGnet (now containing only the positive fraction, which had adhered to the tube wall while placed in the IMAGnet™) was then removed from the BD IMAGnet and the remaining cells resuspended by pipetting up and down at least 10X with a volume of BSA Buffer equal to the volume used in the previous step. The tube was then placed back on the BD IMAGnet™ for another 8 minutes, and the negative fraction (T cell enriched fraction; free floating in the tube) was removed from the tube using a sterile pipette and pooled with the first T cell enriched fraction collection. The tube containing the pooled T cell enriched fraction was then centrifuged at 1200 RPM for

7 minutes at 4°C. Supernatant was removed by vacuum aspiration and the cell pellet resuspended in 400 µL of BSA Buffer by pipetting up and down at least 10 times. The tube was then placed back on the BD IMAGnet™ for 8 minutes. The negative fraction (again, free floating in the tube) was then removed with a 2 mL pipette and placed in a fresh tube. This twice enriched T cell fraction was typically more than 95% pure CD3+ T cells as measured by flow cytometric evaluation, and included both $CD4^+$ and $CD8^+$ T cells. Cells were counted as described in section 3.2.1.

In some cases, T cell enrichment was accomplished using the EasySep® Human T cell enrichment Kit (StemCell Technologies, Vancouver, BC, CA), illustrated in Figure 3-02, with comparable or superior results to the Human T Lymphocyte Enrichment Set-DM. In this protocol, non-T cells were removed from PBMC by positive selection with bi-pecific tetrameric antibody complexes (BTAC) which are directed against the cell surface antigens CD14, CD16, CD19, CD56, glycophorin A as well as dextran. After PBMCs have been labeled with BTAC, the cell suspension is then incubated with magnetic nanoparticles coated with dextran, which allows labeled cells to be removed in a magnetic field. PBMCs from single donors were diluted to a concentration of 5×10^7 cells/mL in BSA Buffer (Table 3-03) in a 12 x 75 mm polystyrene round bottom tube (BD Falcon, Franklin Lakes, NJ, USA) in no more than 2 mL total volume. EasySep® Negative Selection Human T Cell Enrichment Cocktail was then added to the cell suspension at 50 µL cocktail/mL cells and mixed by pipetting up and down several times. The cell suspension was then allowed to incubate for 10 minutes at room temperature (approximately 25°C). Dextran coated magnetic nanoparticles were then added at a

concentration of 100 µL/mL cells, and mixed by pipetting up and down. The cell suspension was then incubated at room temperature for 10 minutes. The cell suspension was then brought up to a total volume of 2.5 mL with BSA Buffer and mixed by pipetting up and down, and placed on the EasySep® Magnet for 5 minutes. The negative fraction (enriched T cells; free-floating in the tube) was then poured off into a fresh 12 x 75 mm polystyrene tube. The tube now contained only the positive fraction adhered to the side of the tube and was taken off the magnet. The positive fraction cells were then resuspended by pipetting up and down with 2.5 mL of BSA Buffer, and placed back on the EasySep® Magnet for an additional 5 minutes. The negative fraction (enriched T cells; free-floating in the tube) was again poured off into the 12 x 75 mm polystyrene tube containing the negative fraction from the previous step. The total pooled negative fraction was then centrifuged for 1500 RPM (450 x g) for 5 min at 25°C. The supernatant was discarded by vacuum aspiration and the cell pellet was resuspended in 2.5 mL of BSA buffer by pipetting up and down. The cell suspension was then placed back into the EasySep® magnet for 5 minutes. The negative fraction (twice enriched T cell fraction) was then poured off into a fresh tube. The twice enriched T cell fraction was then counted as described in section 3.2.1. Approximately 100,000 cells were placed in a new 12x75 mm polystyrene tube for staining with CD3-allophycocyanin, CD14-FITC, and CD19-PE/Cy5 for assessment of T cell purity by flow cytometry.

3.2.4 Isolation of human B cells from PBMC by magnetic positive selection for use in B cell expansion and activation protocols

Human B cells were isolated from PBMC preparations using CD19 Magnetic Particles-DM (CD19-DM, BD Biosciences, San Jose, CA, USA) in conjunction with the IMAGnet™ cell separation magnet (BD Biosciences). CD19 Magnetic Particles-DM consist of a monoclonal antibody that recognizes CD19 conjugated to a magnetic particle that is used for magnetic isolation on the IMAGnet™. Both the 4G7 (mouse IgG1,κ) and HIB19 clones (mouse IgG1,κ) used in two separate preparations of the CD19-DM reagent to obtain viable B cells, however, the 4G7 clone provided superior recovery when compared to HIB19, and was used whenever possible. PBMCs thawed from cryopreserved samples were resuspended in 10 mL of BSA Buffer (Table 3-03) in a 50 mL conical tube (BD Falcon, Franklin Lakes, NJ, USA) and counted as described in section 3.2.1. Typically, an aliquot of 100 million PBMC were removed, placed in a fresh 50 mL conical tube and centrifuged at 1200 RPM (300 x g) for 7 minutes at 4°C to pellet PBMCs. After centrifugation, as much supernatant as possible was removed by vacuum aspiration. CD19-DM particles were added to the PBMC pellet at a concentration of 50 μL per $1x10^7$ PBMCs, and resuspended in particle solution by pipetting up and down. The PBMC:CD19-DM suspension was then incubated in a stationary rack at 4°C in a refrigerator (not on ice) for 30 min. At this point in the protocol, B cells were magnetically enriched with two separate procedures; one that maximized B cell purity and was used exclusively for the experiments described in Chapter 4; and one that maximized B cell recovery and reduced B cell contamination in the B cell depleted PBMC fraction. These procedures are illustrated graphically in Figure 3-03. Human B cells isolated by positive magnetic selection via CD19, as done here, remain un-activated and in a quiescent state, and retain functional properties similar to B cells isolated by

negative magnetic selection (115).

Procedure for maximizing B cell purity

PBMC:CD19-DM particle suspensions were then brought up to a concentration of $5x10^7$ cells/mL with BSA buffer (Table 3-03). Total volume of PBMC-CD19-DM particle suspensions were then removed from the 50 mL conical tube and added to a fresh 12x75 mm polystyrene tube (BD Falcon, Franklin Lakes, NJ, USA). This tube was placed on a BD IMAGnet™ (BD Biosciences, San Jose, CA, USA) and incubated for 10 minutes at 25°C (Figure 3-03). During this incubation period, cells that bound CD19-DM particles are drawn to the magnet-facing wall of the 12x75mm polystyrene tube and adhere to the tube wall. Suspension containing non-labeled cells (free floating non-wall bound), was then removed by aspiration with 2 mL sterile pipette (BD Falcon, Franklin Lakes, NJ, USA) and discarded. The 12x75mm polystyrene tube inside the IMAGnet was then removed from the magnet, and the magnetically selected B cells that had adhered to the tube wall were resuspended in BSA Buffer (Table 3-03) in a volume equal to the volume used in the previous step by pipetting up and down at least 10 times, and placed back on the IMAGnet™ once more for 4 minutes at 25°C. During this incubation period, CD19-DM particle labeled cells are again magnetically drawn to the 12x5mm polystyrene tube wall and adhere. The suspension containing unlabeled cells (free floating, non-wall adhered) was again removed by aspiration with a 2 mL sterile pipette and discarded. The previous two steps were then repeated. Magnetically selected B cells that had adhered to 12x75mm polystyrene tube wall were then resuspended in 5 mL of ice-cold Human Media (Table 3-03) and placed on ice. B cells that have been selected in

this manner are referred to as "magnetically enriched B cells." Live cells were then counted as indicated in section 3.2.1.

Procedure for maximizing B cell recovery, and minimizing B cell contamination in B cell depleted fraction

This procedure was only used in cases where magnetically enriched B cells would be later sorted to purity by FACS. A sterile LD column (capacity: $2x10^8$; Miltenyi Biotec, Bergisch Gladbach, Germany) was placed into an aseptically treated (70% ethanol sprayed) MidiMACS™ magnet (Miltenyi Biotec, Bergisch Gladbach, Germany) that had been mounted onto a MACS®MultiStand (Miltenyi Biotec, Bergisch Gladbach, Germany) and primed with 2 mL of BSA Buffer, by adding BSA Buffer to the top of the column. BSA buffer used during priming was allowed to elute from LD column by gravity, and was collected in a 12x75 polystyrene tube (BD Falcon, Franklin Lakes, NJ, USA). Eluate tube from LD column priming was discarded after BSA Buffer had been allowed to drain through by gravity, and a fresh 12x75 polystyrene tube was placed under the LD column for collection. After a 30-minute incubation with CD19-DM particles (described earlier), PBMC suspensions were brought up to a volume of 500 µL with BSA Buffer, loaded onto the top of the primed LD column, and allowed to pass into the column by gravity. After the PBMC suspension had completely absorbed into the LD column, 1.0 mL of BSA Buffer was loaded on top of the LD column to wash out non-labeled cells. The negative eluate fraction (PBMCs depleted of B cells) was collected in a fresh 12x75 mm polystyrene tube. After the first wash had completely run through the LD column, a second wash of 1.0 mL of BSA Buffer was loaded on top of the LD

column. The negative eluate fraction was again collected. Finally, the LD column was removed from MidiMACS™ magnet and placed on top of a fresh 12x75 mm polystyrene tube. 5 mL of fresh BSA Buffer was applied to the top of the LD column and the positive fraction of B cells was eluted from the column by application of steady pressure using a sterile plunger included with the LD column. Live B cells were then counted as described previously in section 3.2.1.

3.2.5 Sorting of B cells into naïve and memory B cell populations

Magnetically enriched B cells that had been resuspended in BSA buffer and counted as described in section 3.2.1 were centrifuged at 1500 RPM (450 x g) for 5 min at 4°C to pellet the cells. The majority of the supernatant was discarded by vacuum aspiration leaving approximately 100 μL. The cell pellet was resuspended in the remaining supernatant by vortexing. 2 μL of CD19-PECy5 and 2 μL CD27-PE were added per million cells (BD Biosciences, San Jose, CA, USA), and incubated on ice for 15 minutes. Approximately 4.5 mL of ice cold BSA buffer was added to the labeled cells, and centrifuged at 1500 RPM (450 x g) for 5 min at 4°C. The supernatant was discarded by vacuum aspiration, and the cell pellet resuspended in 500 μL of BSA Buffer and placed on ice until sorting. Stained B cells were brought to the UTSW Flow Core facility and sorted into live naïve (CD19+CD27-) and memory (CD19+CD27+) B cell populations on a FACSAria™ (BD Biosciences, San Jose, CA, USA) based on FSC x SSC characteristics and fluorescence intensity (see Figure 3-04 for an example of gating

strategy). Naïve and memory B cells were collected into 12x75 polystyrene tubes in a 4°C cooled chamber containing 1 mL of BSA Buffer each (Table 3-03). Cell counts obtained during sorting procedure were used to calculate the total number of naïve and memory B cells in the sorted populations. Sorted B cell populations were then centrifuged at 1500 RPM (450 x g) for 5 min at 4°C. Supernatants were removed from cells by vacuum aspiration and cell pellets from identical populations were pooled. The cell pellets from sorted populations were then resuspended in 3 mL of human media and centrifuged again at 1500 RPM (450 x g) for 5 min at 4°C. Supernatants were removed from cells by vacuum aspiration and cell pellets resuspended in appropriate volumes of human media for use in subsequent applications.

3.2.6 Labeling of whole PBMCs, B cell depleted PBMCs, and T cells with CFSE to track proliferation

To track cellular proliferation by CFSE dilution, whole PBMCs, B cell depleted PBMCs or purified T cells were labeled using carboxyflouro-diacetate, succinimidyl ester (CFDA-SE; Invitrogen) according to manufacturers instructions and (116). Cells were resuspended at a concentration of $5x10^7$ cells/mL at room temperature in PBS. A 10 mM stock solution of CFDA-SE was prepared in 100% dimethylsulfoxide (DMSO) and aliquoted at 10 μL/tube and stored at -20°C. This stock preparation can be stored for several months without significant detectable loss in activity but must not be thawed multiple times. Stock CFDA-SE was thawed and diluted 1:100 in PBS to create a 100 μM working solution. CFDA-SE working solution was added to cell suspension to create a final concentration of 10 μM CFDA-SE in cell suspension. Cell suspensions were then

incubated at 25°C for 5 minutes. To quench excess un-reacted CFDA-SE, human media was added at a 10X excess volume of labeling reaction volume. The cells were then centrifuged at 1500 RPM (450 x g) for 5 minutes at 4°C. The supernatant was removed by vacuum aspiration and the cell pellet resuspended in 5 mL of human media (Table 3-03) and the cells counted as outlined in section 3.2.1. CFSE labeled cells were again centrifuged at 1500 RPM (450 x g) for 5 minutes at 4°C. The supernatant was removed by vacuum aspiration and the cell pellet resuspended in the appropriate volume of human media required to obtain cell concentrations for subsequent applications.

3.3.0 Generation and analysis of V gene database

3.3.1 Generation of V gene database

Data contained in V gene sequence databases were created from single B cell repertoires derived from the CSF of MS patients listed in Table 3-01, 2 OND patients and 2 HDs, using techniques described elsewhere by our laboratory (117-119). Bench work to obtain these sequences was done by other personnel in the laboratory as described elsewhere (117-119).

3.3.2 Analysis of V_H, V_κ and V_λ sequences

Electropherogram files detailing nested PCR antibody product sequencing generated by the UT Southwestern Medical Center sequencing core were visualized on FinchTV Software (Geospiza, Seattle, WA, USA) or Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were compared manually to germ line V_H, V_K, and V_L sequences listed in IMGT®, the international ImMunoGeneTics

information system® (http://www.imgt.org, (120)). Heavy chain D segments were identified using the Junction Analysis Tool (121). Comparison of sequence variation and mutational characteristics between naïve and memory CSF B cells was not addressed here since cells were sorted based on CD19 expression alone which allowed for direct comparisons to HCPB B cell repertoires.

V_H and V_κ family frequencies were only calculated for those CSF B cell repertoires that consisted of more than 15 rearrangements. Clonally expanded CSF B cells are defined as those single B cells whose heavy and light chain rearrangements were represented two or more times in the repertoire. The non-clonal CSF B cell population is defined as those single B cells whose heavy and light chain rearrangements were not represented more than once in the repertoire. Inclusive CSF B cell populations (which were analyzed here) consist of both non-clonal and clonal CSF B cell immunoglobulin rearrangements. Heavy chain CDR3 regions include the entire D_H segment and J_H segment through codon 102. CDR3 regions may include up to 2 nucleotides from the V_H segment to allow for in frame analysis. The net charges of heavy chain CDR3 regions were analyzed using Lasergene software (DNASTAR Inc., Madison, WI) to translate nucleotide sequence into amino acid sequence and to determine total average.

Heavy chain CDR3 regions include the entire D_H segment and J_H segment through codon 102. CDR3 regions may include up to 2 nucleotides from the V_H segment to allow for in frame analysis. The net charges of heavy chain CDR3 regions were analyzed using Lasergene software (DNASTAR Inc., Madison, WI) or using a web based analysis program (ProteinCalculator v3.3, www.scripps.edu/~cdputnam/protcalc.html) to translate nucleotide sequence into amino acid sequence and to determine total average

charge of the heavy chain CDR3 translated region at pH 7.

3.4.0 Activation of B cells for use as APCs

3.4.1 Culture and maintenance of NIH-3T3 cells expressing hCD40L required for the B cell CD40L activation studies

NIH-3T3 is an immortalized cell line derived from Swiss mouse fibroblasts and can be cultured indefinitely using proper cell culture techniques as described here. NIH-3T3 cells expressing membrane human CD40L ($CD40L^+$3T3 cells, a kind gift of Gordon Freeman) were originally produced through stable transfection by means of electroporation of a linearized plasmid containing the coding region of hCD40L derived from cDNA amplified from activated human T cells, as well as a neo cassette (neomycin phosphotransferase), enabling for selection with the antibiotic G418 and ability to co-stimulate human B cell proliferation in conjunction with IL-4 (122).

$CD40L^+$3T3 cells were cultured according to (123) and personal communication from Gordon Freeman. $CD40L^+$3T3 cells were cultured in G418 Media containing F12/DMEM media (complete contents listed in Table 3-03) at 37°C in an H_2O humidified, 5% CO_2 atmosphere, NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA) in either T25 or T75 Flasks with gas permeable caps (Nalgene, Rochester, NY, USA) and placed in the horizontal position, as $CD40L^+$3T3 cells are an adherent cell type. DMEM or Isocove's modification of DMEM can be substituted for the use of F12/DMEM in the G418 media without noticeable differences in growth or morphology of the 3T3 cells; however 3T3 cells do not grow

well in media containing human serum. To passage 3T3 cells, cell culture media was aspirated from the culture flask and 3 mL of 1x Trypsin-EDTA (Cellgro Mediatech; Manassas, VA, USA) added to each flask and incubated in the horizontal position for approximately 3 minutes at 37°C in a humidified incubator. After 3T3 cells were disassociated from the flask surface, 9 mL of G418 Media was added to quench enzymatic activity of Trypsin; then the entire solution contents transferred to a 15 mL sterile conical tube. $CD40L^+3T3$ cells were centrifuged at 1500 RPM for 5 minutes. 3T3 cells were counted as described in section 3.2.1, and reseeded into new flasks at a density of $1x10^3$-$1x10^4$ cells/cm^2 with G418 Media. 3T3 cells were passaged every 3-4 days to maintain cells at less than 90% confluency. When culturing $CD40L^+3T3$ cells with human lymphocytes, special care was taken to ensure the removal of G418 media, as G418 is toxic to human lymphocytes.

3.4.2 Culture and expansion of activated B cells for use as APCs using the IL-4/CD40L system

On day -1, $CD40L^+3T3$ cells were disassociated from flasks using trypsin-EDTA as described in section 3.2.1 and washed once in G418 media (Table 3-03). $CD40L^+3T3$ cells were then irradiated with 96 Gy using a ^{137}Cs-source. $CD40L^+3T3$ were then washed once more with G418 media, and plated into 6-well or 24-well plates (Costar) at a density of $9x10^4$ cells/cm^2. Irradiated $CD40L^+3T3$ were allowed to adhere to the plate overnight in an H_2O humidified, 37^o, 5% CO_2 atmosphere NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA). Irradiated $CD40L^+3T3$ for human B cell culture can be used at most, up to 72 hours after irradiation.

On day zero, supernatant of cultured irradiated $CD40L^+3T3$ feeder cells was removed by vacuum aspiration, and magnetically enriched B cells in human media (Table 3-03) were added on top of $CD40L^+3T3$ culture monolayer at a density 1×10^6 cells/mL (4 mL per well for 6 well plates, and 1 ml/well for 24 well plates) along with 10 ng/mL recombinant human IL-4 (IL-4; R&D Systems, Minneapolis, MN, USA) and cultured at 37°C in 5% CO_2 atmosphere in a NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA). IL-4 was purchased lyophilized and was resuspended at a concentration of 20 μg/mL in PBS to make stock solutions. Stock solutions of IL-4 were stored in aliquots of 100 μL at -80°C as IL-4 can lose activity if stored at -20° (personal communication from Amy Lovett-Racke). Frozen stock aliquots of IL-4, in general, were thawed and re-frozen once; multiple thaw freeze cycles were avoided.

At day 5 under CD40L/IL-4 culture conditions, non-adherent cells were resuspended by pipetting up and down several times. Non-adherent cells were counted as described in section 3.2.1. The cell suspension was then centrifuged at 1500 RPM (450 x g) for 5 min at 25°C. Supernatant was removed by vacuum aspiration and the cell pellet resuspended at a density of 7.5×10^5 cells/mL with refreshed human media (Table 3-03) containing 10 ng/mL of IL-4. The cell suspension was re-layered onto fresh irradiated $CD40L^+3T3$ feeder cells adhered to tissue culture plates once the supernatant from the feeder cells was removed by vacuum aspiration.

At day 8, non-adherent cells in CD40L/IL-4 culture conditions were resuspended by pipetting, counted and either restimulated under the CD40L/IL-4 culture conditions as done at day 5 or used as APCs in subsequent experiments. This re-stimulation protocol

can induce measurable proliferation of B cells indefinitely, however prolonged stimulation was avoided by culturing B cells for a maximum of 16 days under these conditions.

3.4.3 Assessment of B cell activation and proliferation in CD40L/IL-4 culture conditions

During the 5–16 days of CD40L/IL-4 B cell culture, B cells were periodically assessed for purity and activation status by staining with monoclonal antibodies. An aliquot of 300,000 cells was removed from CD40L/IL-4 or CpG ODN/IL-2 B cell cultures for staining. Typically these aliquots were taken at time-points that coincided with restimulation of CD40L/IL-4 or CpG ODN/IL-2 cultures; at day 5, day 8, day 12, and day 16. Each cell aliquot was transferred to a 12x75mm polystyrene round bottom tube (BD Falcon, Franklin Lakes, NJ, USA), brought up to 300 μL in Blocking Buffer (Table 3-03) and allowed to incubate on ice for 10 minutes. 300 μL of the cell suspension was then split into three aliquots of 100 μL each into separate 12x75mm polystyrene round bottom tubes and labeled “Tube 1”, “Tube 2” and “Tube 3”. Tubes containing B cell aliquots were then stained with the following antibody master mixes at concentrations recommended by the manufacturer: Tube 1) FITC-CD14, PE-CD11c, PECy5-CD19, APC-CD3; Tube 2) FITC-CD80, PE-CD27, PECy5-CD19, APC-HLA-DR; and Tube 3) FITC-IgD, PE-CD138, PECy5-CD19, APC-IgM. All antibodies were obtained from BD Biosciences (Table 3-04). Cells were incubated on ice for 15-30 minutes. To each tube, 4.5 mL of ice-cold FACS Buffer (contents listed in Table 3-03), was added to remove unbound antibody. Staining tubes were then centrifuged at 1500

RPM for 5 minutes at 4°C. Supernatant was removed by vacuum aspiration and the cell pellet was resuspended and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA). 10,000 events from the 3 staining tubes were collected on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA).

3.4.4 Culture and expansion of activated B cells for use as APCs using the CpG ODN/IL-2 system

On day zero, magnetically enriched B cells from single donors were cultured in human media along with 5 μg/mL of the class-B CpG-oligodeoxynucleotide 10103 (CpG-ODN; 5′-T**CG**T**CG**TTTTT**CG**GT**CG**TTTT-3′, Coley Pharmaceuticals, Wellesley, MA, USA), and 20 U/mL recombinant human Interleukin-2 (IL-2; Invitrogen, Carlsbad, CA, USA) in round bottom 96-well plates (Costar Corning, Corning, NY, USA) at a density of 5×10^6 cells/mL in 200 μL/well and cultured at 37°C in 5% CO_2 atmosphere in a NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA). CpG-ODN was purchased lyophilized and resuspended at a stock concentration of 365 μg/mL in PBS (Cellgro Mediatech, Manassas, VA, USA) and stored in 100 μL aliquots at -20°C. Frozen stock aliquots of CpG-ODN, in general, were thawed and re-frozen a maximum of once, or kept at 4°C after thawing for a maximum of 2 weeks. Storage at 4°C is possible because CpG-ODN has a nuclease resistant phosphorothioate backbone.

IL-2 (Invitrogen, Carlsbad, CA, USA) was purchased lyphophilized, and resuspended in 100 mM acetic acid at a concentration of 100 μg/mL and stored in 10 μL aliquots at -20°C. Frozen stock aliquots of IL-2, in general, were thawed and re-frozen no

more than twice. At day 5 under CpG-ODN/IL-2 culture conditions, B cells from each well were resuspended by pipetting up and down several times and pooled into one 15 mL conical tube. Cells were counted as described in section 3.2.1. The cell suspension was then centrifuged at 1500 RPM (450 x g) for 5 min at 25°C. Supernatant was removed by vacuum aspiration and the cell pellet resuspended at a density of 5×10^6 cells/mL with refreshed human media containing 20 U/mL of IL-2 and 5 µg/mL of CpG-ODN and added to round bottom 96-well plates at 200 µL/well and cultured at 37°C in 5% CO_2 atmosphere incubator.

On day 8, CpG-ODN/IL-2 cultured B cells were resuspended by pipetting up and down several times and pooled into one 15 mL conical tube. Cells were then centrifuged at 1500 RPM for 5 min at 25°C. Supernatant was removed by vacuum aspiration and the cell pellet resuspended in 5 mL of human media. The previous step was then repeated as an additional wash to remove excess IL-2 and CpG-ODN. Cells were then resuspended in human media and used as activated APC in B-T culture experiments.

3.5.0 T cell co-culture with activated B cell APCs

3.5.1 Assay for CD40L/IL-4 or CpG-ODN/IL-2 activated B cell APC function: culture with autologous resting T cells

CD40L/IL-4 or CpG ODN/IL-2 activated B cells were irradiated with 15 Gy of radiation with a ^{137}Cs-source to maintain expression of activation markers and simultaneously prevent proliferation of B cells. Purified T cells, obtained as described in section 3.2.3 were left unlabeled or stained with CFSE as described in section 3.2.6. CFSE labeled T cells or unlabeled T cells were placed in 96-well round bottom plates

with either CD40L/IL-4 activated B cells, or CpG ODN/IL-2 activated B cells at T cell:B cell ratio of 1:2 ($2x10^5$:$4x10^5$ cells) or 4:1($4x10^5$:$1x10^5$ cells) with either no exogenous antigen, or with a final concentration in culture of 2 μg/mL gamma-irradiation-inactivated Mumps grade 2 antigen (Mumps; Microbix Biosystems Inc., Toronto, Ontario, Canada), 10 μg/mL human myelin basic protein (hMBP; Sigma, St. Louis, MO, USA), 10 μg/mL glatiramer acetate (GA; Teva Neuroscience, Kansas City, MO, USA), or both 10 μg/mL hMBP and 10 μg/mL GA. Mumps was prepared by manufacturer in 1969B Buffer at 1.3 mg/mL, and stored at -20°C in multiple aliquots (100 μL per aliquot), which were freeze thawed multiple times. Human MBP (hMBP) was received from the manufacturer lyophilized, and resuspended with PBS (Cellgro Mediatech, Manasass, VA, USA) to a concentration of 1 mg/mL, and stored in 100 μL aliquots at -20°C, which were freeze-thawed no more than twice. Research grade GA was received from the manufacturer (TEVA Neuroscience) lyophilized and responded at a concentration of 5 mg/mL in PBS (Cellgro Mediatech, Manassas, VA, USA), stored in 0.1-1 mL aliquots at -20°C, and freeze thawed multiple times. In some cases, bovine myelin basic protein (bMBP, Sigma, St. Louis, MO, USA) was substituted for hMBP with similar results. Each condition was set-up in duplicate or triplicate wells in 200 μL final volume of Human Media (Table 3-03). Cultures were placed at 37°C in an H_2O humidified, 5% CO_2 atmosphere NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA), and cultures containing unlabeled T cells were allowed to incubate for 48 hours, while cultures containing CFSE labeled T cells were allowed to incubate for 7 days.

3.5.2 Examination of T cells subsets for CD69 expression in responses to CD40L/IL-4 or CpG-ODN/IL-2 activated B cell antigen presentation

Cultures were set up as described in section 3.5.1 and contained unlabeled T cells, which were harvested at 48 hours. Harvesting entailed pipetting up and down to resuspended cells, followed by pooling of duplicate or triplicate wells of the same culture conditions into separate non-sterile 12x75 mm polystyrene tubes (BD Falcon, Franklin Lakes, NJ). Tubes were then centrifuged at 1500 RPM (450 x g) for 5 minutes at 4°C. Tubes were removed from the centrifuge and placed on ice. Approximately 250-450 μL of supernatant from each centrifuged tube was removed with a p1000 pipette tip and discarded, leaving approximately 100 μL of supernatant in each tube. The cell pellet in each tube was then resuspended in the remaining supernatant by votrexing. To each tube containing cell suspension, 10 μL of mouse serum (Sigma, St. Louis, MO, USA) was added, followed by vortexing to mix, and then incubation on ice for at least 10 minutes. Then to each tube, an antibody master-mix was added that included FITC-CD69, PE-CD8, PE/Cy5-CD4, and APC-CD3, at concentrations of antibody suggested by the manufacturer at 20 μL per million cells. Tubes were then incubated on ice for 20 minutes. Approximately 4.5 mL of FACS Buffer (Table 3-03) was then added to each tube, followed by centrifugation at 1500 RPM (450 x g) for 5 min at 4°C. The majority of the supernatant from each tube was then removed by vacuum aspiration, leaving approximately 100 μL of supernatant. 100 μL of 2% paraformaldehyde (Electron

Microscopy Sciences, Hatfield, PA, USA) was then added to each tube to fix cells, and the cell pellets resuspended by vortexing. Tubes were then stored at 4°C in a standing rack covered with aluminum foil until collection on a flow cytometer, which typically occurred 7 days or less after fixation. Cells were collected on a FACSCalibur™ (BD Biosciences, San Jose, CA, USA) with at least 20,000 events in the live cell gate (based on FSCxSSC characteristics) and exported as flow cytometry standard (FCS) files for analysis. FCS files were analyzed with Flowjo (Treestar, Ashland, OR, USA), and T cell fractions was quantified as gated live $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, to assess CD4+ and CD8+ T activation respectively. Quantitation of CD69 expression was obtained by gating on CD69+ on CD4+ or CD8+ T cells.

3.5.3 Examination of T cells subset proliferation in response to CD40L/IL-4 or CpG-ODN/IL-2 activated B cell antigen presentation

Cultures set up as described in section 3.5.1 containing CFSE labeled T cells were harvested at 7 days. Harvesting entailed pipetting up and down to resuspended cells, followed by pooling of duplicate or triplicate wells of the same culture conditions into separate non-sterile 12x75 mm polystyrene tubes (BD Falcon, Franklin Lakes, NJ). Tubes were then centrifuged at 1500 RPM (450 x g) for 5 minutes at 4°C. Tubes were removed from the centrifuge and placed on ice. Approximately 250-450 μL of supernatant from each centrifuged tube was removed with a p1000 pipette tip and discarded, leaving approximately 100 μL of supernatant in each tube. The cell pellet in each tube was then resuspended in the remaining supernatant by vortexing. To each tube

containing cell suspension, 10 μL of mouse serum (Sigma, St. Louis, MO, USA) was added, followed by vortexing to mix, and then incubation on ice for at least 10 minutes. Then to each tube an antibody master-mix was added that included PE-CD8, PE/Cy5-CD4, and APC-CD3, at concentrations of antibody suggested by the manufacturer. Tubes were then incubated on ice for 20 minutes. Approximately 4.5 mL of FACS Buffer (Table 3-03) was then added to each tube, followed by centrifugation at 1500 RPM (450 x g) for 5 min at 4°C. The majority of the supernatant from each tube was then removed by vacuum aspiration, leaving approximately 100 μL of supernatant. 100 μL of 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) was then added to each tube, and the cell pellets resuspended by vortexing. Tubes were then stored at 4°C in a standing rack covered with aluminum foil until collection on a flow cytometer, which typically occurred 7 days or less after fixation. Cells were collected on a FACSCalibur™ (BD Biosciences, San Jose, CA, USA) with at least 20,000 events in the live cell gate (based on FSCxSSC characteristics) and exported as FCS files for analysis. FCS files were analyzed with Flowjo (Treestar, Ashland, OR, USA), and the proliferating fraction was quantified on gated live $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells, to assess CD4+ and CD8+ T cell proliferation respectively.

CD40L/IL-4 activated B cells typically induced large amounts of background proliferation in the absence of antigen. Therefore, in order to determine antigen specific proliferation and to normalize the data across different subjects, the percentage of CD4+ and CD8+ T cell proliferation was calculated using a using methodology similar to those established by others (65). We defined the quotient of antigen specific proliferation as:

((percentage of dividing cells observed in presence of antigen / percentage of non-dividing cells in the presence of antigen) / (percentage of dividing cells observed in the absence of antigen / percentage of non-dividing cells in the absence of antigen)) x 100. The normalized proliferation was then calculated by taking the quotient of antigen specific T cell proliferation and subtracting 100. In some cases, normalized proliferation resulted in values less than zero. These values were considered zero for comparison purposes. All fluorescently conjugated monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA) and listed along with the clone names in Table 3-04.

3.5.4 Blocking of MHC II:TCR interaction in mixed B:T cell cultures with monoclonal anti-HLA-DR antibody

The capacity of CD40L/IL-4 activated B cells to process and present specific antigen was tested by blocking MHC II:TCR interactions with an HLA-DR specific antibody. This was done by adding a sodium azide free preparation of the monoclonal anti-HLA-DR antibody, (clone G46-6, BD Biosciences, San Jose, CA, USA) into B:T cell cultures ($5x10^5$ cells per culture condition) at a final culture concentration of 5 μg/mL. Cultures were set up as outlined in Section 3.5.1.

In some cases, CD40L/IL-4 activated B cells were pre-incubated with 20 μL/1 × 10^6 cells of anti-HLA-DR antibody clone L243, (BD Biosciences, San Jose, CA, USA), for 20 minutes at 4°C in 12x75 mm polystyrene tubes (BD Falcon, Franklin Lakes, NJ, USA). Approximately 4.5 mL of ice cold Human media (Table 3-03) was then added to CD40L/IL-4 activated B cells to wash. Tubes were then centrifuged for

1500 RPM (450 x g) for 5 minutes at 4°C. The majority of supernatant was then removed by vacuum aspiration, and the cell pellet was resuspended in the remaining supernatant (approximately 100 μL) by vortexing. Approximately 4.5 mL of ice cold Human media was then added to CD40L/IL-4 activated B cells to wash for a second time. Tubes were then centrifuged for 1500 RPM (450 x g) for 5 minutes at 4°C. The majority of supernatant was then removed by vacuum aspiration, and the cell pellet resuspended in the remaining supernatant (approximately 100 μL) by vortexing. The cell suspension was then brought up to a concentration of $2.5x10^6$ cells per mL with human culture media and cultured as described in section 3.5.1.

3.6.0 Frequency of MBP-reactive CD40L/IL-4 expanded B cells

CD40L/IL-4 expanded B cells were assayed for their ability to bind biotinylated MBP as described with modifications (124). Briefly, 2×10^6 CD40L/IL-4 B cells were washed two times in ice-cold FACS buffer (4% FBS in PBS). Cells were incubated with 10 μg biotin-MBP (Millipore), in a volume of 100 μL on ice for 1 hour. Cells were then washed twice in ice-cold FACS buffer and incubated with either streptavidin-allophycocyanin or streptavidin-PE (both from BD Biosciences) for 30 minutes on ice. Cells were then washed twice in cold FACS buffer and fixed with 1% paraformaldehyde. (Note: fixation should not be done in conjunction with propidium iodide staining). Control stains included CD40L/IL-4 expanded B cells that were stained with streptavidin-PE or streptavidin-allophycocyanin alone. 1×10^6 events were collected on a FACSCalibur (BD Biosciences) and analyzed with Flowjo Software (Treestar). Cells

were gated on live cells based on FSCxSSC characteristics.

3.7.0 Activation of B cells to determine cytokine secretion capacity

Cytokine production was measured in sorted naïve and memory B culture supernatants that had been stimulated with CD40L or CD40L plus anti-IgM/IgG stimulation similar to methods described in (125) or with CpG/ODN and CD40L. Sorted naïve and memory B cells were plated into 96-well flat-bottom plates (Costar Corning, Corning, NY, USA) at $1.5x10^5$ cells per well in 200 μL of human medium (Table 3-03). For CD40L alone stimulation, $CD40L^+$3T3 cells were irradiated with 96 Gy using a ^{137}Cs-source. Irradiated $CD40L^+$3T3 cells were washed by centrifuging twice at 1500 RPM (450 x g) for 5 min at 25°C, each time removing the supernatant through vacuum aspiration and resuspending the cell pellet in 5 mL of human media. Irradiated $CD40L^+$3T3 were then added to sorted B cells at a ratio of 1:15, $CD40L^+$3T3: B cells and plated at 200 μL per well in human media. Plates containing cultures were then incubated at 37°C in an H_2O humidified, 5% CO_2 atmosphere, in an NU-5510 Incubator Direct Heat Sterilization CO_2 Incubator (NuAire, Plymouth, MN, USA). After 48 hours, 160 μL of cell free, culture supernatants were removed by pipette, placed in a fresh 96 well round bottom plate or microcentrifuge tube, and frozen at -20°C for batched cytokine secretion assay by ELISA.

For BCR crosslinking plus CD40L stimulation, $1.5x10^5$ naïve or memory B cells were added to 96 well round bottom plates in a volume of 200 μL of human media containing 0.5 μg/mL of $F(ab)_2$ anti-human IgG and 0.5 μg/mL of $F(ab)_2$ anti-human IgM

both purchased from Jackson Immunoresearch (West Grove, PA, USA). Cells were cultured at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator for 12-24 hours. Cell cultures were then resuspended by pipetting up and down and then transferred to a pre-adhered monolayer of irradiated $CD40L^+$ NIH-3T3 cells at a ratio of 1:15 NIH-3T3 cells to B cells in 96 well flat bottom plates. Cells were then cultured at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator. After 48 hours, 160 μL of cell free, culture supernatants were removed by pipet, placed in a fresh 96 well round bottom plate or microcentrifuge tube, and frozen at -20°C for batched cytokine secretion assay by ELISA.

For CpG-ODN plus CD40L stimulation, $1.5x10^5$ naïve or memory B cells were added to 96 well round bottom plates in a volume of 200 μL of human media containing 5 μg/mL of CpG-ODN. $CD40L^+3T3$ were irradiated with 96 Gy using a ^{137}Cs-source. Irradiated $CD40L^+3T3$ cells were washed by centrifuging twice at 1500 RPM (450 x g) for 5 min at 25°C, each time removing the supernatant through vacuum aspiration and resuspending the cell pellet with 5 mL of human media. Irradiated $CD40L^+3T3$ were then added to sorted B cells at a ratio of 1:15, $CD40L^+3T3$: B cells. Plates containing cultures were then incubated at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator. After 48 hours, 160 μL of cell free, culture supernatants were removed by pipet, placed in a fresh 96-well-round bottom plate or microcentrifuge tubes, and frozen at -20°C for batched cytokine secretion assay by ELISA. The 48h time point was optimal for both IL-10 and LTα secretion measurement (125).

3.8.0 PBMC and Naïve and Memory B Cell antigen presentation assay

3.8.1 PBMC and B-T cell culture set-up

To measure B cell subset APC functionality, $1x10^6$ CFSE labeled T cells were cultured with either no APCs as a control, with $1x10^5$ autologous ex-vivo naïve B cells, or with $1x10^5$ autologous ex-vivo memory B cells, in 1 mL of human media in sterile 12x75 mm polystyrene round bottom tubes with polyethylene caps (BD Falcon, Franklin Lakes, NJ). $2x10^6$ PBMCs cultured in 1 mL of human media (Table 3-03) were set up in parallel also in 12x75 mm polystyrene round bottom tubes with polyethylene caps. Cultures were set up to contain either no exogenous antigen, 5 μg/mL Tetanus Toxoid (TT; Massachusetts Biological Laboratories), 10 μg/mL native bovine myelin basic protein (MBP; Genway Bio-Products), 10 μg/mL glatiramer acetate (GA; Teva Pharmaceuticals), or 10 μg/mL of the recombinant extracellular domain of human oligodendrocyte glycoprotein (MOG; a kind gift from Jeri Anne Lyons). Cells were then cultured at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator.

In some cases, sorted naïve and memory B cells were pre-incubated with 50 μM chloroquine (Cq; Sigma, Saint Louis, MO, USA) along with either 5 μg/mL TT, 10 μg/mL MBP 10 μg/mL GA, or 10 μg/mL MOG, for 4 hours at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator, at a concentration of $1x10^6$ cells in human media (Table 3-03) in sterile 12x75 mm polystyrene round bottom tubes with loose caps to allow gas permeation, prior to culture with autologous T cells. B cells were then removed from 4 hour culture and brought up to 5 mL with room temperature human media (Table 3-03). B cells were then centrifuged at 1500 RPM (450 x g) for 5 min at room temperature. Supernatant was then removed by vacuum aspiration and cell pellets

was resuspended in 5 mL of human media (Table 3-03). Resuspended B cells were again centrifuged at 1500 RPM (450 x g) for 5 min at room temperature. Supernatant was again removed by vacuum aspiration and B cells were brought up to the appropriate volume of human media. $1x10^5$ Cq-treated B cells were then cultured with $1x10^6$ autologus purified CFSE labeled T cells in 1 mL of human media in sterile 12x75 mm polystyrene round bottom tubes with loose caps to allow gas permeation, at 37°C in an H_2O humidified, 5% CO_2 atmosphere incubator.

After 5 days in culture, 750 μL of cell free culture supernatant was removed from each individual culture tube and placed in a 24-well plate (Costar Corning, Corning, NY, USA) and frozen at -20°C for batched cytokine secretion analysis.

3.8.2 Assay of CD4+ and CD8+ T cell proliferation as a measurement of APC functionality in PBMC and B-T cultures

Cell cultures from the previous section were centrifuged at 1500 RPM (450 x g) for 5 minutes at 4°C. Supernatant was removed by vacuum aspiration and cell pellets resuspended in approximately 100 μL of Blocking Buffer (Table 3-03) and incubated on ice for 10 minutes. Cells in the B-T co-cultures were stained with 1.5 μL CD4-PE/CY7, and 6 μL of CD3-allophycocyanin (both from BD Biosciences, San Jose, CA, see Table 3-04), and incubated for 15 minutes on ice. 4.5 mL of FACS Buffer (Table 3-03) was then added to each culture tube, and tubes were centrifuged for 1500 RPM (450 x g) for 5 minutes at 4°C to pellet the cells. The supernatant was removed by vacuum aspiration and the cell pellet resuspended and fixed in 200 μL of a 1% solution of paraformaldehyde

(Electron Microscopy Sciences, Hatfield, PA, USA). At least 100,000 events in the live cell gate (based on FSCxSSC characteristics) were collected for each culture condition on a FACS LSRII™ (BD Biosciences, San Jose, CA, USA) and exported as FCS files using FACSDiva Software (BD Biosciences, San Jose, CA, USA). FCS files were analyzed on Flowjo software (Treestar, Ashland, OR, USA). CD4+ T helper cells were considered $CD4^+CD3^+$, and $CD8^+$ cytotoxic T lymphocytes were considered $CD4^-CD3^+$. Proliferation was quantified into a percentage by dividing the number of T cells (either $CD4^+$ or $CD8^+$) in the CSFE low gate by the total number of T cells in the live gate. In order to normalize the data between assays, the percentage of antigen specific T cell proliferation was subtracted from the percentage of T cell proliferation observed in the absence of antigen. If the normalized proliferation was negative, the antigen specific proliferation was graphed as a zero value, however for statistical comparisons, the actual normalized values were used. A threshold value for positive proliferation was set at 2% similar to previously described techniques (126).

3.9.0 Measurement of cytokine secretion in cell culture supernatants by enzyme linked immunosorbent assay (ELISA)

Interleukin-5 (IL-5), Interferon-γ (IFNγ), Interleukin-10 (IL-10), or lymphotoxin-α (LTα) capture monoclonal antibodies were diluted to 2 μg/mL in 0.1 M Sodium Carbonate, and 50 μL added to each well of Immulon 2HB 96-well plates (Thermo-Fisher Scientific, Pittsburgh, PA, USA) and allowed to bind overnight at 4^oC. Plates were then washed twice with 200 μL of wash buffer (PBS, 0.1% v/v Tween-20) using an automated Wellwash*4 microplate washer, 96 well format (Thermo Scientific, Waltham,

MA, USA). 200 μL of ELISA blocking buffer (Table 3-03) was then added to each well and incubated for 2 hours at room temperature. Plates were washed twice with 200 μL of wash buffer (PBS, 0.1% v/v Tween-20) using the automated Wellwash*4 microplate washer. Samples from supernatants that had been previously frozen at -20°C for batch analysis as well as standards were thawed on ice. In some instances, when the volume of frozen supernatant fell short of the volume required (100 μL) for the ELISA assay, supernatants were diluted 2-5 times their original volume with ELISA Blocking Buffer. In other cases, the required volume was obtained by pooling wells from duplicate culture conditions (identical donor and identical condition). Each cytokine standard was diluted to a high concentration of 2000 pg/mL in ELISA Blocking Buffer. From this high standard, 1000, 500, 250, 125, 62.5, 31, and 15.5 pg/mL standards were created by serial dilution in ELISA blocking buffer, making a total of 7 standard concentrations. 100 μL of each standard concentration (2000-15.5 pg/mL), one blank (ELISA Blocking Buffer) and each sample was added to the washed plate containing bound anti-cytokine antibodies. Standards and samples were then allowed to incubate overnight at 4°C. Plates containing standards and samples were then washed 4 times with 200 μL of wash buffer using an automated Wellwash*4 microplate washer. 100 μL of a 1 μg/mL solution of paired-biotinylated detection anti-cytokine antibody (diluted in ELISA Blocking Buffer, see Table 3-03) was added to each washed well and incubated for 1 hour at 25°C. Plates were then washed 6 times with 200 μL of wash buffer using an automated Wellwash*4 microplate washer. 100 μL of a 2.5 μg/mL solution of streptavidin-HRP (Jackson Immunoresearch, West Grove, PA, diluted in Blocking Buffer) was then added to each washed well and allowed to incubate for 30 minute at 25°C. Plates were then washed 8

times with 200 μL of wash buffer using an automated Wellwash*4 microplate washer. 100 μL of a 100 μg/mL working solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA) substrate was added to each well. TMB was purchased lyophilized, and was resuspended into a 1 mg/mL stock solution in dimethylsulfoxide (DMSO: Sigma, St. Louis, MO, USA), and frozen in 1-2 mL aliquots in 15 mL conical tubes at -20°C. To create a working solution, 1 mL of stock TMB solution was thawed and 9 mL of phosphate-citrate buffer, pH 5.0, and 2 μL of 30% H_2O_2 added according to manufacturer instructions. TMB substrate was allowed to develop for 5-20 minutes in the dark at 25°C. Reaction of TMB substrate in each well was then stopped by adding 100 μL of 1M hydrochloric acid (Sigma, St. Louis, MO, USA) to each well. The optical density (OD) of each well was then read at 450nm, using E-max® plate reader (Molecular Devices, Sunnyvale, CA, USA) with accompanying Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA). All monoclonal antibodies used for capture and detection was purchased from BD Biosciences (San Jose, CA, Table 3-04).

3.10.0 Detection of antigen binding B cells using FITC labeled proteins

3.10.1 Conjugation of whole proteins with FITC

Ovalbumin (OVA; Sigma, St. Louis, MO, USA), TT, MBP, MOG and GA were resuspended in PBS (Cellgro Mediatech, Manassas, VA, USA) at a concentration of 2 mg/mL in 0.05-1 mL and incubated with 10 μM FITC (Pierce, Rockford, IL, USA) for 60 minutes at room temperature. To remove unbound FITC following conjugation reaction, low molecular weight compounds including un-reacted FITC and low molecular weight

species of GA, were removed by size exclusion gel filtration. Zeba spin desalting columns (spin columns; 2 mL capacity, Pierce, Rockford, IL, USA) were prepared for protein desalting according to manufacturer instructions. Spin columns contain a high performance desalting resin with a molecular weight cutoff of 7000 Daltons. FITC conjugated proteins were applied to the top of separate spin columns and allowed to absorb into resin bed. If volume of FITC conjugated protein was less than 350 μL, 40 μL of PBS was added to resin bed and allowed to absorb. Spin columns were recapped loosely, and placed into 15 mL conical tubes (BD Falcon, Franklin Lakes, NJ, USA), and centrifuged for 1000 x g for 2 minutes at 25°C. After centrifugation, effluents that collected in the bottom of the 15 mL conical tube was transferred to a microcentrifuge tube. These were the desalted FITC conjugated proteins. Sodium Azide was added to conjugated protein preparations as preservative at a final concentration of 0.1%. OVA, MOG, and GA FITC conjugates were stable for at least 1 month at 4°C, while MBP and TT conjugates were stable for less than one week.

3.10.2 Quantitation of protein binding B cells by flow cytometry

Cryopreserved PBMCs from HD and RRMS patients were assayed for their ability to bind FITC conjugated proteins as described by others (127) with modifications. $2x10^6$ PBMCs from each donor were resuspended in 80 μL of protein binding buffer (Table 3-03). PBMCs were incubated with either no exogenous antigen, or optimal staining concentrations of FITC conjugated OVA, TT, MBP, MOG, or GA preparations in a final volume of 100 μL on ice for 1 h. Optimal staining concentration of each FITC

conjugated protein preparation were determined by titration. PBMCs were then washed twice in ice-cold buffer and incubated with CD19-PE/CY5 and CD27-PE (BD Biosciences, San Jose, CA) for 30 minutes on ice. Cells were then washed once in ice-cold buffer, and 1 μL propidium iodide (PI; BD Biosciences) was added. $1x10^6$ events in the live cell gate were collected on a C6 flow cytometer (Accuri, Ann Arbor, MI) and analyzed with Flowjo Software (Treestar, Ashland, OR). Cells were gated on live lymphocytes based on FSCxSSC characteristics and exclusion of PI, and further gated into naïve (CD19+CD27-) and memory (CD19+CD27+) B cell subsets. The percent of naïve and memory B cells binding both FITC and biotin preparations of the same proteins were quantified as antigen specific B cells, to ensure specificity of protein binding.

3.11.0 Statistics

Fisher's Exact Test was used to compare the distribution of heavy and light chain rearrangements found in the productive repertoires of the MS patients. Mutational frequencies (MF), Naive:Memory B cell ratios, D segment usage, and V_HCDR3 charges were compared using the χ^2 test. V_HCDR3 lengths were compared using the Mann–Whitney U-test. Statistical differences between MS patient and healthy control groups presented in chapter five were assessed using Mann–Whitney U test. Unless noted otherwise, mean lymphocyte percentages, CD80 MFI, HLA-DR MFI, cytokine secretion, T cell proliferative responses, and B cell protein binding assays were compared between HDs and RRMS patients (between donor group) and naïve and memory B cell responses (repeated measures within donor group) using two-way mixed model analysis of variance

(ANOVA) in chapter six. When overall ANOVA p-values were found to be significant (less than 0.05) a multiple comparison post-hoc analysis with Bonferroni correction was used to determine which means differed significantly, and estimated p-values were reported. Non-significant p-values were reported as exact p-values. In some cases where two-way ANOVA was not appropriate, mean responses were compared using un-paired t-test (between donor group) and paired t-test (within donor group) and exact p-values were reported. Chi-Squared analysis was used to compare number of positive responders between HD and RRMS.

Figure 3-01

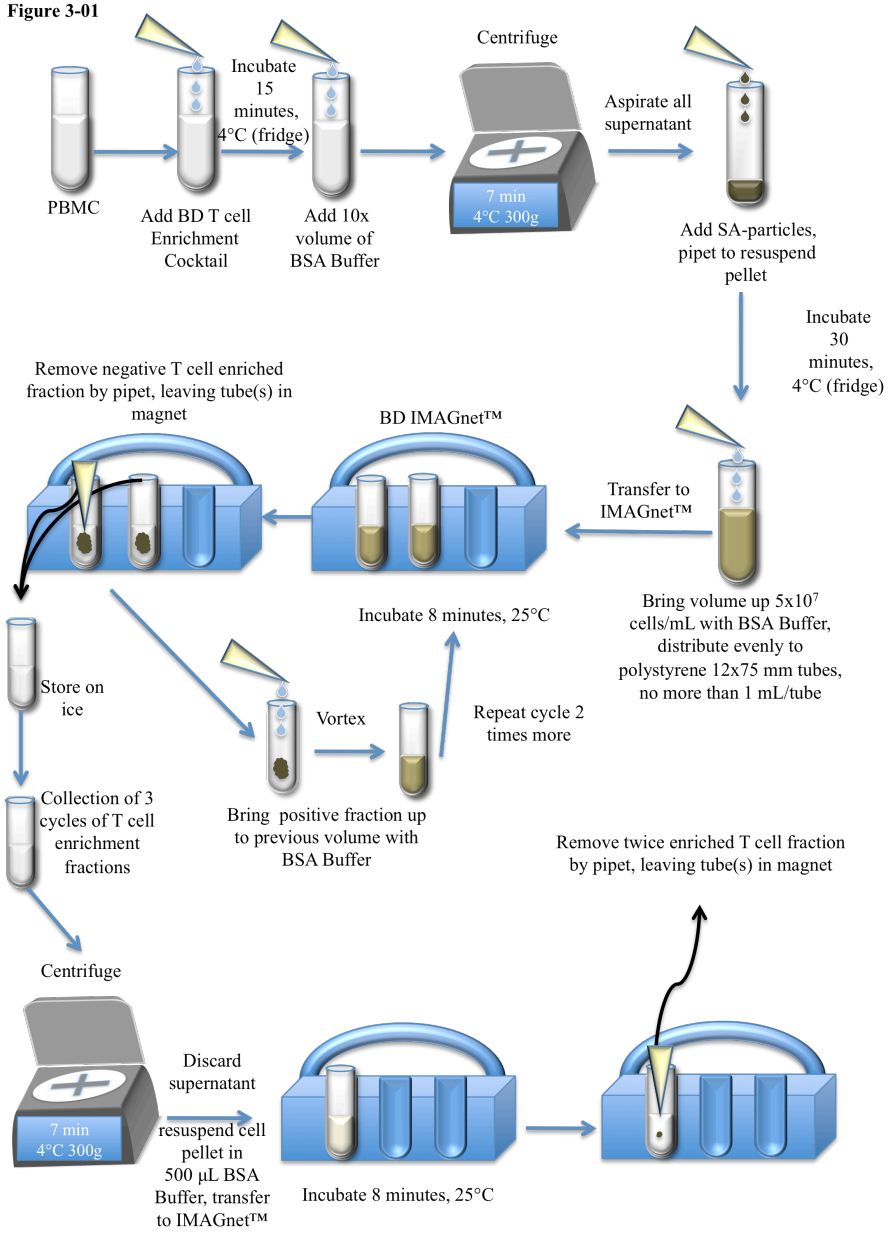


FIGURE 3-01. Procedure for obtaining magnetically enriched T cells using the BD Biosciences IMAG™ Human T cell enrichment kit.

Figure 3-02

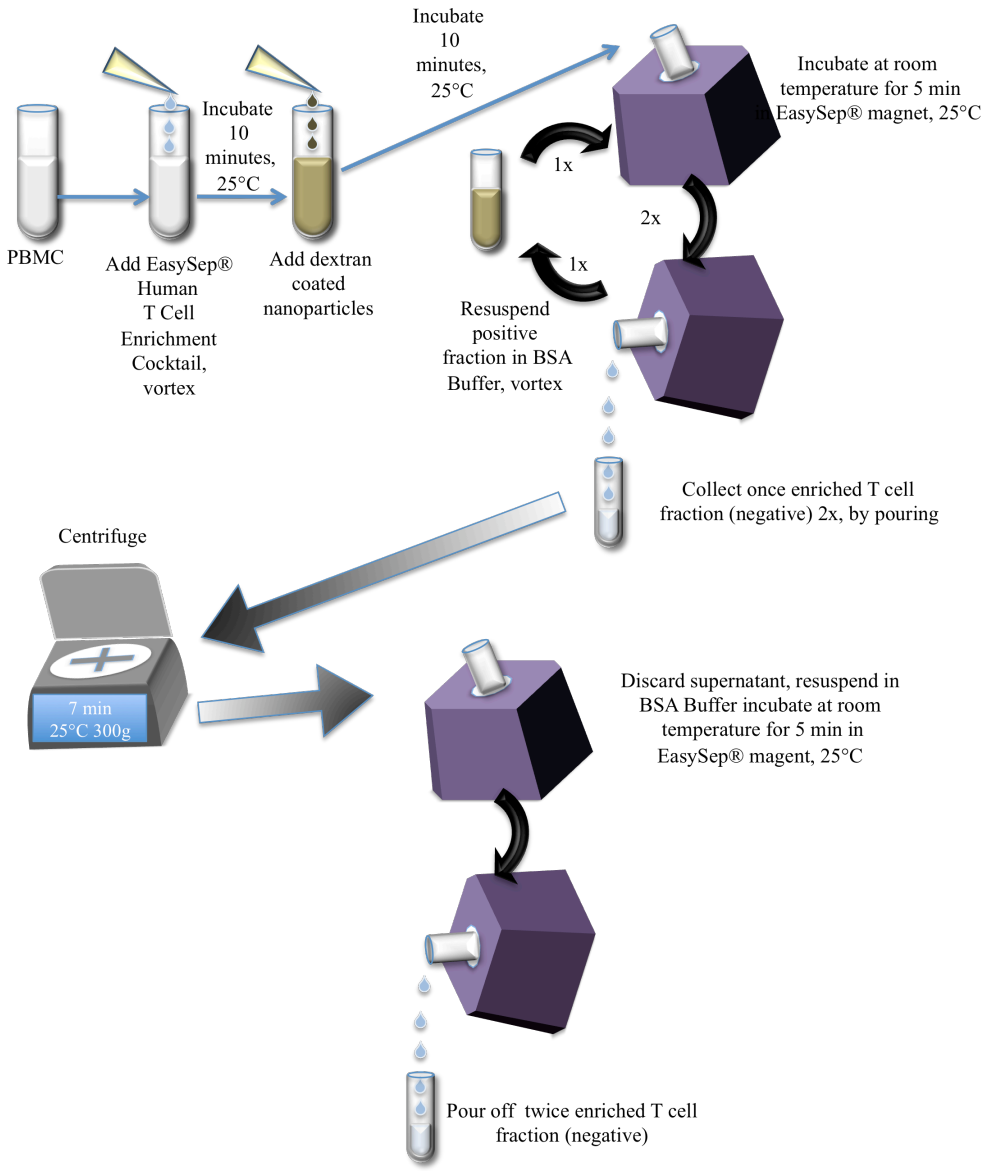


FIGURE 3-02. Procedure for obtaining magnetically enriched T cells using the EasySep® Human T Cell Enrichment Kit from Stem Cell Technologies Inc.

Figure 3-03

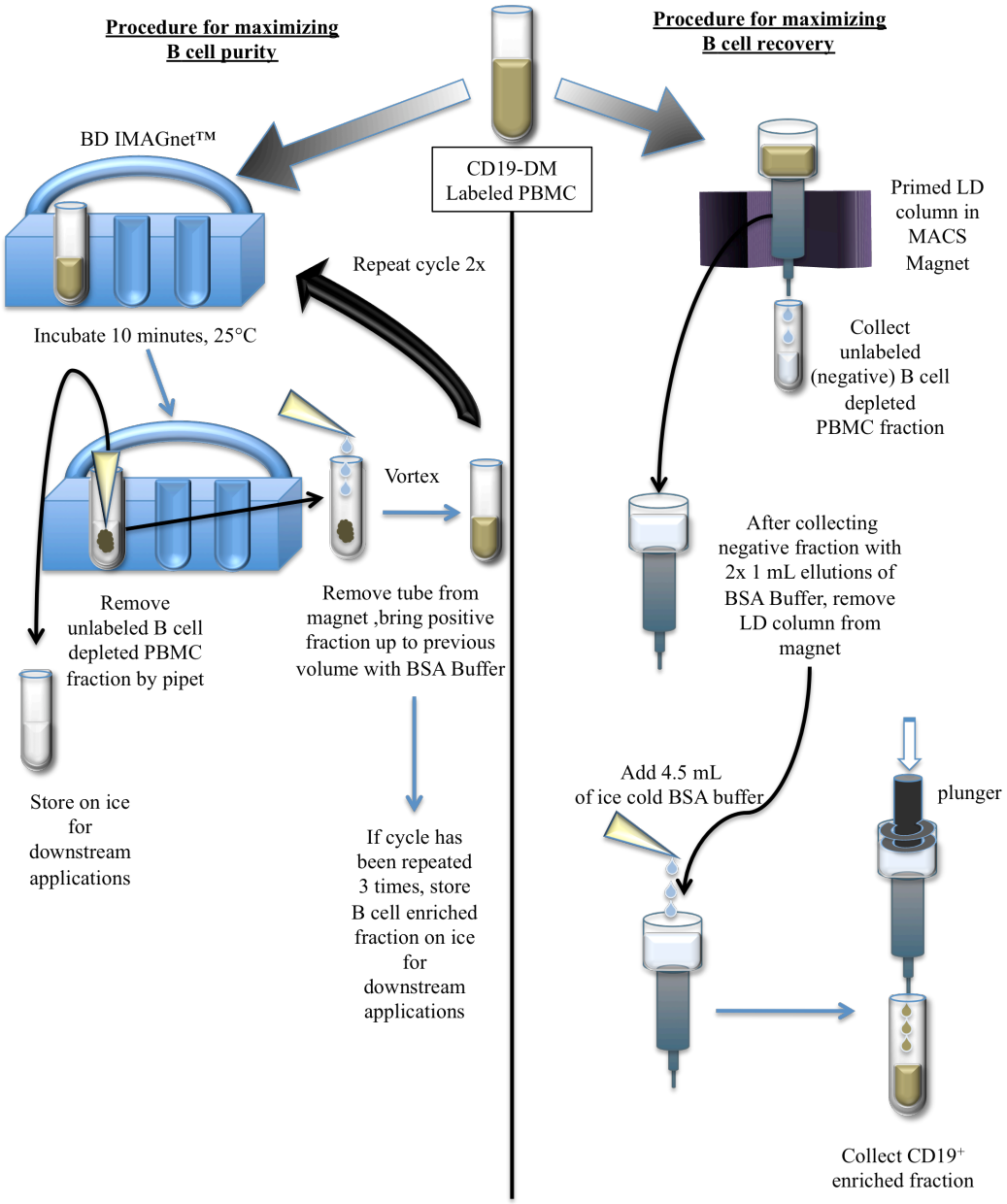


FIGURE 3-03. Procedures for obtaining magnetically enriched B cells from PBMC. Procedure for maximizing B cell purity graphically depicted on left. Procedure for maximizing B cell recovery graphically depicted on right.

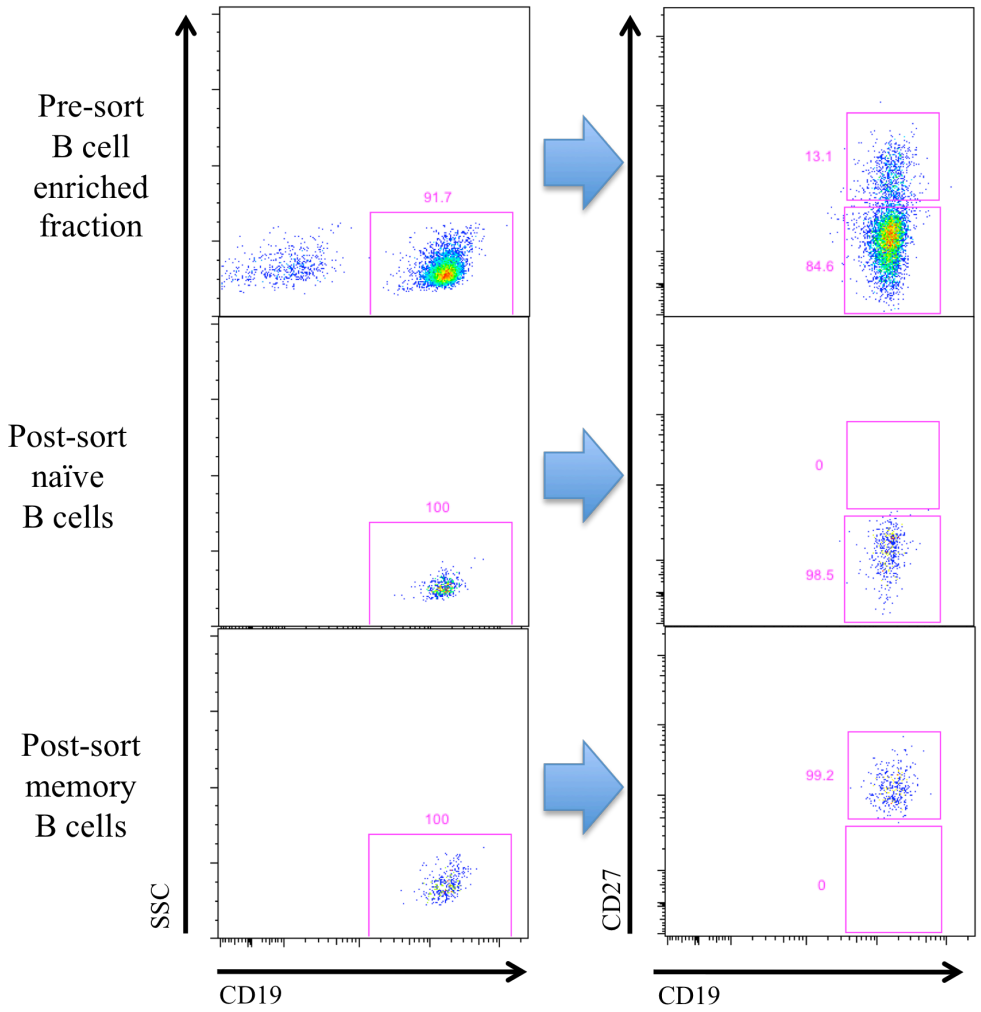


FIGURE 3-04. Typical example of a naïve and memory B cell sort. Magnetically enriched B cells from a healthy donor were sorted by fluorescence activated cell sorting (FACS) into pure (>98%) naïve and memory B cell populations based on expression of CD19+CD27- and CD19+CD27+ respectively.

TABLE 3-01. MS patient characteristics of samples used in chapter four

	MS Type	Time since MS diagnosis	Age/ sex	Exacerbation history	MRI findings	Clonal expansion	OC bands	Ig index	Ig synthesis
M125[b,c,d,e]	RR	< 1 year	32/F	ON	GD+	Yes	No	NL	n.d.
M217[b]	RR	18 years	45/F	Dystonia	WML	n.d.	n.d.	High	n.d.
M354[d,e]	RR	< 1 year	44/F	TM	WML	Yes	Yes	NL	NL
M368[c,d,e]	RR	15 years	41/F	TM	WML	Yes	Yes	High	High
M376[b,d]	RR	20 years	56/F	ON	WML	Yes	No	n.d.	n.d.
M484[d,e]	PP	3 months	46/F	Myelitis	WML	Yes	Yes	High	High
M522[b,c,d]	RR	3 years	35/F	TM	GD+ WML	Yes	Yes	High	n.d.
M558[b]	RR	1 year	29/F	ON	GD+ WML	n.d.	n.d.	n.d.	n.d.
M584[c,d]	RR		44/F	TM	GD+	Yes	n.d.	n.d.	n.d.
M875[b,c,d,e]	RR	1 month	35/F	ON	GD+, WML	Yes	n.d.	n.d.	n.d.
M887[b]	RR	13 years	50/F	ON	WML	No	No	NL	NL
M918[b]	RR	10 years	38/ M	Myelitis	WML	n.d.	No	NL	NL
M927[b]	RR	8 years	31/F	ON	WML	n.d.	No	NL	NL
M944[b]	SP	10 years	53/F	ON	WML	n.d.	Yes	High	High

Abbreviations used: RR, relapsing remitting; SP, secondary progressive; ON, optic neuritis; TM, transverse myelitis; GD+, gadolinium enhancing; WML, white matter lesions.

[a] All patients had CSF white blood cell (WBC) counts in the range of $1x10^3$ to $1x10^4$ per mL, typical of MS patients at UTSW (128).

[b] Patient data used in flow cytometric analysis.

[c] Patient data used in repertoire analysis.

[d] Patient data used in mutation analysis and heavy chain CDR3 charge analysis.

[e] Patient clonal analysis previously published in (13).

TABLE 3-02. Patient characteristics of samples used in chapters five and six

MS patient ID	MS type	Time since diagnosis	Age TP 1	Sex	Treatment TP 2&3	MRI Findings	Exacerbation History by TP 3	EDSS[i] Score TP 1
MS-1[a,b,e,f,g,h]	RR	<2year	23	F	IFN	MRI+	1	1.5
MS-2[a,b,d,f,g,h]	RR	<2year	25	F	IFN, MM	MRI+	2	1.5
MS-3[c]	PP	1 year	n/a.	F	n./a	MRI+	None	n/a
MS-4[a.b,d e]	RR	<2year	43	F	IFN, MM	MRI+	1	1.5
MS-5[a,b g,d,e,f]	RR	<2year	47	F	IFN	MRI+	1	2.5
MS-6[a,b]	RR	<2 years	n/a	M	IFN, MM	MRI+	1	n/a
MS-7[a]	SP	13 years	n/a	F	n/a.	MRI+	1	n./a
MS-8[c]	SP	n.d.	n/a	M	n/a	n.d.	n.d.	n/a
MS-9	n.d.	Nd	n/a	Nd	n/a	MRI+	n.d.	n/a
MS-10[b,d,e,f,g,h]	RR	<2year	47	F	n/a	MRI+	1	2
MS-11[b,d,e,f,g,h]	RR	<2year	40	M	n/a	MRI+	2	0
MS-12[b,d,e,f]	RR	<2year	52	F	n/a	MRI+	1	1.5
MS-13[b,d,e,f]	RR	<2year	41	F	n/a	MRI+	1	1
MS-14[b,f,g]	RR	<2year	41	F	n/a	MRI+	1	2
MS-15[b,f,g]	RR	<2year	32	F	n/a	MRI+	1	1.5
MS-16[b,f,g]	RR	<2year	25	F	n/a	MRI+	1	0
MS-17[b,f,g,h]	RR	<2year	44	F	n/a	MRI+	1	3.5
MS-18[b,f,g,h]	RR	<2year	37	F	IFN	MRI+	1	2.5
MS-19[a,beh]	RR	<2year	35	F	None	MRI+	1	2
MS-20[b,d,e,f,g]	RR	<2year	27	M	None	MRI+	2	1.5
MS-21[b,e]	RR	<2year	34	F	n/a	MRI+	3	1
MS-22 [b,d,e]	RR	<2year	44	F	n/a	MRI+	1	1.5
MS-23[b,d,e,h]	RR	<2year	50	F	n/a	MRI+	1	3
MS-24[b,d,e,h]	RR	<2year	34	F	n/a	MRI+	1	2
MS-25[b,d,h]	RR	<2year	46	M	n/a	MRI+	1	2.5
MS-26[b,d]	RR	<2year	23	F	None	MRI+	2	2
MS-27 [b,d]	RR	<2year	26	F	n/a	MRI+	1	2.5

[a] Post treatment RR patient sample used from TP 2 or TP 3 used in chapter five.
[b] Treatment naïve RR patient sample from TP 1 used in chapter six.
[c] SP or PP sample patient used in chapter five
[d] Sample used in measurement of peripheral B cell percentages, CD80 MFI in chapter six
[e] Sample used in measurement of HLA-DR MFI in chapter six
[f] Sample used in whole PBMC CD4+ and CD8+ T cell proliferation in chapter six
[g] Sample used in measurement of CD4+ T cell proliferation, IFNγ secretion and protein binding assay by flow cytometry
[h] Sample used in measurement of B cell secretion of LTα and IL-10
[i] Expanded Disability Status Scale for MS (129)
Abbreviations used: RR; relapsing remitting, SP; secondary progressive, PP; primary progressive, IFN; Interferon-beta1a, MM; mycophenolate mofetil; MRI+; magnetic resonance imaging reveled detectible lesion, TP; time point, MFI; mean florescence intensity, M; male, F; female, n.d.; data unavailable, n/a; not applicable

TABLE 3-03. Formulation of selected cell culture and buffered media

Media Name	*Contents*	*Filter and Storage Conditions*
Human Media	90% Isocove's Modification of Dubelco's Modified Eagle's Medium without L-glutamine or sodium pyruvate (IMDM; Cellgro Mediatech, Manassas, VA, USA), 10% GemCell™ Human Serum AB (Gemini Bio Products, West Sacramento, CA, USA), 2mM L-glutamine (Cellgro Mediatech, Manassas, VA, USA), and 100 µg/mL Penicillin, 100 IU Streptomycin (Cellgro Mediatech, Manassas, VA, USA).	After combining reagents, filter sterilize with 0.22 cellulose acetate filter (Corning, Corning, NY, USA). Store at 4°C. Protect from prolonged exposure to light. Expires 6 weeks after combining reagents.
G418 Media	45% F12 (Cellgro), 45% Dubelco's Modified Eagle's Medium (DMEM; Cellgro) 10% Fetal Bovine Serum (FBS, Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Cellgro), 2 mM L-glutamine (Cellgro Mediatech, Manassas, VA, USA), 100 µg/mL Penicillin, 100 IU Streptomycin (Cellgro Mediatech, Manassas, VA, USA), and 200 mg/mL G418 (Invitrogen,).	After combining reagents, filter sterilize with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). Store at 4°C. Protect from prolonged exposure to light. Expires 6 weeks after combining reagents.
3T3 Media	45% F12 (Cellgro), 45% Dubelco's Modified Eagle's Medium (DMEM; Cellgro) 10% Fetal Bovine Serum (FBS, Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Cellgro), 2 mM L-glutamine (Cellgro), 100 µg/mL Penicillin, 100 IU Streptomycin (Cellgro).	After combining reagents, filter sterilize with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). Store at 4°C. Protect from prolonged exposure to light. Expires 6 weeks after combining reagents.
BSA Buffer	2mM EDTA, 0.5% Bovine Serum Albumin (BSA; Sigma) 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Cellgro), 2 mM L-glutamine (Cellgro), 100 µg/mL Penicillin, 100 IU Streptomycin (Cellgro).in Phosphate Buffered Saline (PBS; Cellgro Mediatech, Manassas, VA, USA).	After combining reagents, filter sterilize with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). Store at 4°C. Protect from prolonged exposure to light. Expires 6 weeks after combining reagents.
Binding Buffer	10% Fetal Bovine Serum (Gibco Invitrogen) in PBS (Celgro Mediatech, Manassas, VA, USA)	After combining reagents, filter sterilize with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). Store at 4°C. Expires 6 weeks after combining reagents.
Human Freezing Media	40 % Human Serum, 50% Human Media , 10% dimethylsulfoxide (DMSO; Sigma St. Louis, MO, USA)	After combining reagents, filter sterilize with 0.22 µm cellulose acetate filter (Corning, Corning, NY, USA). Store in 1 mL aliquots at -80°C. Expires at the same time as lot of human serum.
FACS Buffer	2-4% Bovine Serum Albumin (Sigma, St. Louis, MO, USA) in PBS (Cellgro Mediatech, Manassa, VA, USA)	Store at 4°C. Expires 6 weeks after combining reagents.
FACS Blocking buffer	10% Mouse Serum (Sigma, St. Louis, MO, USA) in PBS (Cellgro Mediatech, Manassas, VA, USA)	Store at 4°C. Expires 6 weeks after combining reagents.
ELISA Blocking Buffer	1% Bovine Serum Albumin (BSA; Sigma, St. Louis MO, USA) in PBS (Cellgro Mediatech, Manassas, VA, USA)	Store at 4°C. Expires 2 weeks after combining reagents.

TABLE 3-04. Antibodies used for staining and culture

Target	Fluorochrome Conjugate	Clone	Staining Concentration	Vendor	Storage
CD11c	PE	B-ly6	20 μL/1x10^6 cells	BD	4°C
CD138	PE	MI15	20 μL/1x10^6 cells	BD	4°C
CD14	FITC	M5E2	20 μL/1x10^6 cells	BD	4°C
CD19	PECy5	HIB19	2 μL/1x10^6 cells	BD	4°C
CD27	PE	M-T271	2 μL/1x10^6 cells	BD	4°C
CD3	Allophycocyanin	UCHT1	6 μL/1x10^6 cells	BD	4°C
CD4	PEC7	SK3	1.5 μL/1x10^6 cells	BD	4°C
CD4	PECy5	RPA-T4	20 μL/1x10^6 cells	BD	4°C
CD69	FITC	FN50	20 μL/1x10^6 cells	BD	4°C
CD8	Allophycocyanin	RPA-T8	20 μL/1x10^6 cells	BD	4°C
CD8	PE	HIT8a	20 μL/1x10^6 cells	BD	4°C
CD80	FITC	BB1	20 μL/1x10^6 cells	BD	4°C
HLA-DR	Allophycocyanin	L243	20 μL/1x10^6 cells	BD	4°C
HLA-DR	None (GMP)	L243	20 μL/1x10^6 cells	BD	4°C
HLA-DR	None (NA/LE)	L243	5 μL/mL (culture)	BD	4°C
IgD	FITC	IA6-2	20 μL/1x10^6 cells	BD	4°C
IgM	Allophycocyanin	G20-127	20 μL/1x10^6 cells	BD	4°C

CHAPTER FOUR

Results

CEREBROSPINAL FLUID B CELLS FROM MULTIPLE SCLEROSIS PATIENTS ARE SUBJECT TO NORMAL GERMINAL CENTER SELECTION

The following study has been published in the *Journal of Neuroimmunology*. Harp C., Lee J., Lambracht-Washington D., Cameron, E., Olsen G., Frohman, E. Racke M., Monson N., *Cerebrospinal fluid B cells from multiple sclerosis patients are subject to normal germinal center selection,* volume 183, issue 1-2, pages 189-99 and is reproduced here with the permission of *Elsevier*; license number 2264350958879.

Introduction

During the course of many autoimmune diseases (130, 131), including MS (132), activated B cells are able to form ectopic germinal centers in the inflammatory compartment. In addition, during the course of MS, B cells in the CSF show evidence of clonal relationships and intraclonal diversity that is not observed in OINDs such as viral meningitis, (9, 12). We and others have suggested that these specific clones may have been primed in the periphery, but undergo further activation and expansion intrathecally (12). Such studies demonstrate that B cell clones are actively being driven to undergo diversification through somatic hypermutation within in the CNS. Nevertheless the site of antigen driven selection of the non-clonal population remains controversial, as T-independent mechanisms of activation in the CNS have not been explored. The goal of

the work presented in this chapter was to determine if B cells in the CNS show characteristics of antigen driven germinal center selection, or show atypical mutational patterns associated with random selection. Previous findings from our laboratory demonstrated that some clonally expanded cerebrospinal fluid (CSF) B cells from MS patients exhibited high mutation accumulation in their antibody genes, and in most cases, lacked enhanced targeting of mutations to the RGYW/WRCY motifs in heavy and light chain CDRs in comparison to Healthy Control Peripheral Blood (HCPB) B cells (13). These data were surprising since targeting of mutations to CDRs, and more specifically, to RGYW/WRCY motifs within CDRs are characteristic features of classic peripheral germinal center reactions (133-137) driven by antigen encounter. Based on these data, we hypothesized that all the B cells that enter the CNS originate from the periphery, but that only those B cells that recognize neuro-antigens are clonally expanded in that compartment independent of the governance of classical peripheral germinal centers. If this were the case, then the overall CSF B cell antibody repertoires (not including the clonally expanded B cells) should demonstrate mutation patterns typical of GC-selected B cells. To test this hypothesis, we analyzed the antibody gene repertoires from non-clonal CSF B cells of 8 MS patients for mutation characteristics typical of GC-derived B cells. The results of these studies are described in the following section.

RESULTS

Memory B cells are enriched in the CSF B cell pool of MS and OND patients

We had previously reported that extensive clonal expansion and high mutational frequencies in the clonally expanded CSF B cell population from MS patients likely

indicated that most CSF B cells are of a memory phenotype, rather than naïve (13). Indeed, others have now confirmed by flow cytometric methods that the majority of CSF B cells are of a memory phenotype ($CD27^+$) (138, 139). The CSF B cell profiles (memory vs. naïve) from patients analyzed for this study also adhered to this observation. For example, memory B cells in the CSF of MS patients were enriched compared to the peripheral blood from the same MS patient (compare 20.6 ± 4.4% memory B cells in PB to 55.3 ± 7.8% memory B cells in CSF (Figure 4-01). The CSF B cell profiles (memory vs. naïve) in the OND patients were also enriched for memory B cells compared to PB (compare 17.0 ± 2.9% memory B cells in PB to 53.8 ± 13.4% memory B cells in CSF, (Figure 4-01). In fact, memory B cells were so prevalent in the CSF of both the MS and OND patients, that the typical ratios of naive:memory B cells in CSF of both MS and OND patients was inverted in comparison to PB (Table 4-01). Interestingly, the two CIS patients also had a higher propensity towards memory B cell enrichment in their CSF compared to the PB (Figure 4-01).

Heavy chain and Light (Kappa) Chain Family Usage is intact in the CSF B cell pool of MS patients

Since the CSF B cells consisted largely of memory B cells (which have already undergone antigen driven selection), we predicted that their antibody repertoires might be skewed from HCPB, which is largely naïve and not driven by antigen selection. In order to test this hypothesis, we analyzed the frequency of heavy and light chain rearrangement usage from the $CD19^+$ CSF B cell populations of MS (n=8), one CIS and two OND patients.

In HCPB, V_H3 and V_H4 family members are more often utilized in heavy chain rearrangements, followed by V_H1, and the other families (V_H2, V_H5 and V_H6), as shown in Figure 4-02. The CSF B cell repertoires from M522, M584, and M875 followed this pattern (V_H3+ V_H4>1> V_H2= V_H5= V_H6, Figure 4-02). However, M368 utilized V_H1 V genes more often than any other family (V_H1>V_H3>V_H4>V_H2=V_H5=V_H6, Figure 4-02), which is likely attributed to the contribution of several clones utilizing the V_H1 gene, 1–69 in this repertoire. Overall MS CSF V_H family usage was significantly enriched with V_H1 ($p<0.001$, χ^2-test), and V_H4 ($p=0.003$, χ^2-test) at the expense of V_H3 expressing B cells ($p<0.001$, χ^2-test) in comparison to HCPB (Figure 4-03). Additionally, V_H4 ($p=0.017$, χ^2-test) family usage was increased in MS CSF B cells with a concomitant decrease in V_H3 ($p=0.002$, χ^2-test) as compared to OND CSF, suggesting that an increase in V_H4 expressing B cells is characteristic of CSF MS and not just of intrathecal B cells (Figure 4-03). Even though the overall MS CSF repertoire was skewed towards V_H4, the majority of individual MS patient CSF donors did not show this bias as only M522 and M584 had a significant increase in V_H4 usage in comparison to HCPB or OND CSF (Figure 4-02).

In HCPB, J_H4 family members are more often utilized in heavy chain rearrangements, followed by J_H6, and the other families (J_H5, J_H3, J_H2 and J_H1), as shown in Figure 4-02 and Figure 4-03. The frequency of J_H4 usage in the CSF B cells of the OND patients was similar to that of HCPB ($p=0.98$), but there was a significant decrease in J_H4 segment usage in the CSF B cell heavy chains from MS patients (Figure 4-03) in comparison to HCPB (36% vs. 54%, $p=0.0001$). Furthermore, M368, M522, and M584 had an increased frequency of J_H5 in comparison to HCPB. Increases in distal J segment

usage are characteristic of receptor editing (140), thus indicating that the heavy chain repertoire of MS (but not OND) patients may have undergone receptor editing to some degree.

Overall V kappa (κ) and Jκ light chain segment usage in the CSF B cell populations were also similar in MS and OND (Figure 4-03) (118) with some notable exceptions. MS CSF light chains showed an increase in Vκ1 ($p=0.009$, χ^2-test) usage and a decrease in Vκ2 ($P<0.001$, χ^2-test) compared to HCPB. However, no differences were observed between MS CSF and OND CSF (Figure 4-03). MS CSF B cell κ light chain usage of Jκ2 ($P=0.047$, χ^2-test) and Jκ4 ($p=0.004$, χ^2-test) was decreased in comparison to HCPB (Figure 4-03). MS CSF Jκ2 ($p=0.04$, χ^2-test) usage was also decreased at the expense of Jκ3 usage ($p=0.03$, χ^2-test) in comparison to OND CSF.

Enhanced Mutational Frequency of CSF B cell repertoires of MS patients

Mutated V_H rearrangements are characteristic of memory B cells (73). Since the majority of CSF B cells in MS, CIS and OND patients were of a memory ($CD19^+CD27^+$) phenotype, we predicted that the CSF B cell antibody repertoires would have a substantial accumulation of mutations such that the overall mutational frequencies (MF) would be greater than what was observed in HCPB (141-143), since HCPB B cells are largely naive. Indeed, six of the eight heavy chain antibody repertoires from CSF B cells of MS patients had significantly higher mutational frequencies (MF) than HCPB (Figure 4-04 and Table 4-02). In fact, the average MF of all MS patients in this analysis was 5.0, which is 1.5 fold greater than HCPB. This observation is further emphasized by a separate analysis of just those CSF Ig heavy chains that are mutated, which generates a

MF of 5.8, which is 1.8 fold greater than HCPB. This was also the case with CIS 132 and OND 341, which likely reflects the observation that CSF from patients have a high prevalence of memory B cells, rather than naïve. Indeed, over 86% of the heavy chains in these analyses have accumulated considerable mutations, and are thus likely not naïve B cells.

The kappa chain MF comparison was more variable than the heavy chain MF comparisons (Figure 4-04 and Table 4-02). Some of the kappa chain repertoires from the CSF B cells of MS patients demonstrated a higher MF than in HCPB (M368 and M584), but one was similar to HCPB (M522), and still another was lower (M125). The OND population demonstrated a similar lack of consistency, one kappa chain repertoire (OND341) had a similar MF, and OND758 had a lower MF than HCPB. Interestingly, CIS132, had a higher MF than HCPB. The average MF of the MS CSF kappa chain repertoires combined had a slightly higher MF compared to HCPB (2.9 vs. 2.6, p=0.019).

Targeting of mutations to CDRs is intact in the CSF B cell repertoires of MS patients

Mutation targeting to CDRs is characteristic of B cells that have been selected and activated in the context of a germinal center (112, 113, 118, 142-144). However, some of the clonally expanded CSF B cells from MS patients characterized in our laboratory had diminished targeting to CDRs, despite high mutational frequencies overall. We hypothesized that this diminished targeting to CDRs would be unique to clonally expanded CSF B cells from MS patients, such that the CSF B cell repertoire as a whole, would adhere to normal mutation accumulation patterns. In order to test this hypothesis, we calculated the percentage of mutations that were present in CDRs of the

inclusive CSF B cell heavy and kappa chain repertoires from MS patients, comparing them to values we had established in HCPB B cells (Figure 4-05 and Table 4-03).

Frequencies of mutation in heavy or kappa chain CDRs from CSF B cells of all 8 of the MS patients were either similar or greater than values established in HCPB (Figure 4-05 and Table 4-03). For example, 47% of mutations accumulated in the CDRs of heavy chain rearrangements from HCPB. All eight MS CSF B cell heavy chain antibody repertoires demonstrated targeting of mutations to CDRs at levels similar to HCPB or higher (54.7% average for MS CSF V_H rearrangements vs. 47% HCPB V_H rearrangements, p=0.0001). Hence, CSF B cells from MS patients appear to have been selected in the context of a GC. The two OND and one CIS heavy chain antibody repertoires also demonstrated targeting of mutations to CDRs at levels similar to HCPB, indicating that CSF B cells from non-MS patients had also been selected in the context of a germinal center.

All of the kappa chain CSF Ig repertoires whether derived from MS patients or not, demonstrated targeting of mutations into CDRs at similar or higher levels as HCPB (Figure 4-05 and Table 4-03). The demonstration of intact targeting of mutations to the CDRs further confirms that these B cells were subject to normal germinal center selection, even in MS patients whose repertoires may be more biased towards autoreactivity.

Bias towards Replacement Mutations in CDRs of heavy and kappa chain repertoires is intact in the CSF B cell repertoires of MS patients

Mutations resulting in a change in the amino acid sequence (termed

"replacement" mutations), are more likely to accumulate in the CDRs rather than FRs (112, 113, 141, 144), presumably because diversity in CDRs results in greater variability in antigen binding and affinity, whereas diversity in FRs would result in compromised structural integrity of the antibody (145). We reasoned that since targeting of mutations to CDRs was intact in the CSF B cell repertoires, then we would likely observe an accumulation of replacement mutations in the CDRs compared to the FRs as well.

Replacement:Silent (R:S) ("silent" mutations do not result in amino acid change) ratios of mutations within the CDRs and FRs were calculated for each of the eight MS, one CIS and two OND heavy chain repertoires (Figure 4-06 and Table 4-04). As indicated, the heavy chain repertoires from the CSF B cells of all eight MS patients had significantly higher R:S ratios in the CDRs compared to the FRs. The heavy chain repertoires of CIS132 and OND341 also demonstrated higher R:S ratios in the CDRs compared to the FRs. A bias in replacement mutations accumulating into CDRs of kappa rearrangements was also observed (Figure 4-06 and Table 4-04) in all four MS patients, as well as CIS132 and OND758. Therefore, the mechanism that targets replacement mutations into the CDRs rather than in FRs of heavy and light chains is largely intact in CSF B cell repertoires from MS patients, and some non-MS patients.

Targeting of Mutations to RGYW/WRCY motifs in CDRs is intact in the CSF B cell repertoires of MS patients

Mutations in the heavy and light chain repertoires of HCPB B cells are also targeted to particular motifs that follow the amino acid sequence, RGYW or WRCY, and the majority of mutations within CDRs are contained within these motifs (113, 143, 144).

We predicted that since mutations in the CSF B cell repertoires were targeted to CDRs, the next "level" of targeting—that is, to RGYW/WRCY motifs—would also be conserved in the heavy and kappa rearrangements of the CSF B cell repertoires.

Mutations within the RGYW/WRCY motifs in CDRs of HCPB heavy chain rearrangements represent 60% of all mutations in CDRs. In contrast, only 19% of mutations in FRs of HCPB heavy chain rearrangements are within RGYW/WRCY motifs (Table 4-05). This indicates that mutations within RGYW/WRCY motifs are targeted to the CDRs (143). Targeting of mutations to RGYW/WRCY motifs was intact in 5 of the 8 CSF B cell repertoires from MS patients (as well as CIS132 and both OND patients, Table 4-05). In addition, the average percentage of mutations in RGYW/WRCY of CDRs of all 8 MS patients combined was no different than HCPB (compare 57% MS average to 60% HCPB average, p=0.465). However, when only the heavy chain rearrangements from clonally expanded CSF B cells were considered (Figure 4-07 and Table 4-06), the majority of clones demonstrated targeting of mutations to CDRs, but very few of the clones demonstrated targeting of mutations within RGYW/WRCY motifs in the CDRs. Of note, 6 of the 8 MS CSF B cell heavy chain repertoires exhibited an increase in the frequency of RGYW/WRCY mutations in the FRs that was not observed in HCPB (Table 4-05). For example, 42% of FR mutations of M125 were within RGYW/WRCY motifs. This is significantly greater than HCPB, in which only 19% of FR mutations are within RGYW/WRCY motifs (p=0.0001). This increased frequency of FR mutations within RGYW/WRCY motifs was also evident in the MS average (compare 38% MS average to 19% HCPB average, p=0.0001), and cannot be attributed to lack of targeting to CDRs, since this aspect of V_H rearrangement is similar in HCPB

and MS (and OND or CIS) CSF B cells. Targeting of RGYW/WRCY mutations in the CDRs and FRs of kappa chain repertoires from MS, CIS OND CSF B cells was no different than HCPB (Table 4-05).

CSF B cells from MS patients have longer V_H CDR3s than HCPB

It is suspected that there is a breach in B cell selection in SLE B cell repertoires derived from germinal centers such that B cells reactive to several self antigens (i.e. ANA polyreactive B cells) are actually selected into the B cell repertoire rather than removed (146). In fact, ANA polyreative B cells from SLE patients have longer V_H CDR3 lengths than non-reactive mature GC selected B cells (147). It became of interest then to determine if the V_H CDR3 length of CSF B cells from MS patients was also increased. Indeed, the average V_H CDR3 length in CSF B cells from these MS patients was 13.9 ± 0.29 amino acids (AA), which was significantly longer than the average V_H CDR3 length of PB B cells from HCPB (p=.004, Figure 4-08, A and B), but similar to that of ANA polyreactive B cells from SLE patients. Thus, longer V_H CDR3 lengths are not unique to ANA polyreactive B cells, Interestingly, the average heavy chain CDR3 length of CIS 132 was significantly shorter than that of the 8 MS and 2 OND CSF B cell populations. Whether these longer V_H CDR3 lengths of CSF B cells from MS patients can be associated with autoreactivity (as it has been with ANA polyreactive B cells) remains to be determined.

VH CDR3 net charge composition is intact in the CSF B cell repertoires of MS patients

ANA polyreactive B cells also have a high propensity towards accrual of basic

charges in their heavy chain CDR3s, presumably to enhance binding of DNA, which is acidic (147). Hence, one approach to determining if a patient may be prone to this autoimmune state would be to analyze the heavy chain CDR3 charge compositions. We calculated the net charges of the CDR3 regions from the heavy chain rearrangements of the eight MS patients (Figure 4-09) and compared the results to the mature B cell repertoire defined in Wardemann et al. 2003 (147) (henceforth referred to as "mature B cell population"). The majority (70% average) of heavy chain CDR3s from the eight MS CSF B cell antibody repertoires had net acidic charges in the range of −1 to −5. Approximately 80% of the mature B cell population had net acidic charges in the heavy chain CDR3, which was similar to the MS repertoires ($p=0.27$), with a charge range of −1 to −4. Those heavy chain CDR3s that were not net acidic were a mixture of no net charge "0" and some basic net "+" charges. Thus, the net charge composition of heavy chain CDR3s from CSF B cells of MS patients is similar to the mature B cell population. Given these observations, it was of interest to investigate whether the two OND and one CIS patient CSF B cell repertoires aligned with the MS patients, mature B cells and/or ANA polyreactive B cells with regard to net acidic charge of heavy chain CDR3s. Interestingly, the frequency of net acidic charged heavy chain CDR3s from the two OND and CIS patient CSF B cell antibody repertoires was decreased in comparison to the MS patients ($p=0.01$, Figure 4-09). In fact, approximately half of the heavy chain CDR3s had net acidic charges "−", and the other half had no net charge "0". This indicated that heavy chain rearrangements with net acidic charges in their CDR3s are more prevalent in the CSF B cell heavy chain repertoires of patients with clinically definite MS and mature peripheral B cells from healthy donors, compared to the CSF B cell heavy chain

repertoires of non-MS patients. This selection could not be attributed to bias in D segment usage (which constitutes a portion of the CDR3), as the frequency of D segment distributions was no different in the MS patients compared to the OND patients (data not shown). Of note, the only MS heavy chain CDR3 analysis that was similar to the OND/CIS pattern (half net acidic, half net no charge) was M368, an observation that we cannot explain at this time either clinically or by Ig repertoire analysis.

The B cell antibody repertoires from patients with Optic Neuritis (ON, also categorized as Clinically Isolated Syndrome, and which commonly convert to CDMS) have been reported and demonstrate clonal expansion (148). For comparison purposes, we calculated the net charges of the CDR3s from the heavy chain rearrangements of the CD19+ CSF B cell population reported for one of these patients (ON03-5, Table 3 in reference (148)) and compared it to the frequencies of net acidic heavy chain CDR3s from our MS, CIS and OND patients. Interestingly, only 50% (n=32) of heavy chain CDR3s in the CSF B cell population from this ON patient were acidic, indicating a lack of skewing towards net acidic heavy chain CDR3s in this patient, just as we had observed in CIS132 and two OND patients. Furthermore, this frequency was statistically lower than that of MS patients (50% vs. 70%, $p=0.01$), again emphasizing that acidic heavy chain CDR3s are not enriched in CSF B cells from these non-MS patients. Of note, this particular ON patient had only one lesion by MRI. It would be interesting to determine whether the ON patient in the Haubold study that had 17 lesions by MRI (ON03-3) would have a frequency of acidic heavy chain CDR3s that is more similar to MS patients with extensive lesion load.

Discussion

We initially focused on the overall mutational frequencies and targeting of mutations to CDRs because we had observed that clonally expanded CSF B cells isolated from MS patients had high mutational frequencies and a tendency towards diminished mutational targeting, especially as it related to RGYW/WRCY motif targeting within CDRs (13). The mutational analysis of CSF B cells isolated from eight MS, two OND and one CIS patient demonstrated that even the inclusive CSF B cell repertoires from such patients have unusually high mutational frequencies, likely reflecting the higher frequency of memory ($CD27^+$) B cells in CSF compared to HCPB (Figure 4-01). Memory B cells have already been selected based on their antigen specificity, and so it is possible that even those memory B cells that we did not identify as members of a clonal population are also reactive to antigens within the CNS, and we have simply caught them at a moment in time when they have not undergone the final step of activation and differentiation into antibody secreting cells. Indeed, it has recently been reported that expansion of short-lived plasmablasts (which maintain CD19 expression) in the CSF of MS patients correlates with inflammation in MS (138), and that the likely source of these cells is persistent memory $CD19^+$ B cells in the CNS.

Our data suggest that the CSF B cells from MS patients were selected in the context of germinal centers, since CDR mutation targeting characteristic of GC-derived B cells was intact in these populations (see Figure 4-05, Figure 4-06, Table 4-03, Table 4-04, and Table 4-05). However, our previous data had suggested that these targeting mechanisms were not consistently intact in the CSF B cell clones found in the MS patients, especially targeting to RGYW/WRCY motifs within CDRs. Table 4-06 provides

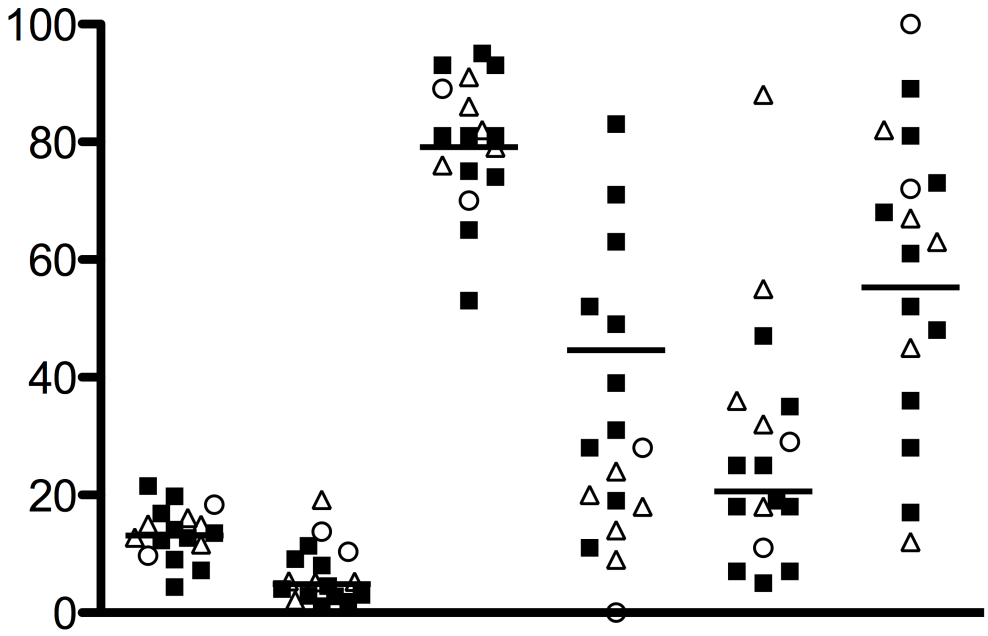
a summary of mutational targeting of heavy chain CSF B cell clonal repertoires from MS patients which illustrates that although the majority of clones do demonstrate targeting of mutations to CDRs, targeting of these CDR mutations within RGYW/WRCY motifs was not as impressively observed as it is in the overall CSF B cell repertoires from these same patients, or in HCPB. Although targeting of mutations to RGYW/WRCY motifs within CDRs is only one measurement, it is a very well established readout of GC-dependent mutation accumulation (111-113, 134, 149-154). Thus, its diminished impact on these clonally expanded CSF B cells from MS patients supports the possibility that clonal expansion of self-reactive CSF B cells may occur independent of classical germinal centers. This is not an incongruous concept, since others have reported that self-reactive B cells in SLE mouse models do expand outside the context of GCs (155). In addition, Corcione et. al. (156) have proposed that one possible mechanism of CSF B cell repertoire generation is by activated B cells directly differentiating into short-lived plasma cells without passing through germinal centers, which would parallel current opinions within the SLE community. Of note, the B cell repertoires that we studied here would have included these short-lived plasma cells, which are CD19+.

Alternatively, (and more likely) clonal expansion of CSF B cells occurred in the context of GCs, but perhaps these particular GCs do not confer the same restriction on B cell selection (including particular mutation patterns) as classical peripheral secondary lymphoid follicles confer on B cells. Serafini et al. have documented that GC-like follicles can be detected in the meninges of MS patients that contain severe demyelinating lesions, and such ectopic (or non-classical) GCs may be permissive repositories for clonal expansion of autoreactive B cells in the CNS. Unfortunately, we

cannot determine from this analysis whether the CSF B cells we analyzed here matriculated from follicles within the CNS, or even whether germinal centers could be detected in any of these patients. However, we can confirm that the CSF B cell repertoire in these MS patients adheres to mutation patterns consistent with GC-derived B cells, and that in some cases, clonally expanded CSF B cells from some MS patients do not adhere to mutation patterns consistent with GC-derived B cells, especially as they relate to RGYW/WRCY targeting within CDRs. Also recall that the frequency of net acidic CDR3 heavy chains from the two OND patients, one CIS patient, and the Haubold optic neuritis patient was somewhat decreased in comparison to CDMS, and mature B cells. This observation suggests that those B cells with net acidic charges in their heavy chain CDR3s may have an advantage to enter and reside in CNS of patients with CDMS, but not in the CNS of patients who are either "preclinical" for MS, or have some other neurological disease. It is possible that these CSF B cells also have a higher propensity to bind proteins with net basic charges, but antigen-antibody interactions are more likely dependent on the position of particular acidic residues, and not on net acidic charge. Perhaps there is something different in the milieu of patients with clinically definite MS that permits enrichment of these cells in clinically definite MS patients, such as the degree of inflammation and/or lesion load within the CNS. Preventing enrichment of CSF B cells in MS patients that may be poised to recognize basic proteins prevalent in the CNS may provide an avenue to dampen potential contributions of B cells to the pathogenesis of MS.

In any case, these results demonstrate that the overall B cell repertoire in the CSF of MS patients is derived from a classic germinal center reaction, and may have

matriculated from the periphery. The next step then, was to examine the functional capacity of peripheral B cells from MS patients to present neuro-antigens to autologous T cells. This would not prove that neuro-antigen specific CSF B cells matriculate from the periphery, but would reveal whether the peripheral B cell pool harbors neuro-antigen specific B cells that can influence T cell responses in MS patients.



	CD19$^+$		Naïve (CD27$^-$)		Memory (CD27$^+$)	
	PBL	CSF	PBL	CSF	PBL	CSF
MS	13.1 ±1.8%	4.9 ±1.1%	79.1 ±4.4%	44.6 ±7.8%	20.6 ±4.4%	55.3 ±7.8%
OND	14.1 ±0.9%	7.5 ±3.4%	82.8 ±3.0%	45.8 ±13.5%	17 ±2.9%	53.8 ±13.4%
CIS	14.1 ±6.2%	12.1 ±2.4%	79.5 ±13.4%	14 ±19.8%	20 ±12.7%	86 ±19.8%

FIGURE 4-01. Frequency of B cell subsets in PB and CSF of MS, OND, and CIS patients. Cells isolated from PB and CSF of MS patients (filled box symbol, $n = 10$), OND patients (open triangle symbol, $n = 5$), and CIS patients (open circle symbol, n = 2) were analyzed by flow cytometry for expression of CD19 and CD27. Total CD19$^+$ cells (defined as a percentage of total lymphocytes) were analyzed for expression of CD27 to determine the percentage of naïve (CD27$^-$) and memory (CD27$^+$) subsets within the CD19$^+$ population. Mean frequencies (± standard error) of each subset are as indicated below the *x*-axis. Solid bars in graph indicate MS mean frequency.

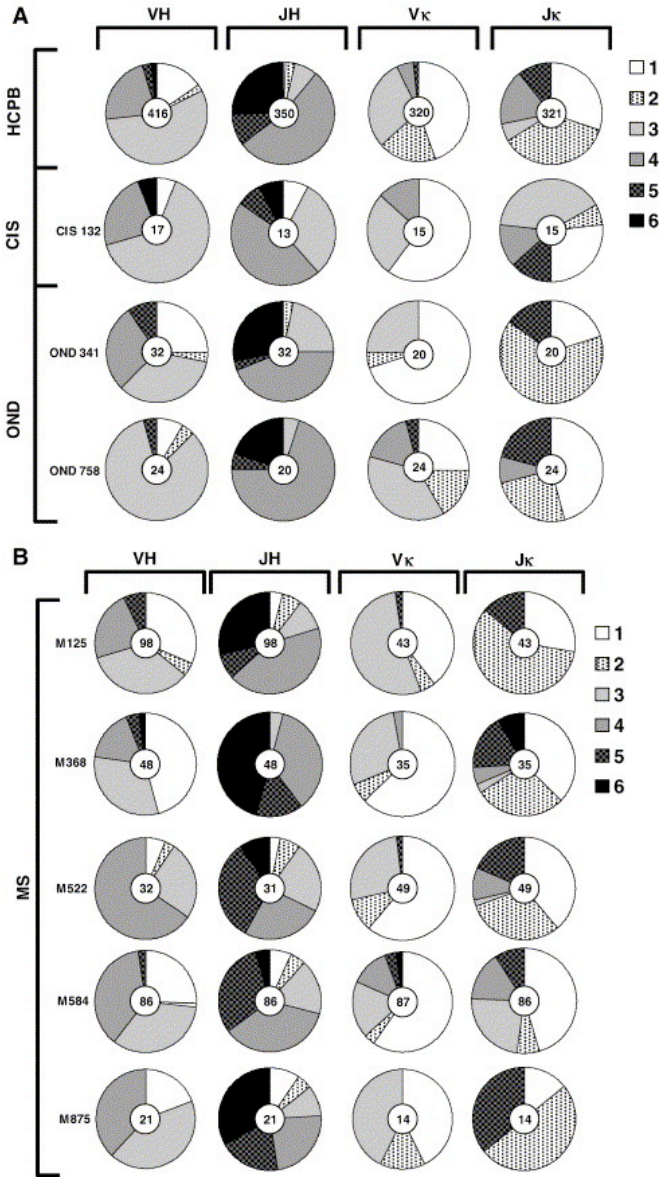


FIGURE 4-02. Heavy and Kappa Variable and Junction Chain usage in HCPB, CIS CSF, OND CSF, and MS CSF B cells.

Antibody rearrangements were analyzed from single B cells from HCPB, and single CSF B cells from one CIS and two OND patients (A) and five MS patients (B) as indicated and as described in Chapter 3. Heavy (H) and Kappa (κ), Variable (V) and Junction (J) segment usage in HCPB and CSF $CD19^{+}$ B cells are presented as a percentage of total sequences obtained from each patient sample. Center oval "n" indicates number of productive sequences obtained and analyzed. H chain V- and J-segment data is summarized on the left, κ chain V- and J-segment data on the right.

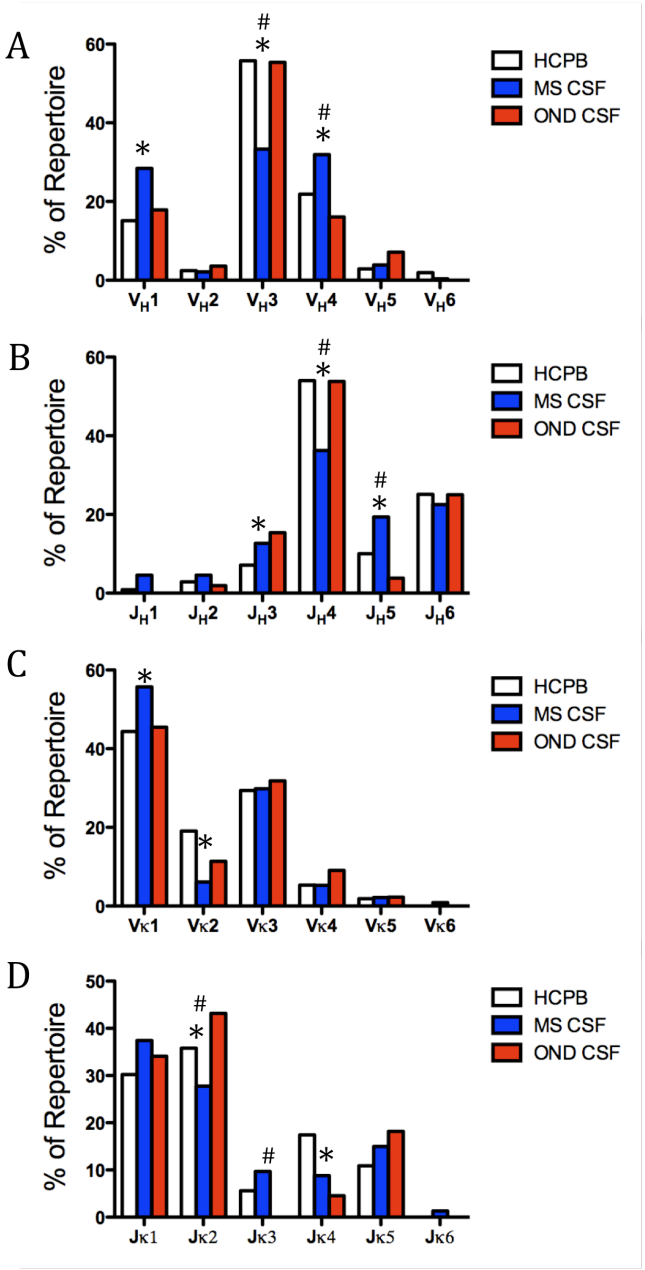


FIGURE 4-03. Certain VH, JH, Vk and Jk family usages in MS CSF B cells are skewed from HCPB and OND CSF. Antibody rearrangements from individual donors (represented in Figure 4-02) in the HCPB, OND, and MS CSF B cell databases were combined and compared according to V_H (A), J_H (B), Vκ (C), and Jκ (D) family usage, presented as a percentage of the total repertoire. * = $p \leq 0.05$, χ^2-test, MS CSF vs. HCPB. # = $p \leq 0.05$, χ^2-test, MS CSF vs. OND CSF.

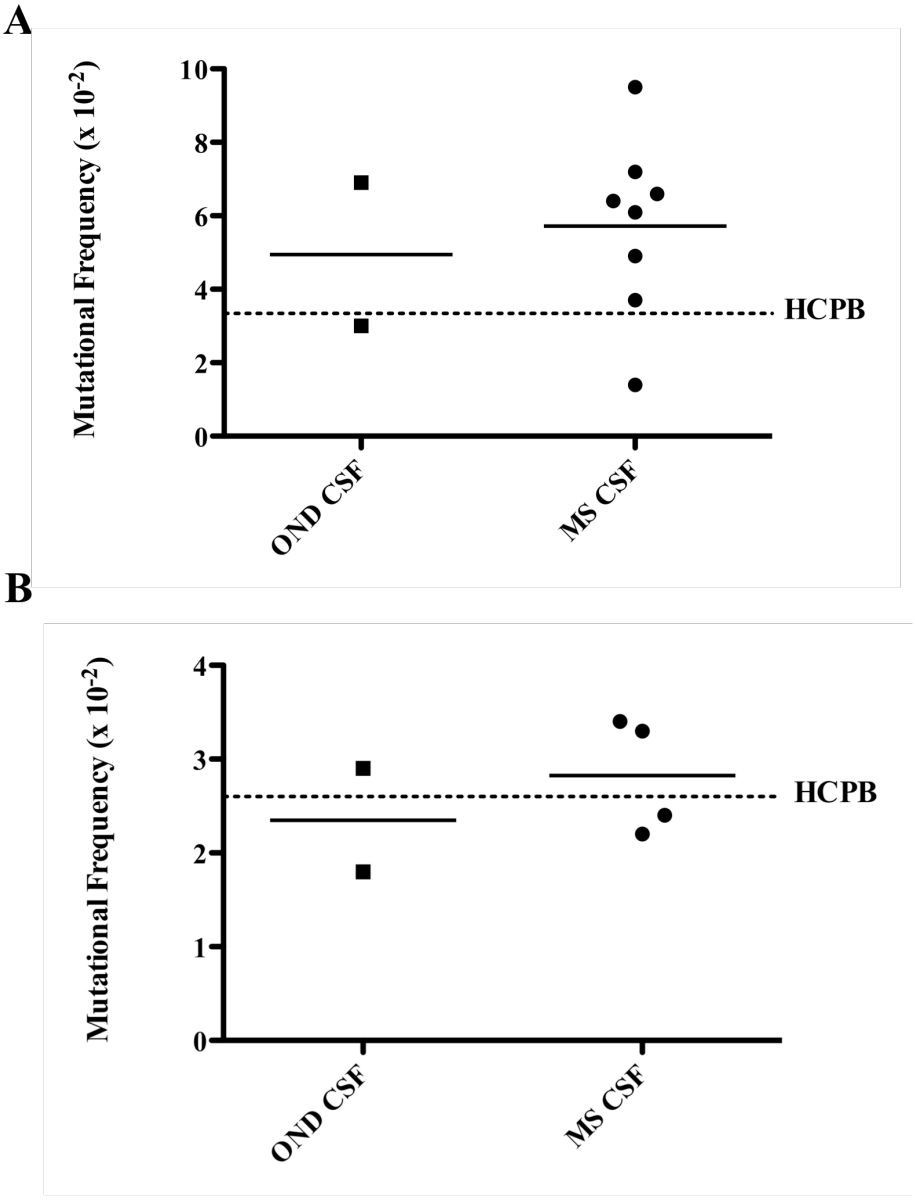


FIGURE 4-04. Mutational frequency of CSF B cell variable genes. Variable gene sequences obtained from single B cells isolated from the CSF of OND and MS patient CSF were compared to un-mutated germline Variable gene sequences. Mutational frequency was calculated by averaging the number of nucleotide mutations in CSF B cell genes divided by the total number of nucleotides within the variable gene and multiplied by 100 in the Heavy chain (A) and Kappa light chain (B). Dotted line represents the average mutational frequency in HCPB.

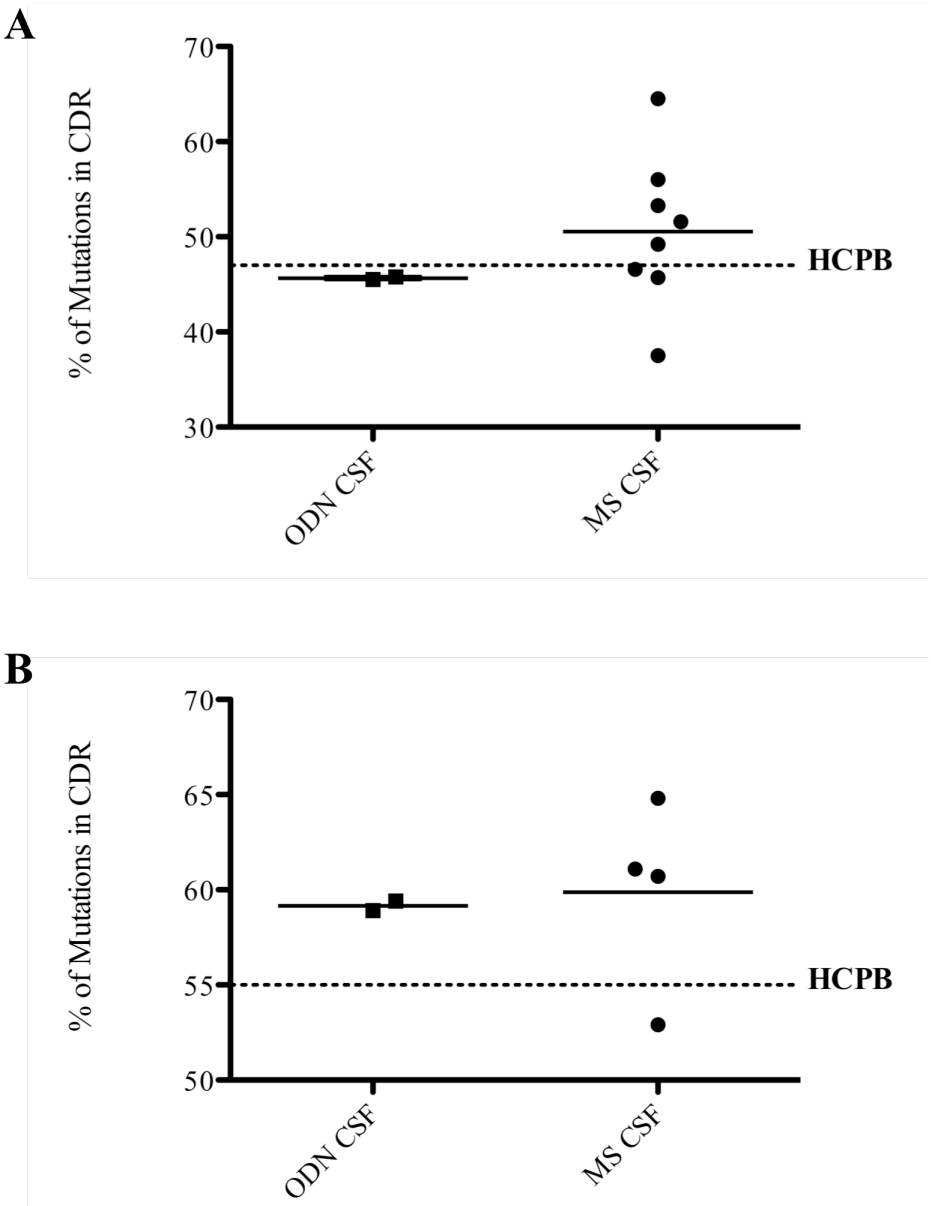


FIGURE 4-05. Percentage of mutations within CDRs of CSF B cell Variable genes. Mutations in Variable gene sequences obtained from single B cells isolated from the CSF of OND and MS patient CSF were analyzed for targeting to the CDR. The percentage of mutations in the CDR was calculated by dividing the number nucleotide mutations in the CDR regions divided by the total number of nucleotide mutations and multiplied by 100 in the Heavy chain (A) and Kappa light chain (B). Dotted line represents the average percentage of mutations in the CDR in HCPB.

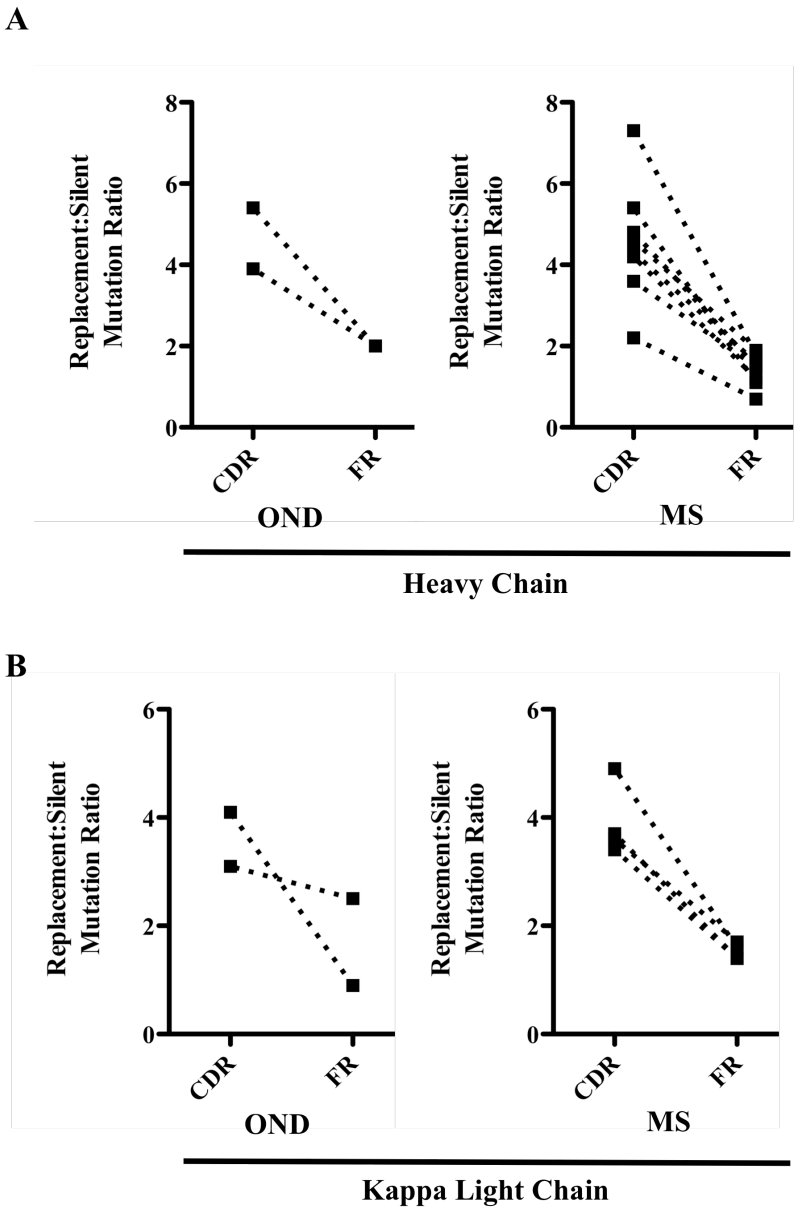


FIGURE 4-06. Replacement:Silent mutation ratio in CSF B cell Variable genes. Nucleotide mutations that resulted in an amino acid change during translation (replacement mutation, R) were compared to nucleotide mutations that did not result in an amino acid change during translation (silent mutation; S) in both the CDR and FR Heavy Chain (A) and Kappa light chain (B) Variable genes from OND and MS CSF B cells. The number of replacement mutations in the CDR was divided by the number of silent mutations in the CDR to obtain an R:S ratio. This was also done for the FR. Dotted lines connect the same patient.

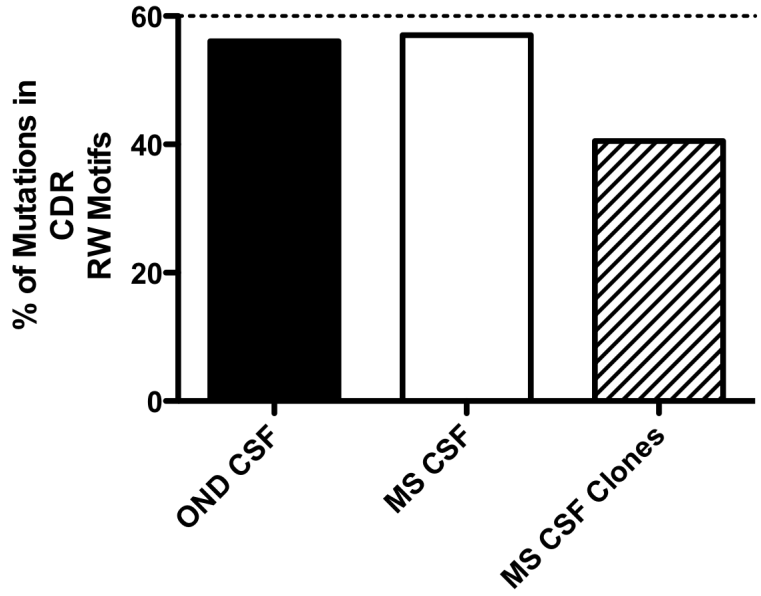


FIGURE 4-07. Overall percentage of mutations in RGWY/WRCY (RW) motifs. The percentage of mutations within CDR RW motifs in heavy chain variable genes from CSF B cells was compared between the overall OND, the overall MS CSF repertoire and the MS CSF clonal population.

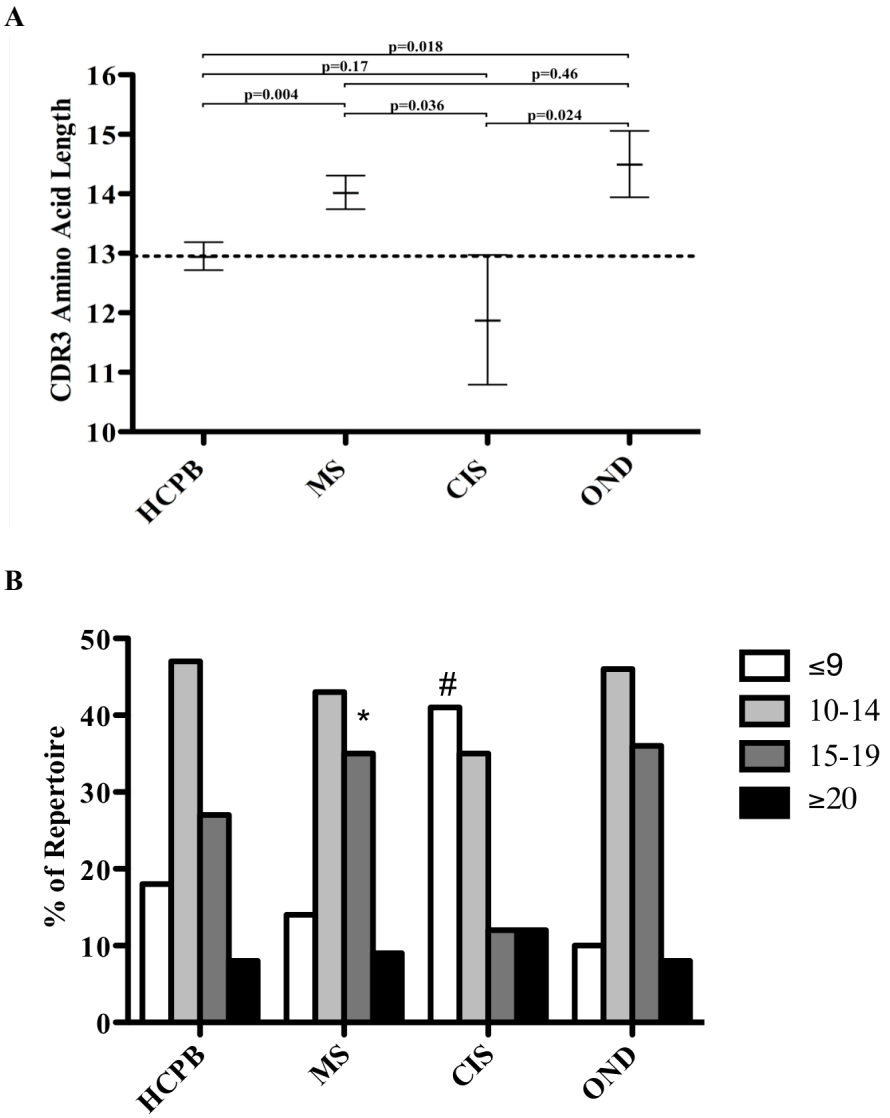


FIGURE 4-08. Heavy Chain CDR3 amino acid length. (A) Mean CDR3 lengths from heavy chain sequences of CSF B cells from (n=317), eight MS (n=302), one CIS (n=17) and two OND (n=50) patients and HCPB (n=317) were determined and plotted (dotted line represents, HCPB mean, error bars +/− standard error, p values calculated using Mann Whitney t-test). (B) Sequences from panel A were separated into 4 groups of varying length and plotted according to group length. "*" indicates significant deviation from HCPB ($p \leq 0.05$, χ^2-test), "#" indicates significant deviation from all groups ($p \leq 0.05$, χ^2-test).

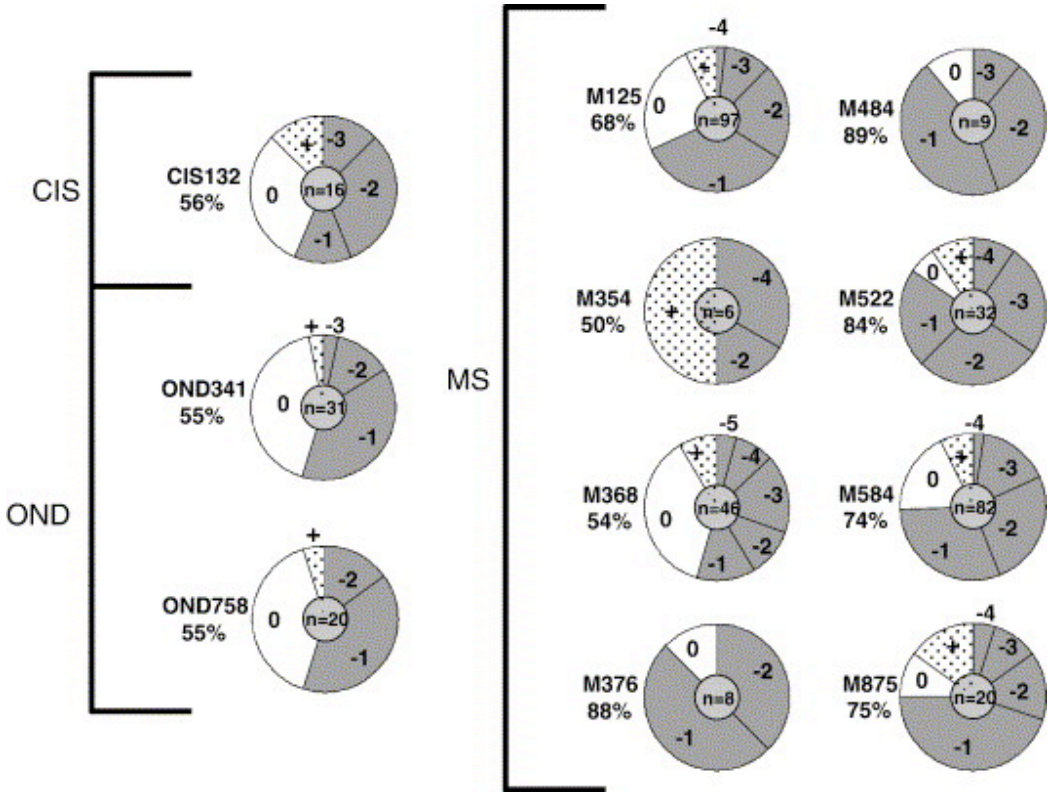


FIGURE 4-09. Heavy Chain CDR3 total amino acid charge. Amino acid sequence of heavy chain CDR3s of the CSF B cells isolated from MS, CIS and OND patients were analyzed to determine total CDR3 charge at pH 7. Charges are grouped from each patient repertoire and listed as a proportion of total sequences that were analyzed from each patient. Center circle "n" indicates number of sequences included in the analysis.

TABLE 4-01. Naïve:Memory Ratios of B cells from Flow Cytometric Analysis

	MS	OND	Comparison of MS to OND
PB Naïve B:Memory B	3.8	4.8	p=0.51
CSF Naïve B:Memory B	0.8	0.9	p=0.84
Comparison of PB to CSF	p= 0.0001	p= 0.0001	

TABLE 4-02. Overall mutational frequencies from heavy and kappa chain CSF B cells.

	Heavy chain			Kappa chain		
	n	MF ($\times 10^{-2}$)	HCPB COMP	n	MF ($\times 10^{-2}$)	HCPB COMP
HCPB		3.34	NA		2.6	NA
CIS132	17	4.8	↑	15	3.8	↑
OND341	32	6.9	↑	20	2.9	=
OND758	18	3	=	16	1.8	↓
M125	100	4.9	↑	43	2.2	↓
M354	6	6.1	↑	1	n.d.	n.d.
M368	48	6.6	↑	35	3.4	↑
M376	8	7.2	↑	6	n.d.	n.d.
M484	9	9.5	↑	8	n.d.	n.d.
M522	32	6.4	↑	49	2.4	=
M584	86	3.7	=	87	3.3	↑
M875	21	1.4	↓	14	n.d.	n.d.
AVE MS		5	↑		2.9	↑

Abbreviations: HCPB COMP, Comparison to HCPB; MF, Mutational Frequency; n, number of sequences

TABLE 4-03. Percent mutations in CDRs of heavy and kappa chains from CSF B cells.

	Heavy chain			Kappa chain		
	n	%M CDR	HCPB COMP	n	%M CDR	HCPB COMP
HCPB	484	47	NA	754	55	NA
CIS132	182	52.2	=	124	58.1	=
OND341	452	45.8	=	129	58.9	=
OND758	123	45.5	=	64	59.4	=
M125	891	53.3	↑	224	60.7	=
M354	73	46.6	=	2	n.d.	n.d.
M368	643	56	↑	263	52.9	=
M376	95	51.6	=	37	n.d.	n.d.
M484	164	45.7	=	75	n.d.	n.d.
M522	439	49.2	=	250	64.8	↑
M584	650	64.5	↑	632	61.1	↑
M875	64	37.5	=	7	n.d.	n.d.
MS Overall		54.7	↑		60.1	↑

Abbreviations: HCPB COMP, Comparison to HCPB; %M CDR, percent of total mutations in CDR regions; n, number of sequences

TABLE 4-04. Replacement:silent ratios in heavy and kappa chains.

	Heavy chain			Kappa chain		
	CDR	FR	Target	CDR	FR	Target
CIS 132	3.4	1.4	CDR	3.1	1.3	CDR
OND341	5.4	2	CDR	3.1	2.5	=
OND758	3.9	2	=	4.1	0.9	CDR
M125	3.6	1.5	CDR	3.6	1.7	CDR
M354	7.3	1.8	CDR	n.d.	n.d.	n.d.
M368	4.3	1.6	CDR	4.9	1.6	CDR
M376	4.8	1.5	CDR	n.d.	n.d.	n.d.
M484	4.6	1.9	CDR	n.d.	n.d.	n.d.
M522	4.2	1.2	CDR	3.4	1.4	CDR
M584	5.4	1.1	CDR	3.7	1.4	CDR
M875	2.2	0.7	CDR	n.d.	n.d.	n.d.
AVE MS	3.6	1.4	CDR	3.8	1.2	CDR

Abbreviations: CDR, complementary determining region; FR, framework region

TABLE 4-05. Percent mutations in RGYW/WRCY motifs of heavy and kappa repertoires from CSF B cells.

	Heavy chain				Kappa chain			
	CDR		FR		CDR		FR	
	%M in RW	HCPB COMP	%M in RW	HCPB COMP	%M in RW	HCPB COMP	%M in RW	HCPB COMP
HCPB	60	NA	19	NA	60	NA	49	NA
CIS 132	58.3	=	28.2	=	59.7	=	38.8	=
OND 341	53.9	=	39.2	↑	60.7	=	49	=
OND 758	47.7	=	33.3	=	36.1	=	47.8	=
M125	64.3	=	41.9	↑	65.2	=	53.4	=
M354	42.4	↓	14.7	=	n.d.	n.d.	n.d.	n.d.
M368	63.3	=	35.2	↑	54	=	51.6	=
M376	51.4	=	47.4	↑	n.d.	n.d.	n.d.	n.d.
M484	58.2	=	40	↑	n.d.	n.d.	n.d.	n.d.
M522	62.4	=	45.3	↑	53.8	=	30.7	↓
M584	45.4	↓	32.3	↑	55.4	=	44.7	=
M875	36.8	↓	31.4	=	n.d.	n.d.	n.d.	n.d.
AVE MS	57.7	=	37.9	↑	56.5	=	45.4	=

Abbreviations: CDR, complementary determining region; FR, framework region; %M in RW, % of mutations in RGYW/WRCY motifs; HCPB COMP, comparison to HCPB

TABLE 4-06. Mutation targeting summary of heavy chain rearrangements from CSF B cell clones

	N	Percent	n	Percent
M125	6/7	85	2/7	29
M368	5/7	71	2/7	29
M522	4/8	50	3/8	38
M584	10/15	67	8/15	53

CHAPTER FIVE
Results

IMPACT OF CD40L-ACTIVATED PERIPHERAL BLOOD B CELLS ON T CELL ACTIVATION IN MULTIPLE SCLEROSIS

The following study has been published in the journal, *Clinical Immunology*. Harp C.T., Lovett-Racke A.E., Racke M.K., Frohman E.M., Monson N.L., *Impact of myelin-specific antigen presenting B cells on T cell activation in multiple sclerosis,* volume 128, issue 3, pages 382-391, and is reproduced here with the permission of *Elsevier*; license number 2264350746440.

Introduction

The role of B cells in the context of MS pathogenesis has remained controversial because it is unclear what the function of B cells is in MS with regards to antigen presentation, cytokine secretion or autoantibody secretion. It has been hypothesized that the population of B cells most relevant to disease pathogenesis are those present in the CNS and not in the periphery. This is partly because antibody secreting B cells that extravasate into the CNS parenchyma and perivascular regions of the CNS can directly mediate inflammation in the absence of blood brain barrier breakdown, can contribute directly to the inflammatory cytokine mileu in the CNS as well as direct activation of CNS T cells through antigen presentation (157). The origin of these CNS B cells and how they managed to expand in response to neuro-antigens is unknown. Germinal center like

structures have been observed in meninges of SPMS patients (132, 158), but it has not been determined if B cells emanating from this type of reaction in the CNS show atypical or typical antigen driven selection. Ectopic germinal centers have also been detected in the synovium of rheumatoid arthritis patients, however B cells derived from these germinal centers do not show evidence of antigen driven affinity maturation (130). In the previous chapter, I presented evidence that the overall B cell antibody repertoire excluding B cell clones, showed evidence of antigen driven selection typical of B cells selected in the context of peripheral germinal center reactions. This data suggested that the CSF B cell pool may have matriculated from the periphery where they underwent primary affinity maturation in a peripheral germinal center reaction in a T-dependent manner. Antigen selected B cells that are derived from a peripheral B cell germinal center reaction develop into long lived circulating memory B cells, or long lived plasma cells (159), which happen to dominate the CNS of patients with MS. This data warranted investigation of whether the peripheral circulating B cell pool of MS patients can function as neuro-antigen specific antigen presenting cells in the promotion of neuro-antigen specific T cell activation. Thus, the focus of this study was to evaluate the ability of B cells from MS patients to be competent APCs in a neuro-antigen specific manner.

Compared to activated DCs and/or monocytes, *ex vivo*, human peripheral blood B cells (PBBC) do not constitutively express the level of costimulatory molecules necessary to be efficient APCs and do not survive in culture for long periods of time without stimulation. Therefore we chose to activate B cells from MS patients and healthy donors using a classic antigen independent *in vitro* stimulation approach with CD40L and IL-4 to study their ability to be efficient neuro-antigen APCs to autologous T cells. The

results of these studies are summarized in the next section.

Results

Both CD40L/IL-4 and CpG ODN/IL-2 stimulation sufficiently activate human B cells to express costimulatory molecules and upregulate MHC II

PBBC were stimulated with CD40L/IL-4, to mimic T-dependent B cell activation, then evaluated for upregulation of CD80 and MHC II (HLA-DR) which are classical markers of B cell activation and are required to instigate potential B:T cell interactions. We found that 8 days of stimulation were required for maximum expression of CD80 and HLA-DR, which was maintained for the duration of *in vitro* stimulation using this approach. For some patient samples, the number of B cells that were required to perform the experiment could not be obtained on day 8 of stimulation because of the low starting number of B cells obtained from the *ex vivo* samples. This required us to increase the time of stimulation for some patients up to 16 days, with negligible change in the activation status of the B cells. After 8–16 days in culture with CD40L/IL-4, MS PBBC readily upregulated the costimulatory molecule CD80 based on evaluation of the mean fluorescence intensity (MFI) of CD80 by flow cytometry (0.61 ± 0.05 on Day 0 vs. 1.59 ± 0.05 on Day 8–16, $p \leq 0.0001$, Figure 5-01, A), as well as on HD PBBC (0.58 ± 0.06 on Day 0 vs. 1.57 ± 0.10 on Day 8–16, $p \leq 0.0002$, Figure 5-01, A). These results were confirmed by earlier reports detailing that the expression of CD80 is detectable on the majority of PBBC after CD40L/IL-4 stimulation *in vitro* (160). While

HLA-DR is constitutively expressed on the majority of PBBC *ex vivo*, CD40L/IL-4 stimulation *in vitro* resulted in the upregulation of MHC II molecules on both MS PBBC (2.25 ± 0.13 on day 0 vs. 3.13 ± 0.06 on day 8–16, $p \leq 0.004$) and HD PBBC (2.17 ± 0.19 on day 0 vs. 3.11 ± 0.05 on day 8–16, $p \leq 0.01$) based on flow cytometric MFI of HLA-DR (Figure 5-01, B).

Previous data from other laboratories have suggested that T cell stimulation such as CD40L/IL-4, activates human memory B cells more robustly than naïve B cells (77, 161). In our hands, both naïve ($CD19^{+}CD27^{-}$) and memory B cells ($CD19^{+}CD27^{+}$) upregulated CD80 and HLA-DR in response to CD40L/IL-4, although memory B cells highly upregulated HLA-DR compared to naïve B cells based on MFI ratios (Table 5-01). In addition, analysis of the naïve:memory (N:M) B cell ratio *ex vivo* indicated that the percentage of memory B cells from MS PBBCs following CD40L/IL-4 stimulation *in vitro* for 8–16 days, remained similar to the *ex vivo* percentage (compare 26.1 ± 4.2% to 23.9 ± 7.3%, $p = 0.90$, data not shown). In most cases however, the percentage of naïve B cells tended to increase at the expense of memory B cells at later time points (Table 5-02), even though statistical significance was not met. CD40L/IL-4 stimulation did not result in the formation of $CD138^{+}$ plasma cells (data not shown).

In order to mimic T independent B cell activation through TLR9 stimulation, we also generated CpG ODN/IL-2 activated B cells from the same donors that we had generated CD40/IL-4 activated B cells. CpG ODN/IL-2 PBBCs from both MS and HD upregulated CD80 (0.45 ± 0.08 day 0 vs. 1.01 ± 0.08 day 8, $p \leq 0.0003$, Figure 5-01, A). However, the increase in CD80 expression by log MFI upon CpG ODN/IL-2 stimulation

was significantly lower than with CD40L/IL-4 stimulation (1.01 ± 0.08 vs. 3.13 ± 0.06, $p \leq 0.0003$, Figure 5-01). HLA-DR expression by log MFI of PBBCs was slightly upregulated on MS PBBCs following CpG ODN/IL-2 stimulation (2.23 ± 0.14 on day 0 vs. 2.68 ± 0.14, Figure 5-01 B), but not to the same extent as MS PBBCs following CD40L/IL-4 activation and expansion (compare 2.68 ± 0.14 with CpG ODN/IL-2 and 3.13 ± 0.06 with CD40L/IL-4, $p \leq 0.02$, Figure 5-01 A and B). This mild upregulation of CD80 and HLA-DR upon CpG ODN/IL-2 stimulation was observed on both memory and naïve B cells (Table 5-01), and the *ex vivo* N:M B cell ratio *after in vitro stimulation* was maintained (Table 5-02, $p = 0.056$).

CD40L/IL-4-activated B cells but not CpG ODN/IL-2 activated B cells induce autologous $CD4^+$ T cell activation and proliferation

To test the capacity of these CD40L/IL-4 and CpG ODN/IL-2 activated B cells to stimulate recall responses by autologous T cells in an antigen specific manner, T cells from both HD donors and MS patients were incubated with mumps antigen and either autologous CD40L/IL-4 or CpG ODN/IL-2 activated B cells. As an early marker of activation, we chose to examine CD69 expression on $CD4^+$ T cells at 48 h which predicts if recall antigens will trigger proliferation in a mixed lymphocyte reaction (MLR) (162). Basal CD69 expression on ex vivo CD4+ and CD8+ on T cells derived from HD and MS patients is listed in Table 5-03. However, since CD69 upregulation does not correlate with the magnitude of proliferation, the extent of $CD4^+$ T cell proliferation was examined by CFSE dilution at 7 days. T cells cultured alone or in the presence of mumps antigen did not upregulate CD69 or proliferate (data not shown). Upregulation of CD69 (Figure

5-02, A) and robust $CD4^+$ T cell proliferation (Figure 5-02, B) was apparent in all samples, although to varying degrees, when stimulated in the presence of CD40L/IL-4 B-APC + mumps. In contrast, CpG ODN/IL-2 activated B cells induced poor $CD4^+$ recall responses to mumps based on CD69 expression (Figure 5-02, A) and proliferation (Figure 5-02, B).

CD40L/IL-4-activated B cells induce upregulation of CD69 and proliferation of autologous $CD4^+$ T cells through neuro-antigen specific presentation

PBBC generated through CD40L/IL-4 activation (B-APC) were capable of presenting mumps recall antigens to autologous T cells from the PB of both HD donors and MS patients. Therefore, our next objective was to determine whether these B-APCs could present myelin basic protein (MBP), a well-characterized CNS autoantigen in MS, to autologous T cells. We predicted that a proportion of both HD and MS patient T cells would respond to B-APC presenting MBP as both HD and MS patients are known to have a circulating pool of MBP-reactive T cells (36, 163). In addition, glatiramer acetate (GA), a well-known mimic of MBP used as a therapeutic agent to treat MS was included either alone or in combination with MBP to study how GA may effect B-APC presentation of MBP to autologous T cells. B:T MLRs were setup as described in Materials and Methods. Positive responses were defined using a normalization formula to adjust for background proliferation (Figure 5-03). CD69 expression and CFSE dilution were used to assay $CD4^+$ T cell activation and proliferative responses in the presence of B-APC.

CD69 was upregulated on the $CD4^+$ T cell subset from all HD donors and MS patients in response to B-APC + mumps (Figure 5-04, A). CD69 was also upregulated on the $CD4^+$ T cell subsets of 4/6 MS patients (MS-1, MS-2, MS-5, MS-6) and 2/4 HDs (HD3, HD4) in response to B-APC + MBP (Figure 5-04, A). $CD4^+$ T cells from 4/6 MS patients (MS-1, MS-4, MS-5, MS-6) and 3/4 HDs (HD2, HD3, HD4) responded to B-APC + GA by upregulation of CD69, and $CD4^+$ T cells from 3/6 MS patients (MS-3, MS-5, MS-6) and all 4 HDs responded to B-APC + MBP + GA by upregulation of CD69 (Figure 5-04, A). Proliferation as measured by CFSE dilution (Figure 5-04, B) demonstrated that $CD4^+$ T cells from all 6 MS patients and 5 HD donors proliferated in response to B-APC + mumps recall antigen, although to varying degrees. $CD4^+$ T cell proliferation in response to B-APC + MBP occurred in 3/6 MS patients (MS-1, MS-2, MS-6). Of those 3 MS patients that proliferated in response to B-APC + MBP, 2 of them (MS-1, MS-6) also responded by proliferation to B-APC + GA, which is a random copolymer of amino acids that was originally designed to have similar properties of MBP (164). Interestingly, MS-2, which had responded to B-APC + GA, had the most robust response to B-APC + MBP, while MS-3 and MS-4 did not respond to B-APC + MBP, but did respond by proliferation to B-APC + GA. Thus, responses to MBP presented by B-APC are not predictive of responses to GA presented by CD40L/IL-4 PBBC and vice versa. Addition of anti-HLA-DR antibody (which effectively blocks antigen presentation by B cells in the context of MHC II) suppressed $CD4^+$ T cell proliferation in all cases, indicating that B:T cell contact was at least partially required for T cell stimulation (Figure 5-07 and Table 5-04).

Frequency of MBP-reactive B cells does not correlate with CD4$^+$ T cell antigen specific proliferation

To examine whether the frequency of MBP-reactive B cells was a contributing factor to the variation in CD4$^+$ T cell proliferation in response to MBP alone, we adapted methodology currently being validated to assess the frequency of antigen specific B cells in patient samples where it is important to monitor memory B cell frequencies against particular infectious agents. The frequency of MBP-reactive B cells in 6 MS patients ranged from 0 to 3.92 ± 0.68% (Figure 5-05). None of the B-APC populations generated from HDs that we tested bound to MBP with detectable frequency (data not shown). Interestingly, MS-2 and MS-3 had the highest frequency of MBP-reactive B cells, but only CD4$^+$ T cells from MS-2 responded by proliferation to MBP. Thus, functional readout of T cell responses to MBP does not correlate with enriched frequencies of MBP-reactive B cells in the population.

CNS-antigen specific CD8$^+$ T cell proliferation in response to CD40L/IL-4 B-APCs is dependent on CD4$^+$ T cell proliferation

We also investigated whether CD8$^+$ T cells included in the B:T MLRs, proliferated in these conditions, and whether this effect was related to CD4$^+$ T cell activation. CD8$^+$ T cells from 5/6 MS patients and all HDs were able to proliferate in the presence of B-APC + mumps + CD4$^+$ T cells (Figure 5-06). CD8$^+$ T cell proliferation was observed in 4/6 patients (MS-1, MS-2, MS-4, MS-6) in response to B-APC + MBP, only one of which (MS-1) also responded to GA. In fact, CD8$^+$ T cell proliferation in

response to B-APC + GA was suppressed in 5/6 MS patients, consistent with others' work demonstrating that $CD8^+$ T cell proliferation in response to total APC + GA is suppressed in GA treatment naïve MS patients, compared to HDs (165). In addition, $CD8^+$ T cells that proliferated in response to MBP alone also proliferated in response to the combination of MBP + GA. This observation supports the concept that the combination of B-APC + MBP + GA does not mediate suppression of $CD8^+$ T cell proliferation in response to MBP (165). $CD8^+$ T cell proliferation was readily blocked by anti-HLA-DR antibodies (Figure 5-08 and Table 5-04), indicating that $CD4^+$ T cells are required for $CD8^+$ T cell responses to occur.

Discussion

In order to study B–T cell interactions in a neuro-antigen dependent fashion, we took advantage of a well-characterized *in vitro* culture system where large numbers of activated $CD80^+$ B cells could be generated using a combination of CD40L and IL-4 and subsequently used as APCs in vitro (160, 166, 167). Purification of B cells prior to CD40L/IL4 stimulation *in vitro* typically allowed for appropriate expansion and activation in 8 days and also limited skewing of the original *ex vivo* naïve:memory B cell ratio. We also generated CpG ODN/IL-2 activated B cells from the same donors in order to determine if B cells activated through TLR stimulatory signals would be as effective as B-APCs activated through T-dependent signals (CD40L/IL-4). However, in contrast to previous studies using allogeneic T cells (168), CpG ODN/IL-2 B cells did not efficiently perform as APCs to autologous T cells as evidenced by lack of T cell proliferation in response to CpG ODN/IL-2 B cells presenting mumps (Figure 5-02, A). This negligible

recall response to mumps could be attributed to the sub-maximal upregulation of CD80 and HLA-DR by CpG ODN/IL-2 stimulated B cells in comparison to CD40L/IL-4 activated B-APCs, implicating CD40L/IL-4 B cells as more potent APCs than CpG ODN/IL-2 activated B cells in both HD donors and MS patients. Furthermore, there is some evidence to suggest that CpG/IL-2 stimulation results in the differentiation of B cells into antibody secreting cells. For example, it was recently demonstrated that stimulation of naïve B cells with CpG ODN for 8 days resulted in a substantial increase in IgM secretion by naïve B cells, suggesting that longer-term CpG ODN stimulation results in the differentiation of naïve B cells into ASCs (168). This was in contrast to the demonstration that short term stimulation of naïve B cells (2 days) with CpG ODN resulted in a significant up regulation of CD80 and HLA-DR compared to resting B cells, and significantly increased the alloantigen presentation capacity of these B cells to stimulate CD4+ and CD8+ T cell proliferation (168). Perhaps one reason that I observed little antigen specific T cells proliferation in response to CpG ODN/IL-2 stimulated B cells was that the majority of these cells had differentiated into ASCs.

We were successful in generating large numbers of CD40L/IL-4 activated B cells from 6 MS patients and 5 HD donors within a relatively short time frame of stimulation (8–16 days). These B cells highly upregulated CD80 and HLA-DR which are necessary for efficient antigen presentation by B cells, and indeed supported robust $CD4^+$ T cell proliferation in an antigen specific manner. All 11 populations of CD40L/IL-4 B-APCs from both HD donors and MS patients supported $CD4^+$ T cell proliferation to mumps recall antigen, as expected, since mumps immunization is fairly uniform in the

population. However, $CD4^+$ T cell proliferation to MBP was more variable such that some, but not all MS patients and HD donors could respond. This finding is consistent with previous reports demonstrating that MBP-reactive T cells can be detected in both HD donors and MS patients (163, 166). Interestingly, MS-2, the patient with the most active disease in this study had the highest $CD4^+$ T cell proliferation to B-APC + MBP among MS patients included in this study, and comparatively had one of the highest percentages of MBP binding B-APCs. This finding warrants further study of B-APC function in patients undergoing exacerbations. In contrast, the single PPMS patient included in this study, MS-3, had the highest percentage of MBP binding B-APC, but $CD4^+$ T cell proliferation in response to B-APC + MBP was not observed. This finding is consistent with others demonstrating that T cell responses to some neuro-antigens are reduced in PPMS patients (169), and may indicate a regulatory function of B cells in PPMS patients in particular. One would predict then, that if B cells from PPMS patients are enriched for regulatory function, that B cell depletion would either have no effect on PPMS patients, or worsen their disease. Recent data indicates that Rituximab, a B cell depleting antibody, does not have efficacy in patients with PPMS (170) supporting the hypothesis that B cells in PPMS patients may not be pathogenic, but are instead enriched for regulatory function, and warrants further study.

Others have demonstrated that CD40L/IL-4 activated B cells can be used to expand autologous, PBMC primed, MBP-specific T cell lines (171). However, our study is unique in that we have demonstrated that B cells can efficiently activate resting MBP-specific T cells from some MS patients, and to our knowledge, is the first to demonstrate

that human B cells can effectively induce resting autologous T cells to become activated and proliferate in the presence of MBP.

Previous reports by others demonstrated that T cells from both HD donors and MS patients proliferate in response to GA in a standard MLR where irradiated autologous PBMCs are used as the APC population (165, 172). Our observations are consistent with these earlier reports and emphasize that CD40L/IL-4 B-APCs can directly mediate T cell responses to GA. It is likely that we did not observe uniform $CD4^+$ T cell responses in these B:T MLRs because the APC population for this study did not include other professional APCs (such as dendritic cells) that are more likely to uptake the multiple peptide variations included in the GA reagent and present them to autologous T cells. B cell antigen uptake is likely more restrictive, especially in the context of a peptide antigen, such as GA.

Interestingly with the exception of MS-5, a positive response to the combination of MBP + GA only required response to one of the antigens alone. For example, MS-2, which responded to MBP + GA, also responded to MBP alone, but not to GA alone. Thus, the combination of MBP + GA does not instigate a suppressive effect on $CD4^+$ T cell proliferation in response to MBP, or vice versa. Thus, we were not surprised to observe proliferation responses by $CD4^+$ T cells from HD5 to MBP + GA given that this donor had a robust $CD4^+$ T cell proliferative response to MBP alone. Similarly, one would predict that HD2 would proliferate in response to MBP + GA since this donor had a robust proliferative response to GA alone. H1, H3, and H4 also had a robust proliferative response to GA alone, suggesting that CD40L/IL-4 B-APCs from HD

donors do not instigate a suppressive effect on $CD4^+$ T cell proliferation in response to GA, paralleling what we observed consistently by $CD4^+$ T cells from MS patients.

Considering that MS is a disease of the central nervous system, the discovery that peripheral B cells can efficiently present CNS antigens to autologous T cells is important because it demonstrates that peripheral B cells specific for CNS antigens could potentially home to the CNS and act as efficient neuro-antigen specific APCs. Previous reports from our laboratory support this concept since the B cell receptors on the majority of CSF derived clonally expanded memory B cells (which would have the proper APC machinery) recognize MBP (173). In this respect, we would predict that peripheral B cells might also have a profound effect on CNS-derived T cells, and is substantiated by the efficacy of emerging therapeutics that directly affect B cells (14, 128, 174).

Of note, activation and proliferation of T cells in response to CD40L/IL-4 B-APCs + MBP was not uniformly observed in all MS patients. One interpretation of this observation is that the autoreactive T cell pool in those patients that did not respond does not include MBP-reactive T cells, but harbors autoreactive T cells to other CNS-derived antigens not included in this study. A second possibility is that B cells have negligible influence on autoreactive T cell responses, even in those cases where MBP-specific B cells and T cells are present. It is also possible that MBP-specific T cell stimulation by B-APCs + MBP occurs in a BCR independent manner as has been observed in murine models (94). Finally, B cells in these patients may be operating as regulatory B cells (B_{regs}). For example, naive B cells produce the regulatory cytokine IL-10, whereas memory B cells produce the majority of pro-inflammatory cytokines, including tumor

necrosis factor-α and lymphotoxin-α (125). In addition, many inflammatory and autoimmune mouse models have described situations where B_{regs} arise (175, 176). Such data warrants further exploration of the discordance between the frequency of MBP-reactive peripheral B cells and ability to support T cell activation and proliferation.

What is also of interest is that $CD8^+$ T cell proliferation did not necessarily correlate with $CD4^+$ T cell proliferation. For example, $CD4^+$ T cells from MS-2 proliferated in response to CD40L/IL-4 B-APCs + MBP + GA, but $CD8^+$ T cells did not. This data suggests that the $CD4^+$ T cells may be secreting some factor that does not allow $CD8^+$ T cell proliferation in the presence of $CD4^+$ T cell proliferation. What effect this may have on clinical exacerbations is unknown at this time, but may point to an important aspect of regulation not previously observed. Characterization of the cytokine milieu present in B:T MLR cultures may address this issue.

One additional interesting finding in the CD40L/IL-4 in vitro culture system was that in the absence of exogenous antigen, activated B cells elicited high levels of CD4+ and CD8 + T cell proliferation. This was surprising because even in a highly activated state, an antigen-presenting cell must present cognate ligand on HLA molecules in order to initiate T cell proliferation and activation. These findings would suggest three possible hypotheses as to the reason that T cell proliferation was observed in the absence of apparent cognate ligand to activated B cells: 1) A subset of T cells in MS patients can be induced to proliferate through bystander activation in the absence of cognate antigen recognition, 2) a subset of T cells in the peripheral pool of MS patients are specific for self antigens presented by autologous CD40L/IL-4 activated B cells, or 3) CD40L/IL-4

activated B cells are presenting mouse fibroblast antigens, acquired during culture with $CD40L^{+}$3T3 cells. The majority of CD4+ and CD8+ T cell proliferation observed in the absence of antigen was reduced in the presence of HLA-DR blocking antibody, demonstrating that in this system, T cell proliferation is dependent on antigen recognition in the context of HLA-DR. Although I provide no evidence here that T cells in this system are not responding to self antigen, I propose that the most likely explanation for the phenomenon of antigen-non specific T cell proliferation is that CD40L/IL-4 activated B cells in the current in vitro activation system, acquire and present mouse antigens. It has been formally demonstrated by other groups that B cell chronic lymphocytic leukemia (B-CLL) cells can acquire membrane bound molecules such as CD40L and OX40L from fibroblasts transfected to express these molecules, through a process of bystander membrane transfer between fibroblast and B-CLL (177, 178). These data would suggest that in the current CD40L/IL-4 activation system, B cells acquire significant amounts of non-self mouse antigens through 3T3 membrane acquisition. Although this hypothesis was not formally tested in the experiments described here, it may be possible to prevent the exposure of human B cells to mouse antigens in the CD40L/IL-4 system by stimulating B cells with humanized anti-CD40 $F(abs')^2$ fragments as an alternative to $CD40L^{+}$3T3 feeder cells.

In summary, our results show that activated peripheral B cells can function as CNS-antigen specific B-APCs *in vitro*. The consequence of such potential would be that peripheral B cells affect disease outcome by channeling T cell responses to antigens recognized by the peripheral B cell population. Such a mechanism could explain efficacy of newly emerging therapeutic agents that target B cells on diseases of the CNS with

suspected humoral involvement.

Although the work presented in the current chapter clearly demonstrates that PBBCs expanded in the CD40L/IL-4 system are functionally capable of presenting both control (TT) and myelin related (MBP and GA) proteins to autologus T cells, and that a subset of CD40L/IL-4 activated B cells bind MBP directly, it is unclear if the antigen specificity of these populations of B cells was relevant to the ability to stimulate antigen specific autologus T cell proliferation. PBBCs are extremely heterogenous both because of the highly diverse use of distinct BCR genes but also in terms of antigen experience and affinity maturation. PBBC expanded using the CD40L/IL-4 system included both antigen naïve B cells as well as antigen experienced memory B cells. The studies presented in this chapter did not take into account the antigen experience of B cell subsets in these patients which should be addressed in order to determine if antigen experience determines the outcome of neuro-antigen specific T cell activation. Therefore, in the next chapter I examine the functional role of naïve and memory B cells to be antigen specific APCs to autologus T cells.

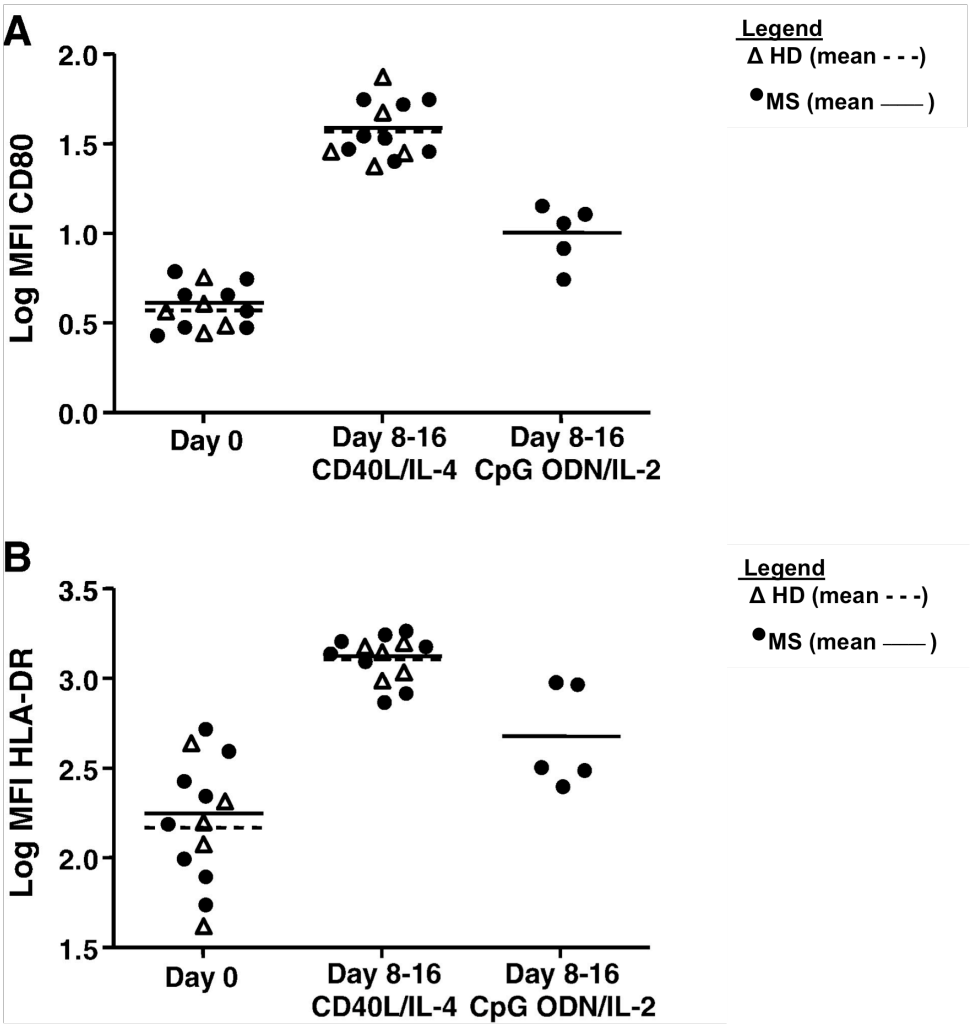


FIGURE 5-01. Costimulatory molecule expression following *in vitro* stimulation of CD19+ PBBC. *In vitro* expression of CD80 (A) and HLA-DR (B) were evaluated by flow cytometry prior to activation at day zero, and after PBBCs were stimulated for 8–16 days of culture in either the CD40L/IL-4 or CpG ODN/IL-2 culture systems. Closed circles and open triangles represent expression from MS patients (MS 1–9) and HDs (HD1–5) respectively. The solid and dotted lines represent the MS and HD averages respectively.

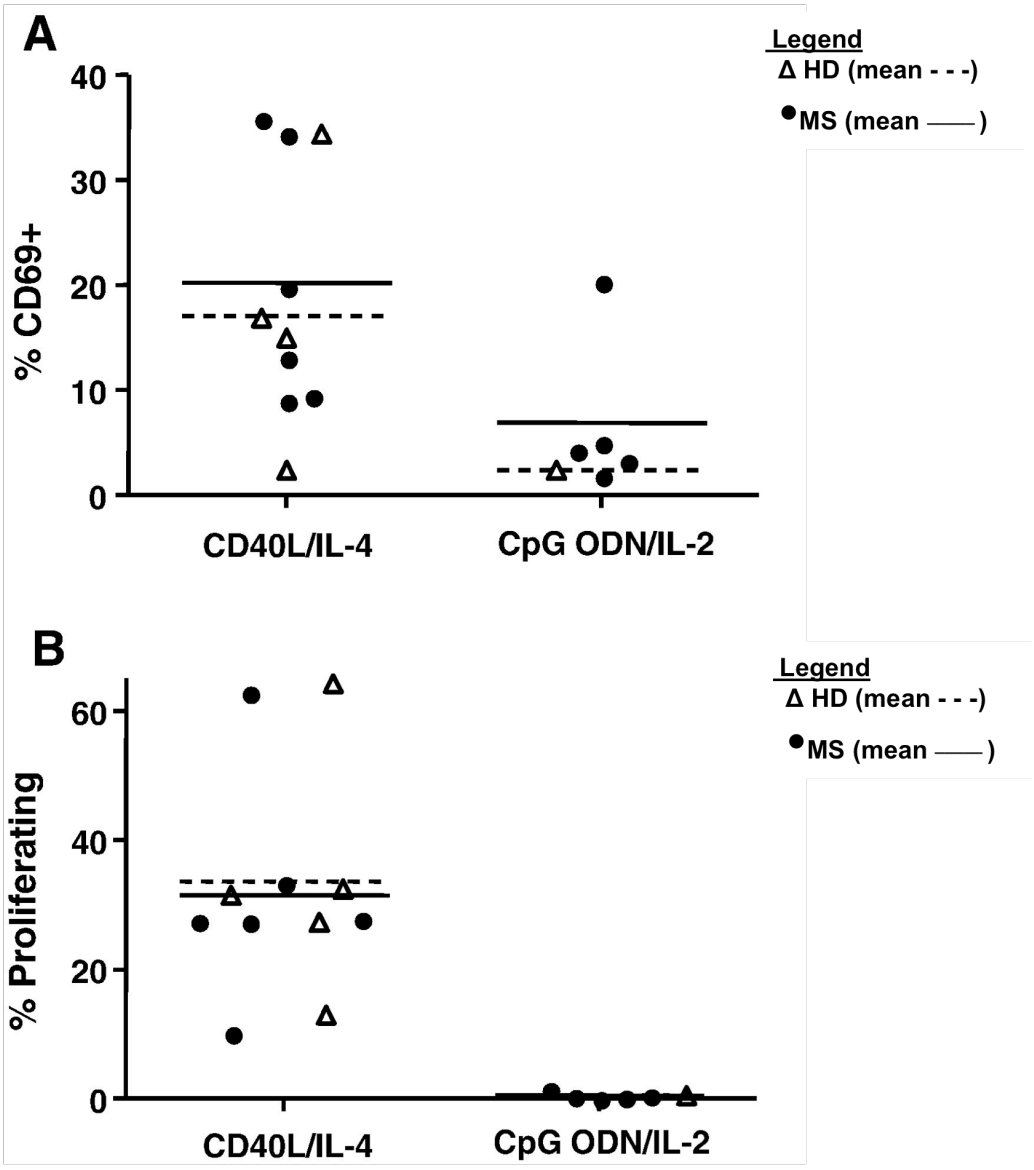


FIGURE 5-02. $CD4^+$ T cell proliferation in the presence of activated PBBC and mumps recall antigen. The percentage of $CD4^+$ T cells expressing the early activation molecule CD69 (A) was assayed by flow cytometry at 48 h after purified $CD3^+$ T cells were incubated in the presence of mumps and *in vitro* stimulated PBBCs. The percentage of $CD4^+$ that had proliferated after 7 days (B) was assayed by flow cytometry using CFSE dilution. Results normalized to background CD69 expression (A) or proliferation (B) observed when no exogenous antigen was present.

A

$$\left[\left(\frac{\text{Pf(Ag)\%}}{\text{nPf(Ag)\%}}\Big/\frac{\text{Pf(Max Ag)\%}}{\text{nPf(Max Ag)\%}}\right)\times 100\right]-\left[\left(\frac{\text{Pf(No Ag)\%}}{\text{nPf(No Ag)\%}}\Big/\frac{\text{Pf(Max Ag)\%}}{\text{nPf(Max Ag)\%}}\right)\times 100\right]=\text{Normalized Proliferation (\%)}$$

B

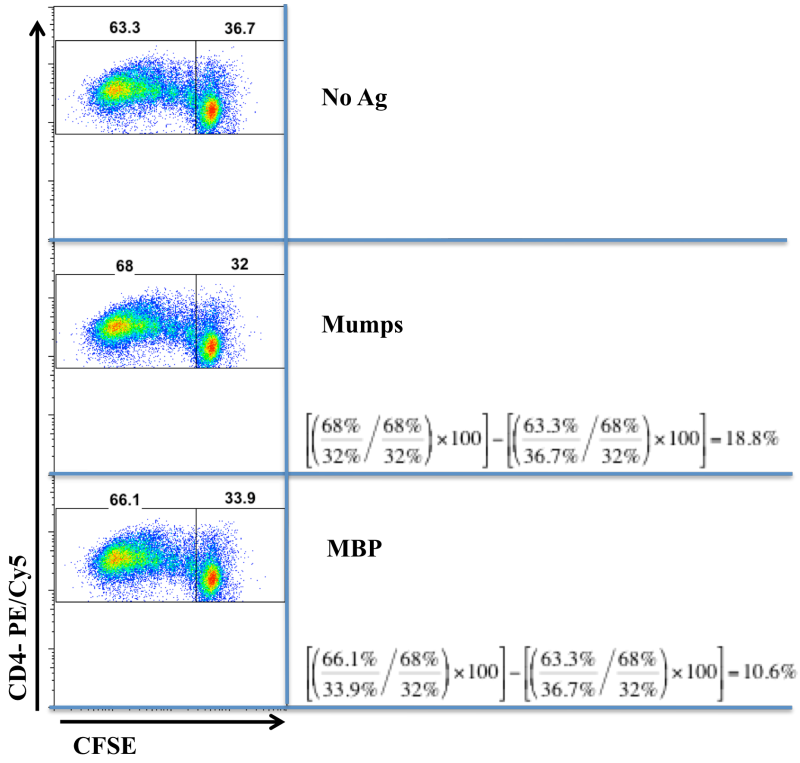


FIGURE 5-03. Example of gating and calculation used to determine normalized T cell proliferation. (A) Formula used to calculate normalized proliferating fraction used in Figures 5-03 and 5-05. (B) Purified CFSE labeled T cells from MS-1 (TP3) were cultured with autologous CD40L/IL-4 expanded B APCs in the presence of the indicated antigens for 7 days and T cell proliferation was measured as described in section 3.5.3. Cells were gated on live CD3+CD4+ cells. Gates depicted in each dot plot are the raw proliferating (CFSE low) and non-proliferating (CFSE high) fractions. Raw proliferating fractions are reported in Figure 5-02. Raw percent proliferating and non-proliferating fractions are used in formula to determine normalized proliferation reported in Figures 5-03 and 5-05. Abbreviations used: Pf(no Ag); proliferating fraction (%) in the absence of exogenous antigen, nPf(no Ag); nonproliferating fraction (%) in the absence of exogenous antigen, Pf(Ag); proliferating fraction (%) in the presence of exogenous antigen, nPf(Ag); non-proliferating fraction (%) in the presence of exogenous antigen, Pf(Max Ag); proliferating fraction (%) in the presence of positive control (mumps) antigen, nPf(Max Ag); non-proliferating fraction (%) in the presence of positive control (mumps) antigen.

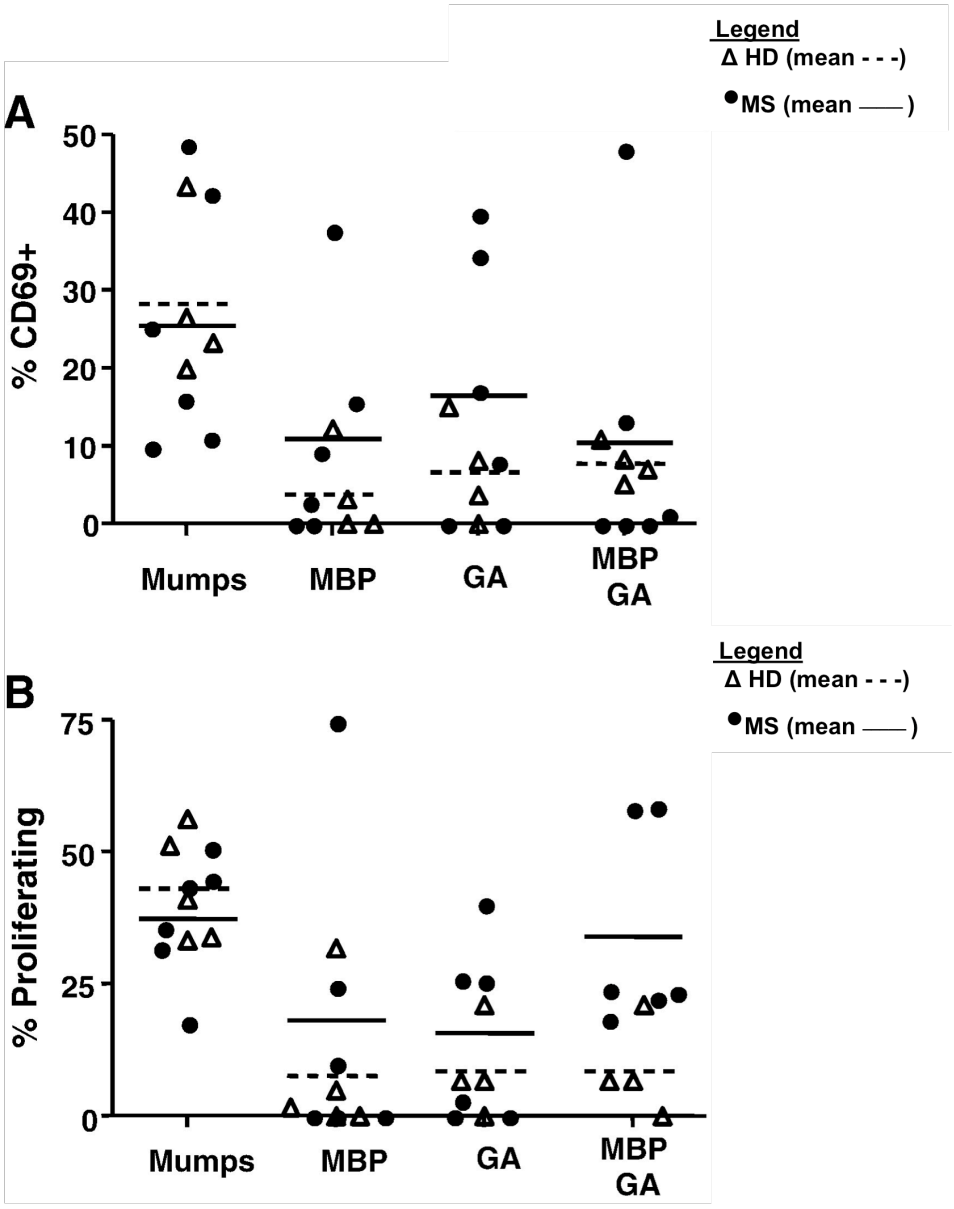


FIGURE 5-04. CD40L/IL-4 B-APC stimulate CNS-antigen specific CD4$^+$ T cells. The percentage of CD4$^+$ that expressed CD69 at 48 h (A) and proliferation as measured by CFSE dilution (B) was assayed from MS patients (MS 1–6), and HDs (HD 1–5) after incubation with mumps, MBP, GA, or MBP + GA. Results are expressed as a function of non-antigen specific CD69 expression and proliferation observed when no exogenous antigen was present. Values greater than zero were considered positive responses and negative values were considered non-responders.

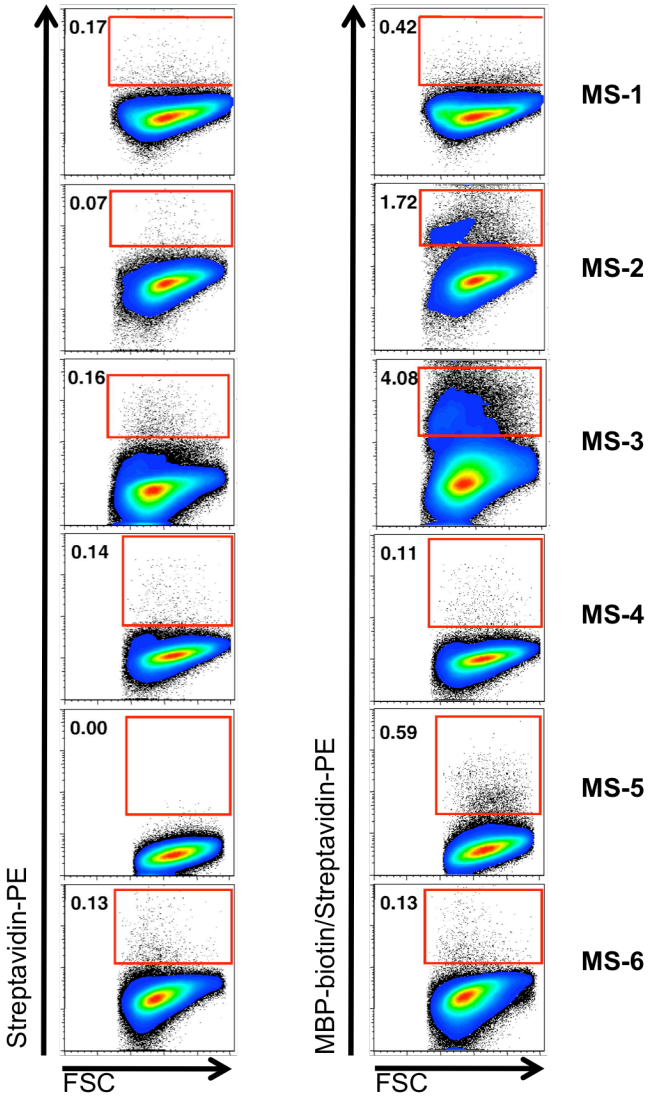


FIGURE 5-05. A subset of CD40L/IL-4 B-APCs from MS patients directly bind MBP. CD40L/IL-4 stimulated PBBC were maintained for a period of 12–53 days from MS patients (MS1-6). CD40L/IL-4 stimulated PBBC were assessed for their ability to bind MBP-biotin detected with streptavidin-PE by flow cytometry.

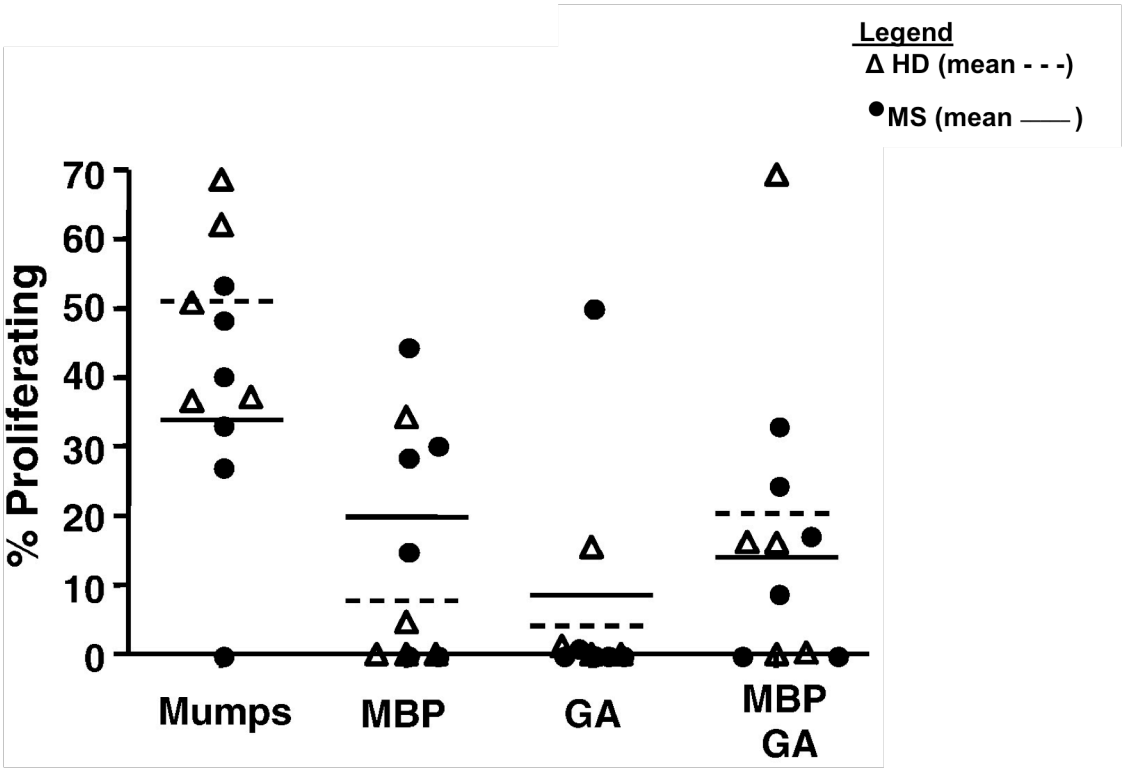


FIGURE 5-06. CD8$^+$ T cell proliferation in CD40L/IL-4 PBBC: T cell MLR. CD8$^+$ T cell proliferation was measured by CFSE dilution at day 7 in a CD40L/IL-4 B:T MLR. Results are expressed as a function of non-antigen specific CD69 expression and proliferation observed when no exogenous antigen was present. Values greater than zero were considered positive responses and negative values were considered non-responders.

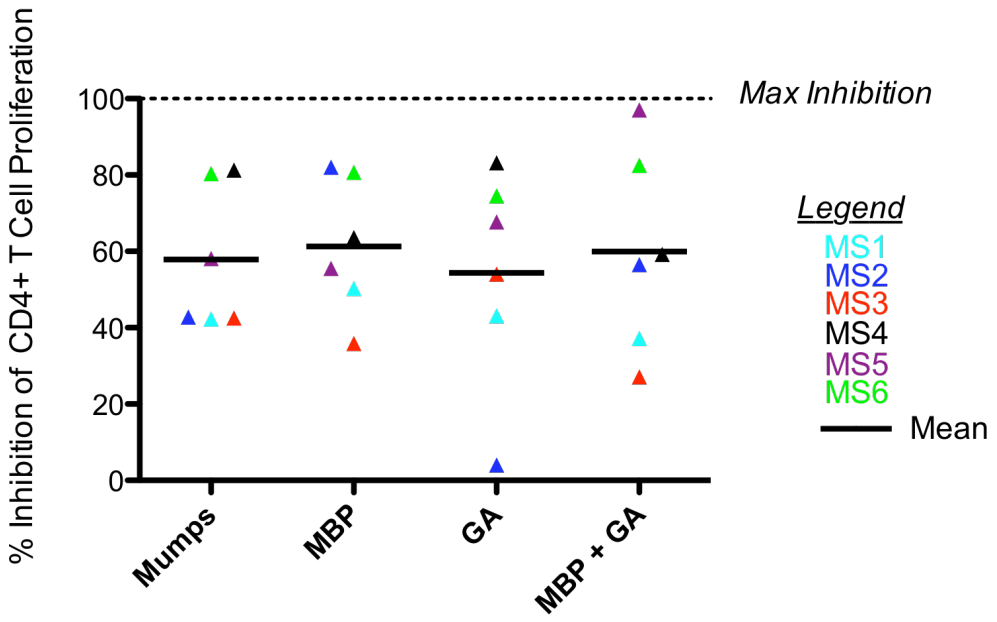


FIGURE 5-07. Percentage inhibition of MS CD40L/IL-4 PBBC induced antigen specific CD4+ T cell proliferation by anti-HLA-DR antibody. Anti-HLA-DR antibody added into B-T cultures at 5 μg/mL or pre-incubated with B cells as described in materials and methods, inhibited the majority of PBBC induced CD4+ T cell proliferation in all antigen assays tested. Dotted line represents maximum possible inhibition (100%). Individual MS patients are coded by color in the legend. Thick solid line represents mean inhibition.

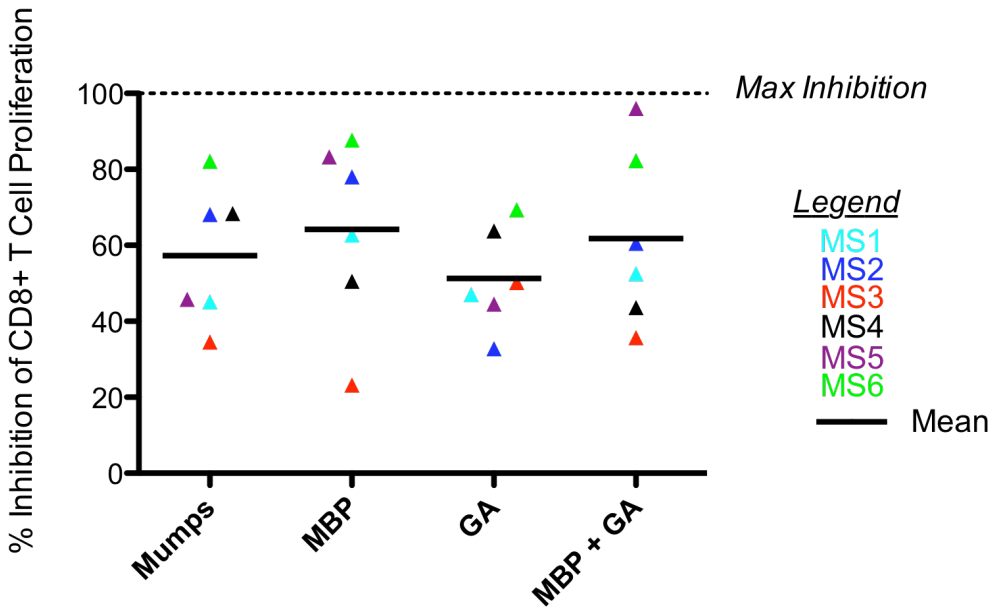


FIGURE 5-08. Percentage inhibition of MS CD40L/IL-4 PBBC induced antigen specific CD8+ T cell proliferation by anti-HLA-DR antibody. Anti-HLA-DR antibody added into B-T cultures at 5 μg/mL or pre-incubated with B cells as described in materials and methods, inhibited the majority of PBBC induced CD8+ T cell proliferation in all antigen assays tested. Dotted line represents maximum possible inhibition (100%). Individual MS patients are coded by color in the legend. Thick solid line represents mean inhibition.

TABLE 5-01. CD80 and HLA-DR ratios in each system.

	CD40L/IL-4 activated B cells		Cpg/IL-2 activated B cells	
CD80[a]	Naïve MFI ratio (D8-16:D0)	Memory MFI ratio (D8-16:D0)	Naïve MFI ratio (D8-16:D0)	Memory MFI ratio (D8-16:D0)
MS-1	15.7	12.7	5.3	4.6
MS-2	10.5	13.6	4.5	3.8
MS-3	9.3	6.1	N.d.	N.d.
MS-4	13.3	14.3	N.d.	N.d.
MS-5	4.8	4.7	4.4	3.2
MS-6	2.4	3	N.d.	N.d.
MS-7	N.d.	N.d.	3.5	2.4
HD-1	8.5	4.7	N.d.	N.d.
HD-2	13.9	9.7	N.d.	N.d.
HD-3	N.d.	N.d.	N.d.	N.d.
HD-4	10.6	7.1	N.d.	N.d.
HD-5	7.7	13.9	5.8	3
HLA-DR[b]				
MS-1	1.6	5.9	21	6.3
MS-2	6	37.6	2.4	1.9
MS-3	2.5	9.9	N.d.	N.d.
MS-4	4.4	38.8	N.d.	N.d.
MS-5	0.8	3.7	1.3	1.6
MS-6	7.1	28.3	N.d.	N.d.
MS-7	N.d.	N.d.	12.8	2.2
HD-1	9.3	20	N.d.	N.d.
HD-2	19.8	117.6	N.d.	N.d.
HD-3	N.d.	N.d.	N.d.	N.d.
HD-4	6.7	13.9	N.d.	N.d.
HD-5	17.5	53.5	1.3	1.6

[a] The percentage of B cells expressing CD80 following at least 8 days of CD40L/IL-4 activation was 52–60%. The percentage of B cells expressing CD80 following at least 8 days of CpG/IL-2 activation was 16–21%.

[b] The percentage of B cells expressing HLA-DR following at least 8 days of CD40L/IL-4 or CpG/IL-2 activation was 86–100%.Abbreviations used in this table: MFI, mean fluorescence intensity; MS, multiple sclerosis; HD, healthy donor; N.d., not done.

TABLE 5-02. Naïve:memory B cell ratios in each system.

	Day 0	Day 8–16 CD40L/IL-4	Day 8–16 CpG/IL-2
MS patient			
MS-1	6.8	12.8	1
MS-2	4.5	7.9	4.2
MS-3	2.9	10.7	N.d.
MS-4	1.1	2.1	N.d.
MS-5	8.3	11.3	5.5
MS-6	2.4	1.5	N.d.
MS-7	4.6	N.d.	3.1
MS-8	1.5	0.5	N.d.
MS-9	2.8	2.3	N.d.
MS-10	3.1	9.3	N.d.
Average	3.7	6.5	3.45
SE	1	1.9	0.64
healthy donor			
HD-1	2.7	2.6	N.d.
HD-2	0.8	1.6	N.d.
HD-3	3.3	11.6	N.d.
HD-4	3	14.1	N.d.
HD-5	0.4	0.7	0.3
Average	2	6.1	N.d.
SE	0.7	3.1	N.d.

Abbreviations used in this table: MS, multiple sclerosis; SE, standard error; HD, healthy donor. N.d., not done.

TABLE 5-03. Basal expression levels of CD69 on HD and MS CD4+ and CD8+ T cells.

	CD4 T Cells Basal %CD69+		CD8 T Cells Basal %CD69+	
	MS (N=6)	HD (N=5)	MS (N=6)	HD (N=5)
	1.6%	1.1%	8.3%	1.2%
	1.0%	0.9%	0.7%	0.7%
	1.6%	0.8%	8.5%	2.6%
	3.0%	1.0%	3.2%	1.3%
	6.4%	2.8%	5.0%	2.2%
	3.1%		6.5%	
mean	2.8%	1.3%	5.3%	1.6%
SEM	*0.9%*	*0.6%*	*1.4%*	*0.5%*
p-value (Student's t-test)	0.15		0.03	

Abreviations used: SEM; standard error of the mean, MS; multiple sclerosis, HD; healthy donor, N; number of donors

TABLE 5-04. Percent inhibition of $CD4^+$ and $CD8^+$ T cell proliferation with anti-HLA-DR blocking antibody.

CD4+ T cell proliferation

	Mumps	MBP	GA	MBP + GA
MS-1	42.3	50.3	43.1	37.2
MS-2	42.8	82	4	56.5
MS-3	42.5	35.9	54	27.1
MS-4	81.3	63.6	83.2	59.2
MS-5	58.1	55.5	67.7	97
MS-6	80.4	80.7	74.5	82.5
Average	57.9	61.3	54.4	59.9
SE	8.4	8	12.8	11.8

CD8+ T cell proliferation

	Mumps	MBP	GA	MBP + GA
MS-1	45.1	62.7	47	52.5
MS-2	68.1	78	32.8	60.5
MS-3	34.5	23.2	50.2	35.7
MS-4	68.4	50.5	63.8	43.6
MS-5	45.8	83.3	44.5	96
MS-6	82.1	87.7	69.4	82.3
Average	57.3	64.2	51.3	61.8
SE	8.1	10.9	6	10.4

Abbreviations used in this table: MBP, myelin basic protein; GA, glatiramer acetate; MS, multiple sclerosis; SE, standard error.

CHAPTER SIX
Results

MEMORY B CELLS FROM RELAPSING REMITTING MULTIPLE SCLEROSIS PATIENTS ELICIT FUNCTIONAL RESPONSES BY CD4+ T CELLS IN RESPONSE TO NEURO-ANTIGENS

At the time of this writing the following study was in preparation for submission to a peer-reviewed journal. Christopher T. Harp, Sara Ireland, Laurie S. Davis, Bonnie Cassidy, Petra D. Cravens, Olaf Stuve, Amy E. Lovett-Racke, Todd N. Eagar, Benjamin M. Greenberg, Michael K. Racke, Lindsay G. Cowell, Nitin J. Karandikar, Elliot M. Frohman, Nancy L. Monson. *Memory B cells from relapsing remitting multiple sclerosis patients elicit functional responses by CD4+ T cells in response to neuro-antigens.*

Introduction

The CD40L/IL-4 system used to expand and activate B cells i*n vitro* in the previous chapter provided an excellent model for assessing B cell antigen presentation *in vitro* and conclusively demonstrated for the first time that under strong *in vitro* activation conditions, B cells could be efficient neuro-antigen presenting cells in the context of MS. However, the assays used in the previous chapter were not designed to examine if *ex vivo* resting B cells could be efficient neuro-antigen presenting cells. In addition, CD40L/IL-4 stimulation results in poly-clonal activation of B cells, which makes it difficult to isolate the contribution of antigen specific B cells in the activation of autologous T cells.

A pathogenic role of B cells in the pathogenesis of relapsing remitting multiple sclerosis (RRMS) was recently substantiated, as therapeutic depletion of B cells with

Rituximab resulted in reduction in disease activity as measured by gadolinium enhancing lesions on MRI (14); and reappearance of B cells in the periphery correlated with a return in RRMS symptoms (15). It is also becoming evident that preventing B cells from entering the CNS may be an important target of disease modifying therapy, since RRMS patients treated with the selective adhesion molecule inhibitor natalizumab, a monoclonal antibody to VLA-4 present on a variety of leukocytes, show a disproportionate increase in the number of circulating B cells (179).

Although B cells serve varied functions in the immune system including the production of antibodies, lymphoneogenesis, cytokine secretion and antigen presentation, it remains largely elusive as to which of these functions play a central role in the pathogenesis of RRMS. For instance, it does not appear likely that antibody secretion is central to RRMS disease activity, given that treatment with Rituximab confers benefit in reducing both clinical and radiographic disease manifestations without concomitant reduction of circulating antibody since CD20, the target of Rituximab, is not present on plasma cells (14). However, Rituximab treatment of RRMS patients does result in a significant depletion of B cells within the CSF (16) and cerebral perivascular spaces (180). A marked decrease in T cell frequency in the CSF has also been observed (16), indicating a potential role of B cells in regulating T cells within CNS. This finding was similarly observed in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), in which depletion of B cells after EAE induction resulted in a reduction of antigen-specific effector T cells within the CNS (92). Interestingly, a decline in T cell frequency in the CSF of pediatric opsoclonus-myoclonus patients

receiving Rituximab was not observed (181), indicating that T cell regulation by B cells may be a unique feature of MS patients.

At first glance, these results are surprising since the most extensively studied consequence of B-T cell interactions is the induction of T cell tolerance or expansion of regulatory T cells (182-184). For example in mice, antigen-specific naïve B cells induce naïve T cells to proliferate and differentiate into regulatory T cells, unlike dendritic cells, which induce effector T cell subset development (96). In addition, HEL-specific B cells fail to initiate significant T cell proliferation or IL-2 and IFNγ secretion (185). Further, on a per cell basis, human B cells are less potent antigen presenting cells (APCs) than dendritic cells *in vitro* (88). Ultimately, these findings have suggested that B cells are unlikely to play a significant role as APCs in the induction of effector T cell responses. Nevertheless, these studies did not characterize APC function in the memory B cell pool. Importantly, other in vitro investigations have demonstrated that human memory B cells are potent APCs in the context of both allo-antigen (186) and exogenous foreign antigen (77). Interestingly, circulating memory B cells are also reduced in RRMS patients during mitoxantrone (125) and IFN-beta therapy (187). The relationship of these reductions to therapeutic efficacy remains unknown.

In this section I focused on the potential impact of memory B cells from RRMS patients that dominate the B cell pool in the CNS of RRMS patients (138, 188), and whether pathogenic mechanisms are mediated through the process of neuro-antigen presentation to effector T cells. Here I demonstrate for the first time that memory B cells from the peripheral blood of RRMS patients are able to induce significant neuro-antigen specific T cell proliferation and IFNγ secretion in comparison to peripheral memory B

cells from healthy donors. My findings may be germane to advancing our understanding of the relationship of compartment specific memory B cell responses and the pathobiologic underpinnings of MS. The results of these findings are summarized in the following section.

RESULTS

RRMS naïve and memory B cell subsets are phenotypically similar to HD

Recent studies on an untreated RRMS patient cohort similar to ours found no significant differences in the percentages of circulating naïve and memory B cells between HDs and RRMS patients (187). Nevertheless, we wanted to confirm this phenotype in our cohorts as patients with other autoimmune disorders have a decrease in the total percentage of circulating memory B cells in the peripheral blood (PB) compared to HDs (189), supporting the idea that these cells are at the site of inflammation during the course of autoimmune inflammation, and not in circulation. The HD and RRMS patients in our cohort showed similar percentages of memory B cells (4.42±0.56% vs. 4.07±0.56%, p=0.12, Figure 6-02A, example of gating used in Figure 6-01A) and naïve B cells (13.70±2.01% vs. 9.66±1.20%, p=0.12, Figure 6-02A, example of gating used in Figure 6-01A) within the peripheral blood, similar to what was previously reported (187). Also, as previously observed (188), naïve B lymphocytes dominate the PB of both HDs (13.70±2.01% naïve B cells vs. 4.42±0.56% memory B cells, $p<0.001$, Figure 6-02A), and RRMS patients (9.66±1.20% naïve B cells vs. 4.07±0.56% memory B cells, $p<0.001$, Figure 6-02A), with average naïve:memory ratios of 3.27±0.37 and 2.68±0.40 in HDs and RRMS patients, respectively.

Costimulatory molecule expression can influence the potency of B cell antigen presentation, and is differentially regulated between naïve and memory B cells in HDs (77, 186). In addition, one study had demonstrated that CD80+ B cells expand in the peripheral blood of MS patients undergoing exacerbation (97). Therefore, we examined memory and naive B cell subsets for their relative expression of CD80 (B7.1) in our RRMS cohort and compared subset expression to HDs using mean fluorescence intensity (MFI) of CD80 expression by flow cytometry. Memory B cells expressed significantly higher levels of CD80 than naïve B cells in both HD (6.51±0.49 vs. 3.99±0.44, $p<0.001$, Figure 6-02B example histogram in Figure 6-01B) and RRMS patients (6.88±0.45 vs. 4.03±0.25, $p<0.001$, Figure 6-02B, example histogram in Figure 6-01B). CD80 MFI naïve:memory ratios in HDs and RRMS patients were similar (0.60±0.02 and 0.59±0.02, respectively).

HLA-DR is a major component of human MHCII and is constitutively expressed on resting B cells and other APCs, but is highly upregulated upon activation (190). Given the increased expression of CD80 on memory B cells in our cohorts, we hypothesized that HLA-DR expression may also be significantly increased on memory B cells as compared to naïve B cells in both HD and RRMS. Instead, we observed that the memory B cell compartment expressed significantly lower levels of HLA-DR compared to the naïve B cell compartment in both HD (217.6±38.7 vs. 394.2±65.4, $p<0.001$, Figure 6-02C, example histogram in Figure 6-01C) and RRMS patients (98.2±16.9 vs. 198.8±33.7 $p<0.01$, Figure 6-02C, example histogram in Figure 6-01C). However, the relative ratio of naïve:memory B cell HLA-DR expression was similar in the two cohorts

(2.00±0.10 vs. 2.12±0.14) indicating that even though naïve and memory B cells from the HD cohort in general had higher HLA-DR expression than RRMS, the relative ratio of expression between naïve and memory B cells in both cohorts was similar.

Memory B cells from both HD and RRMS secrete pro-inflammatory LTα

Memory and naïve B cells from HDs secrete pro- and anti-inflammatory cytokines, respectively, when polyclonally stimulated in vitro (125), potentially indicating a reciprocal role in modulating T cell responses through antigen presentation during the course of RRMS. LTα is a prototypic inflammatory cytokine critical in the development of some EAE models of MS (191), and IL-10 is a prototypic anti-inflammatory cytokine that inhibits human T cell proliferation (192). Therefore, we chose to examine LTα secretion as a measure of pro-inflammatory activity and IL-10 secretion as a measure of anti-inflammatory/regulatory activity in both our HD and RRMS patient cohort. Accordingly, CD40L stimulation served as a model of T cell dependent bystander activation (125), and BCR + CD40L stimulation a model of cognate antigen recognition followed by T cell help. As an additional stimulus, TLR9 agonist (CpG ODN) + CD40L stimulation was included as a model of innate ligand stimulus plus T cell help (193).

Memory B cells produced significantly more LTα than naïve B cells when stimulated with CD40L alone in both HDs (483±49 pg/mL vs. 125±36 pg/mL, $p<0.001$, Figure 6-03A) and RRMS patients (314±72 pg/mL vs. 78±20 pg/mL $p<0.01$, Figure 6-03A). There was no significant difference between the HD and RRMS cohorts when we compared LTα production in the memory B cell compartment or the naïve B cell

compartment (p=0.0502). Similar response patterns were observed with the combination of BCR and CD40L stimulation, such that LTα secretion by memory B cells was greater than LTα secretion by naïve B cells in both HDs (455±36 vs. 56±18 pg/mL, p<0.001, Figure 6-03B) and RRMS patients (325±61 vs. 80±22, p<0.001, Figure 6-03B). No significant differences in LTα production between memory or naïve B cells from HDs or RRMS patients (Figure 6-03B) were observed (p=0.29). These data suggest that the addition of BCR stimulation at this dose does not induce greater LTα secretion than what is observed with CD40L alone in either the naïve or memory B cell compartment in HDs and RRMS patients.

Although naïve B cells from HDs and RRMS patients secreted similar amounts of LTα, naïve B cells from RRMS patients stimulated with CD40L alone produced significantly more IL-10 than naïve B cells from HDs stimulated with CD40L alone (89±30 pg/mL vs. 9±9 pg/mL, p<0.01, Figure 6-04A). In fact, naïve B cells from RRMS patients stimulated with CD40L alone produced significantly more IL-10 than memory B cells from the same RRMS patients (89±30 pg/mL vs. 13±10 pg/mL, p<0.01, Figure 6-04A), suggesting that a subset of naïve B cells from RRMS patients may be functioning as regulatory cells not present in HDs. Upon addition of BCR stimulation, we observed an insignificant decrease in the amount of IL-10 produced by RRMS naïve B cells stimulated with BCR and CD40L stimulation in combination versus CD40L stimulation alone (89±30 pg/mL vs. 28±9 pg/mL, p=0.09, un-paired t-test, Figure 6-04A and Figure 6-04B, respectively). However, we did not observe any significant IL-10 production by

B cells from HDs stimulated with either CD40L alone or BCR and CD40L stimulation in combination.

TLR9 can be detected on the surface of memory B cells, and is maintained intracellularly in naïve B cells (80), and may play a predominant role in the development of RRMS (194). Thus, we hypothesized that cytokine secretion by memory and naïve B cells in response to TLR9 signaling plus CD40L stimulation might be dysregulated in RRMS. When memory and naïve B cells from HDs were stimulated with a TLR9 agonist (CpG ODN) in combination with CD40L stimulation, LTa production increased in memory B cells compared to naïve B cells (2027±227 pg/mL vs. 942±213 pg/mL, p=0.03, paired t-test, Figure 6-03C). However, there was no difference between HD and RRMS in regards to LTa production by naïve B cells in response to TLR9 signaling in combination with CD40L stimulation (942±213 pg/mL vs. 501±364 pg/mL, un-paired t-test, Figure 6-03C). The limited number of memory B cells available in some RRMS donors prevented us from performing these experiments on our current cohort.

When memory and naïve B cells from HD's were stimulated with TLR9 agonist in combination with CD40L stimulation, IL-10 production was increased in the memory B cell compartment as compared to naïve B cells (174±59 vs. 92±62 pg/mL, p=0.01, paired t-test, Figure 6-04C). IL-10 production by naïve B cells from RRMS patients stimulated with TLR9 agonist and CD40L was not statistically different compared to IL-10 production by naïve B cells from HD's. However, naïve B cells from 3 of the 6 RRMS patient samples exhibited notably high IL-10 production compared to naïve B cells from HD's in response to TLR9 agonist plus CD40L stimulation.

MOG specific CD4+ and CD8+ T cell proliferation in PBMCs is greater in RRMS patients than HDs.

Whole PBMC cultures containing heterogeneous APC populations are often used to assess the magnitude of antigen specific T cell proliferation *in vitro*. To measure T cell reactivity in response to control and neuro-antigens present in HDs and RRMS patients, we cultured whole PBMCs with tetanus toxoid (TT), glatiramer acetate (GA), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG), and measured CD4+ and CD8+ T cell proliferation 5 days later (see section 3.8.0 for setup). Mean control antigen TT specific CD4+ T cell proliferation in these whole PBMC cultures (compare 13.7±4.9% vs. 16.7±5.4%, p=0.69, Figure 6-05, A) and number of donor responders (9/11 vs. 9/11, p=1.0 Figure 6-05, A) was comparable between HDs and RRMS patients respectively. Mean neuro-antigen MBP specific CD4+ T cell proliferation and number of responders in these whole PBMC cultures was also similar (proliferation: 3.7±1.6% vs. 5.6±2.1%, p=0.50, Figure 6-05, C: number of responders: compare 5/11 vs. 7/13, p=0.68, Figure 6-05, C) in HDs and RRMS patients respectively. Mean GA specific CD4+ T cell proliferation and number of responders was also similar between HDs and RRMS patients (proliferation: 12.4±4.0% vs. 11.0±3.5% respectively, p=0.79, Figure 6-05, E; and number of responders: 9/12 vs. 9/13 respectively, p=0.75, Figure 6-05, E). In contrast, mean neuro-antigen MOG specific CD4+ T cell proliferation in whole PBMC cultures was significantly greater in RRMS patients as compared to HDs (11.1±2.4 vs. 3.0±1.0 respectively, p=0.007, Figure 6-05, G), and a greater number of RRMS patients also responded to MOG by CD4+ T cell proliferation compared to HDs (compare 12/13 vs. 6/12 respectively, p=0.02, Figure 6-06, G). Interestingly, mean MOG

specific CD8+ T cell proliferation (compare 1.4±0.6% vs. 6.0±2.0, p=0.045, Figure 6-05, H) and number of donors responding (compare 8/11 vs. 3/11 p=0.03, Figure 6-05, H) was also significantly greater in RRMS patients than HDs respectively, whereas CD8+ T cell proliferation and number of responders was similar in RRMS patients and the HDs when TT, MBP, or GA were included in the cultures (Figure 6-05, B, D, and F).

Memory B cells elicit more antigen specific CD4+ T cell proliferation than naïve B cells

In vivo depletion of B cells in the context of RRMS results in a reduction of gadolinium enhancing lesions and a reduction in the number of T cells within the CSF (14), which substantiates a role for B cells in MS as mediators of T cell expansion, activation and pathogenicity. Since the majority of B cells found in the CSF of RRMS patients are short-lived plasma blasts and memory B cells (138, 188), we hypothesized that within the memory B cell pool of RRMS patients, there is a sub-population of memory B cells that operate as potent neuro-antigen presenting cells that supports neuro-antigen reactive T cell expansion and activation. To test this hypothesis, we investigated the potential of primary ex vivo memory and naïve B cells to elicit autologous T cell proliferation to one control antigen (TT), two candidate neuro-antigens (MBP and MOG), and GA, which is an FDA approved therapeutic agent in the treatment of MS that has similar biochemical properties to MBP (47). Purified memory or naïve B cells were incubated with purified autologous T cells, and T cell proliferation was measured by CFSE dilution as described in the materials and methods. An example of the output data is provided in Figure 6-06. Memory and naïve B cells from this patient did not induce CD4+ T cell proliferation in the absence of antigen. Both naïve and memory B cells

induced CD4+ T cell proliferation in response to TT, MOG, and GA, while only memory B cells induced CD4+ T cell proliferation in response to MBP. In this particular case, memory B cells induced greater MOG and GA specific CD4+ T cell proliferation than their naïve counterparts. These same assays were performed on 11 MS patients and 10 HDs and the results depicted in Figure 6-07.

Because HD and RRMS are presumably immunized to tetanus at the same frequency and have similar frequencies of residual memory B and T cells, we expected that CD4+ T cell proliferation in response to TT when memory B cells were included in the cultures would be similar in HDs and RRMS patients. Indeed, CD4+ T cell proliferation in response to TT above the threshold of 2% when memory B cells were the APC was similar between HDs and RRMS patients ($p=0.16$, χ^2-test, Figure 6-07A). Also as expected, memory B cells elicited greater mean CD4+ TT specific proliferation than naïve B cells in HDs (6.3±2.4% vs. 3.4±1.6%, $p<0.05$, Figure 6-07A). However, CD4+ TT specific proliferation of CD4+ T cells from RRMS patients was similar in co-cultures containing memory B or naïve B cells (5.3±2.3% vs. 3.4±1.7%, $p>0.05$, Figure 6-07A).

In contrast, memory B cells from some of the RRMS patients, but not from any of the HDs, elicited CD4+ T cell proliferation in response to MBP (4/9 vs. 0/10 responders, respectively, $p=0.018$, χ^2-test, Figure 6-08A). Memory B cells from RRMS patients were also more likely to elicit CD4+ T cell proliferation than memory B cells from HDs in response to MOG, although this trend did not meet statistical significance (5/10 vs. 1/9 responders respectively, $p=0.069$, χ^2-test, Figure 6-08B). In contrast, memory B cells from HDs and RRMS patients had enhanced capacities to elicit CD4+ T cell proliferation in response to GA in comparison to naïve B cells (3.3±1.0% vs.

1.02±0.4%, $p<0.01$; and 3.6±0.7 vs. 0.86±0.32, $p<0.01$, respectively, Figure 6-07B). Interestingly, naïve B cells from RRMS patients or HDs could not elicit CD4+ T cell proliferation in response to MBP or GA, but naïve B cells from RRMS patients could elicit CD4+ T cell proliferation in response to MOG more readily than naïve B cells from HDs ($p=0.02$, χ^2-test, Figure 6-08B).

Chloroquine (Cq) is an inhibitor of lysosomal maturation, and prevents processing of exogenously acquired antigens and presentation to T cells. In order to determine if B cell meditated T cell proliferation was due to antigen processing and presentation, naïve and memory B cells were pretreated with or without Cq as described in Chapter 3. Pre-treatment with Cq resulted in attenuation of the majority of T cell proliferation in the presence of either naïve or memory B cells (Figure 6-09), indicating that processing of antigen by B cells was required for induction of T cell proliferation.

Memory B cells from RRMS patients induce greater antigen specific IFNγ secretion than memory B cells from HDs

CD4+ T cells proliferated in response to both neuro-antigen and control antigens in vitro, and so our next goal was to determine whether IFNγ secretion (as a measure of Th1 responses) was affected when memory or naïve B cells were the source APC in these culture conditions (Figure 6-10 and Figure 6-11). In whole PBMC cultures from HDs, TT does not typically induce a significant IFNγ response (195, 196). In agreement with these data, both memory and naïve B cells from our HD cohort induced little IFNγ production in B-T cell co-culture supernatants in response to TT (85±33 pg/mL vs. 68±40 pg/mL, $p=0.38$, Figure 6-10A). However, both memory and naïve B cells from the RRMS

patient cohort induced levels of IFNγ above the set threshold in response to TT, and memory B cells from RRMS patients in comparison to HDs, produced greater IFNγ (503±157 pg/mL vs. 85±31 pg/mL respectively, $p<0.05$, Figure 6-10A) and were more likely to induce IFNγ secretion than memory B cells from HDs (0/7 vs. 6/9, respectively, $p=0.006$, χ^2-test, Figure 6-10A).

Because we observed a significantly greater number of memory B-T cell co-cultures from RRMS patients that resulted in CD4+ T cell proliferation to MBP compared to HDs, we expected to find that IFNγ secretion would also be concomitantly increased in response to memory B cells from RRMS patients presenting MBP to autologous T cells. Indeed, we observed that only memory B cells from a subset of RRMS patients induced IFNγ secretion in B-T cell co-cultures with MBP, while memory B cells from HD did not (3/9 vs. 0/7 responders respectively, $p=0.09$, χ^2-test, Figure 6-11A). Naive B cells from either HDs or RRMS patients did not induce significant IFNγ secretion in response to MBP (Figure 6-11A). These results would indicate that in at least a subset of RRMS patients, IFNγ secretion in response to MBP is induced by memory B cells but not naïve B cells. However, it should be noted that the amount of IFNγ secretion did not correlate with the frequency of CD4+ T cells that proliferated in these culture conditions (Table 6-01). For example, in our assay, IFNγ secretion was detected in the highest amount in patient MS-14 culture containing memory B cells presenting MBP (1935 pg/mL) but CD4+ T cell proliferation was not observed (Table 6-01). Similarly 10.52% of CD4+ T cells were in the proliferating fraction in MS-10 cultures

containing memory B cells presenting MBP, but no IFNγ secretion was observed (Table 6-01).

Glatiramer acetate induces both IFNγ and IL-5 cytokine secretion in T cell lines derived from HDs naïve to GA (172). Therefore, we hypothesized that primary ex vivo T cells from HD and our GA-treatment naïve RRMS patient cohort would also secrete IFNγ and IL-5 in response to both memory and naïve B cells presenting GA. Instead, no significant IFNγ was produced in response to GA when either naïve or memory B cells from HDs were APCs (0/8 vs. 0/8 responders respectively, p=1, χ^2-test, Figure 6-10B). Only 2/11 donors from the RRMS cohort produced significant amounts of IFNγ (Figure 6-10B) and IL-5 (data not shown) in response to GA presented by either memory or naïve B cells. Interestingly, the RRMS patient that produced the highest level of IFNγ detected in response to memory B cells presenting GA (MS-14) also produced the highest level of IFNγ in response to memory B cells presenting MBP (Table 6-01).

In a previous study, IFNγ secreting T cells from untreated MS patients were increased in response to purified whole MOG in comparison to control patients (55). Similar to that observation, both memory and naïve B cells from a subset of RRMS patients, but not HDs, induced IFNγ secretion in the B-T cell co-cultures in response to MOG when memory (4/10 vs. 0/6 responders, p=0.09, χ^2-test, Figure 6-11B) or naïve B cells (3/10 vs. 0/6 responders, p=0.13, χ^2-test, Figure 6-11B) were used as APCs. Yet there was no significant difference in the mean concentration of IFNγ detected in RRMS B-T cell co-cultures containing memory or naïve B cells presenting MOG (460±230 vs. 532± 414 pg/mL, p=0.9). Again, as observed in the case of MS-2, high IFNγ secretion

did not necessarily correlate with a higher fraction of proliferating CD4+ T cells in response to MOG (Table 6-01).

MBP and GA binding B cells are enriched in the memory B cell compartment

In order to determine if we could correlate the extent of CD4+ T cell proliferation that occurred through B cell antigen presentation and IFNγ secretion to the frequency of antigen specific B cells present in memory and naïve B cell populations in our donors, we chose to make use of a technique that has been utilized since the 1970s to isolate antigen specific antibody producing B cells using fluorescent conjugated antigen and identifying them by flow cytometry (197). This technique was also used to affirm that individual B cells expressed antibody of monospecificity (198), and has been more recently utilized to identify and quantify virus specific B cells in humans (199), and TT specific B cells in TT immune individuals (124). Others have used whole protein tetramers to identify antigen specific B cells as well (200). In a previous study, we used this technique to identify MBP specific B cells in CD40L activated B cell cultures to identify antigen specific B cells (190). For the purposes of the current study, we chose to directly conjugate amine reactive FITC to the antigens that we had used in the B-T culture assays, yielding, TT-FITC, MBP-FITC, MOG-FITC, and GA-FITC. OVA-FITC was also generated for use as a negative control, as we predicted no individual donors should have a large number of circulating OVA specific B cells. As a note, we have not directly assessed if any of the antigen binding that we have observed using this technique is due to direct binding to the B cell through the B cell receptor, and therefore refer to cells that are positive for bound antigen as “antigen binding cells” rather than antigen specific B

cells. As described in previous studies (201), we observed that whole GA-FITC bound to all B cells (Figure 6-12, B), making it an unsuitable reagent to identify GA specific B cells using this technique. Intact GA-FITC also bound readily to the HLA-DR^{+} U266 plasma cell line (Figure 6-12, E) but not to the HLA-DR- Jurkat T cell line (Figure 6-12, D). However, by gel filtering GA-FITC to remove low molecular weight species of GA (<7000 Da) prior to use in staining B cells, GA-FITC bound only a small subset of memory B cells (Figure 6-12, C) making it suitable for use in identifying antigen binding B cells.

An example of the gating strategy used to identify OVA, and GA antigen binding B cells is shown in Figure 6-13A and Figure 6-13B respectively. The number of naïve and memory B cells that bound TT were greater than the number that bound the control antigen OVA in both HD and RRMS patients (Figures 6-13C). In contrast to TT-binding B cell frequencies, MBP binding B cells were significantly enriched in the memory B cell compartment in comparison to the relative number of naïve B cells that bound MBP in both HDs (2342±678 vs. 329±90 per $1x10^6$ cells, $p<0.01$, Figure 6-13D) and RRMS patients (3931±652 vs. 1255±309 per $1x10^6$ cells, $p<0.001$, Figure 6-13D). The frequency of MBP binding memory B cells was similar between HDs and RRMS patients ($p=0.053$, Figure 6-13D), although only memory B cells from RRMS patients were able to elicit CD4+ T cell proliferation.

In contrast to the relative enrichment of MBP binding B cells in the memory B cell compartment in both HD and RRMS patients, no such relative enrichment was observed with MOG binding B cells (Figure 6-13F), even though B cells from RRMS patients and not HDs were able to elicit CD4+T cell proliferation in response to MOG.

GA has many similar biochemical properties to MBP including undefined secondary structure in solution and overall positive charge, so we expected to find that the number of B cells that bound GA would be enriched in the memory B cell compartment as well. Indeed, GA binding B cells were also significantly enriched in the memory B cell compartment in comparison to the relative number of naïve B cells that bound GA in both HDs (3095±579 vs. 517±166 per $1x10^6$ cells, $p<0.05$, Figure 6-13E) and RRMS patients (5010±1250 vs. 735±235per $1x10^6$ cells, N=11, $p<0.001$, Figure 6-13E). Interestingly, in addition to memory B cells containing higher frequencies of GA binding, memory B cells were also able to elicit CD4+T cell proliferation more readily in response to GA than their naïve B cell counterparts.

DISCUSSION

In this study, we investigated the phenotypic attributes and antigen-presenting role of memory vs. naïve B cells from MS patients. While B cells from MS patients were phenotypically similar to those from healthy subjects and also showed similar patterns of responses to polyclonal stimuli, we found that memory B cells from a subset of MS patients were uniquely suited to presenting myelin antigens to autologous CD4+ T cells. At the same time, ability to elicit control antigen-specific responses were similar between MS patients and HDs. Thus, the differences were significantly centered around CNS-specific T cell responses, which are relevant to the pathogenesis of MS.

Our findings cannot be attributed to an enrichment of neuro-antigen reactive memory B cells in RRMS patients, since the neuro-antigen binding assay indicated that the frequencies of neuro-antigen binding memory B cells are similar in HDs and RRMS

patients. We cannot rule out, however, that other features of memory B cells from RRMS patients render them more capable of inducing neuro-antigen specific functional responses than their HD counterparts. Our findings are also unlikely to be attributable to general enrichment of neuro-antigen specific T cells in the RRMS patients, since it has been well established that the overall frequency of MBP specific T cells and MOG-specific responses by T cells is similar between HDs and RRMS patients (28 and 49). However, in these assay systems a mixture of APCs is used to elicit T cell responses, almost always including dendritic cells. Prior reports also indicate that neuro-antigen-reactive memory T cells are more prominent in RRMS patients than in HDs (36, 39). Thus, it is possible that B cells preferentially elicit functional responses from memory T cells, which may be most relevant to the underlying disease process.

The finding that neuro-antigen specific B cells are enriched in the peripheral blood suggests that perhaps neuro-antigen specific B cells were primed in the periphery and have an effector function in the periphery as well. B cells could influence the pathogenesis of MS by priming T cells in secondary peripheral lymphoid organs, which then migrate to the CNS. B cells residing in the CNS may further promote CNS T cell activation, although this has not been formally tested.

GA-specific memory B cells, which are cross-reactive with MBP, may elicit the activation of MBP specific CD4+ T regulatory cells (Tregs). In vitro treated $CD4^{+}CD25^{high}$ Tregs show increased IL-10 production in response GA treatment (202). In addition it was recently demonstrated that while untreated MS patients have reduced numbers of peripheral CD4+ Tregs in comparison to HDs, GA treatment restores both the number and functionality of these Tregs (203). Perhaps this would suggest that memory

B cells contribute most significantly to the expansion of CD4+ Tregs in patients undergoing GA therapy.

Our data suggest that memory B cells from RRMS patients (but not HDs) may promote the activation of Th1 specific clones since the CD4+ T cells often secrete IFNγ in response to memory B cells presenting neuro-antigens. Of note, this response did not correlate with CD4+ T cell proliferation. Interestingly, significant IFNγ and IL-5 secretion was consistently observed in cultures where memory B cells from RRMS patients were presenting Tetanus Toxoid (TT) to T cells, suggesting that memory B cells are capable of stimulating both Th1 and Th2 TT reactive clones. Also of note, IFNγ secretion in response to memory B cells from RRMS patients presenting TT was enhanced compared to memory B cells from HDs presenting TT (Figure 6-10A). This is significant as it would suggest that TT specific clones which do not typically secrete IFNγ or show a predominant Th1 profile in HDs (196, 204-206) have been skewed towards a Th1 phenotype in some RRMS patients. In fact, it was recently demonstrated that female RRMS patients show an exaggerated IFNγ response to TT in PBMCs as compared to control females (207). Our data may suggest that memory B cells from RRMS patients are likely candidates influencing this phenomenon.

In contrast to the increase in IFNγ secretion in response to memory B cells presenting neuro-antigens or TT, IFNγ secretion in response to memory B cells presenting GA was rarely observed (Figure 6-10B). Yet CD4+ T cell proliferation in the presence of memory B cells and GA was robust in the majority of RRMS patients (Figure 6-07B). CD4+ T cell proliferation in the presence of memory B cells and GA was also

robust in the majority of HDs, yet IFNγ was not observed in the majority of these cultures either. This data would suggest that memory B cells from RRMS patients and HDs naïve to GA therapy maintain their capacity to elicit CD4+ T cell proliferation in response to GA, but that in most cases, are not able to drive T cell effector functions such as IFNγ secretion. This was somewhat unexpected as the majority of GA-specific T cell lines derived from GA-naïve MS patients secrete IFNγ readily (64, 208). However, GA-specific T cell lines generated from MS patients that have been treated for several months with GA secrete significantly less IFNγ (64). We would not expect that IFNγ reactive T cell clones were absent in the MS patient cohort samples used in these studies since the patients were treatment naïve and in early stages of disease. It would be interesting to determine whether memory B cells from RRMS patients benefiting from GA therapy would additionally lose their capacity to elicit CD4+ T cell proliferation in response to GA, and the duration of GA therapy that is required to initiate this effect. Of note, it has been documented that GA may not need to be processed since it binds directly to HLA-DR on the surface of APCs (201). We were able to purify sufficient numbers of memory B cells from one patient to test whether GA processing by memory B cells was required to induce CD4+ T cell proliferation. Indeed, pretreatment of memory B cells with chloroquine, a lysosomotropic agent that prevents antigen processing, prior to co-culture with T cells and GA, reduced CD4+ T cell proliferation below the detection threshold in response to GA.

The finding that TLR9 plus CD40L stimulation, compared to CD40L alone, or CD40L plus BCR crosslinking, results in greater IL-10 and LTα secretion in both memory and naïve B cells from HDs, as well as naïve B cells from RRMS patients is

intriguing. Previously it was demonstrated that TLR9 plus CD40L stimulation synergistically acts to promote the production of IL-10, IL-6, TNFα, and IL-12p70 from whole peripheral B cell populations in comparison to TLR9 or CD40L stimulation separately (193). Additionally, while T cell help and BCR crosslinking are sufficient to drive low levels of naïve B cell proliferation, TLR9 stimulation is the third signal required for full activation of naïve B cells (209). Our data would suggest that memory B cells are more responsive to TLR9 stimulation than their naïve B cell counterparts, perhaps due to the constitutive expression of TLR9 by memory B cells in comparison to naïve B cells, which required pre-stimulation to express TLR9 (78). Perhaps TLR9 expression on memory B cells from RRMS patients is greater than that observed on memory B cells from HDs, which might explain the differences observed between HDs and RRMS patients in response to memory B cells presenting either MOG or MBP, however this remains to be examined.

Since sera from a subset of RRMS patients and HDs contain MBP and MOG reactive antibodies (210), we predicted that a subset of B cells from RRMS patients and HDs would bind to MBP and MOG, and also have the capacity to elicit CD4+ T cell proliferation in response to these neuro-antigens. Indeed, memory B cells from RRMS patients and HDs were able to bind to MBP and MOG, which substantiate this prediction. However, memory B cells from RRMS patients, but not from HDs, were able to elicit CD4+ T cell proliferation in response to MBP or MOG. As mentioned earlier in the discussion, this finding may be attributable to features of memory B cells from RRMS patients that are not present on memory B cells from HDs or some differential enrichment of MBP- or MOG-specific memory T cells in the RRMS patients in comparison to HDs.

However, naïve B cells from some RRMS patients, which bind to MOG at similar frequencies as memory B cells from RRMS patients, are also able to elicit CD4+ T cell proliferation in response to MOG. This finding is unique to MOG-reactive naïve B cells from RRMS patients since MBP-reactive naïve B cells from the same patients do not elicit appreciable CD4+ T cell proliferation in response to MBP. One possibility is that MOG-specific memory T cells enriched in RRMS patients can be reactivated by both memory and naïve B cells. Previous studies indicate that naïve B cells can elicit memory T cell activation (182). However, this hypothesis would apply only to MOG-specific responses, since naïve B cells do not elicit CD4+ T cell proliferation in response to MBP.

A second possibility is that MOG may be serving as a molecular mimic for an antigen recognized equally well by both the memory and naïve B cell repertoire, explaining the ability of both of these subtypes of B cells to bind and present MOG to T cells. For example, it has been demonstrated that MOG serum antibodies cross-react with epitopes present in the milk protein, butyrophilin (211). Additionally mice transgenic for IgH and IgL recognizing a conformationally specific epitope of MOG undergo receptor editing to endogenous light chains on both MOG sufficient and MOG deficient genetic backgrounds indicating some form of cross-reactivity of the MOG reactive antibodies to a distinct self protein (212). MOG could also potentially act as an auto-stimulatory TLR agonist in the context of RRMS as ribonucleoprotein particles do in the context of SLE (213). Along these same lines, chloroquine, in addition to inhibiting antigen processing and presentation of intact protein antigens, also inhibits the full activation of intracellular TLRs such as TLR7 and TLR9, by inhibiting acidification of endosomal/lysosomal compartments (214, 215). Therefore an alternate explanation for the inhibition of B cell

mediated antigen specific CD4+ T cell proliferation observed with chloroquine could be the inhibition of intracellular TLRs.

Speculation as to the development of neuro-antigen specific memory B cells in the context of RRMS is of great interest given the fact that autoreactive B cells are typically negatively selected during development (147), or are prevented from accessing a germinal center reaction (216). Our results suggest that in the subset of RRMS patients whose memory B cells promoted CD4+ T cell expansion to MBP or MOG in vitro, either peripheral mechanisms of B cell tolerance failed or tolerance has been overcome through sensitization. Tolerance for example can be overcome by sensitization in a mouse model where B cells that express BCR specific for an endogenous neo-antigen are negatively selected during B cell development but none-the-less can develop a memory B cell repertoire that recognizes this neo-antigen after immunization (217). In addition, it was demonstrated that inherently autoreactive VH4-34 expressing B cells are not excluded from a germinal center reaction and can develop into memory B cells in the context of SLE (216, 218). Whether this same principle explains the enrichment of neuro-antigen specific B cells in RRMS patients remains unexplored.

In conclusion, these studies identify MBP and MOG specific memory B cells as potent activators of myelin specific T cells from RRMS patients, but not HDs. Thus, memory B cells may promote the exacerbation of RRMS by reactivating T cells in the periphery. Such studies provide a mechanistic explanation for why specific depletion of B cells from RRMS patients is beneficial to patients and may indicate the need to investigate depletion of specific subsets (memory B cells) as a therapeutic strategy.

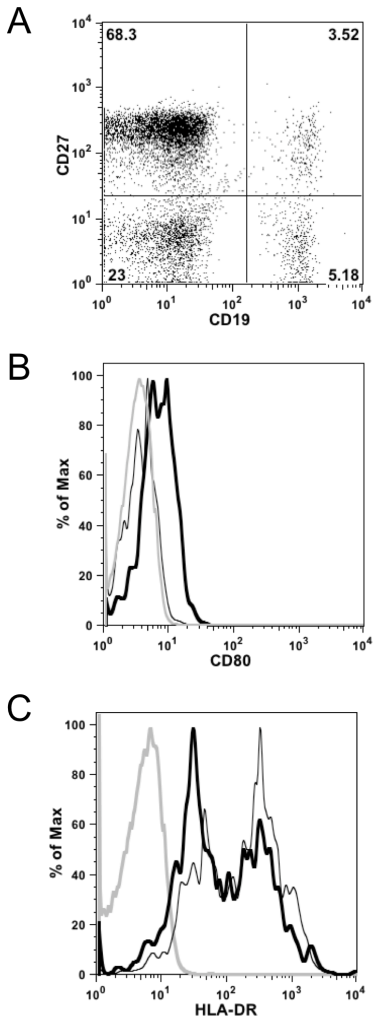


FIGURE 6-01. Example gating strategy and expression of CD80 and HLA-DR on naïve and memory B cells from a HD. PBMCs from HD-4 were stained with CD80-FITC, CD27-PE, CD19-PECy/5, and HLA-DR-APC antibodies (Table 3-04) as described in materials and methods, and at least 20,000 events were collected on a FACSCalibur™ (BD Biosciences, San Jose, CA, USA) after four color compensation with single flourochrome stained cells. (A) Gated live lymphocytes were displayed with CD27 vs. CD19. Gated CD27+CD19+ cells were considered memory B cells, and CD27-CD19+ cells were considered naïve B cells. Percentages in each gate are listed in corner of dot plot. (B) CD80 expression was calculated by determining mean fluorescence intensity (MFI) of gated naïve (thin black line, MFI=3.65) and memory B cells (thick black line, MFI=6.75) and compared to isotype control antibody staining (grey line, MFI=3.28) on a histogram. (C) HLA-DR expression was calculated by determining MFI of gated naïve (thin black line, MFI=195) and memory B cells (thick black line, MFI=65.3) and compared to isotype control antibody staining (grey line, MFI=4.64) on a histogram.

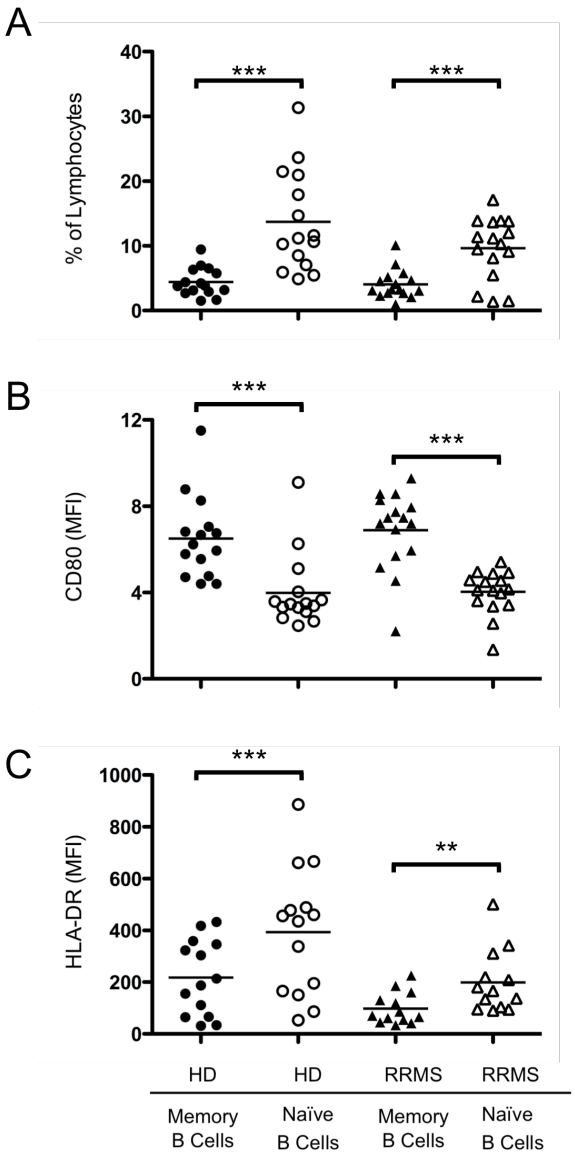


FIGURE 6-02. Memory B cells from RRMS patients and HD exhibit an activated phenotype but express less HLA-DR. The percentage of memory (CD19+CD27+) and naïve (CD19+CD27-) B cell subsets from the peripheral blood of RRMS patents and HDs were quantified by flow cytometry in a live lymphocyte gate based on FSCxSSC characteristics (A). The expression of CD80 (B) and HLA-DR (C) on memory and naïve B cells were determined by quantifying MFI on gated naïve and memory B cell subsets. Individual points represent individual subjects, and black bars represent mean values within group. ** = $p<0.01$, *** = $p<0.001$, multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed-model ANOVA.

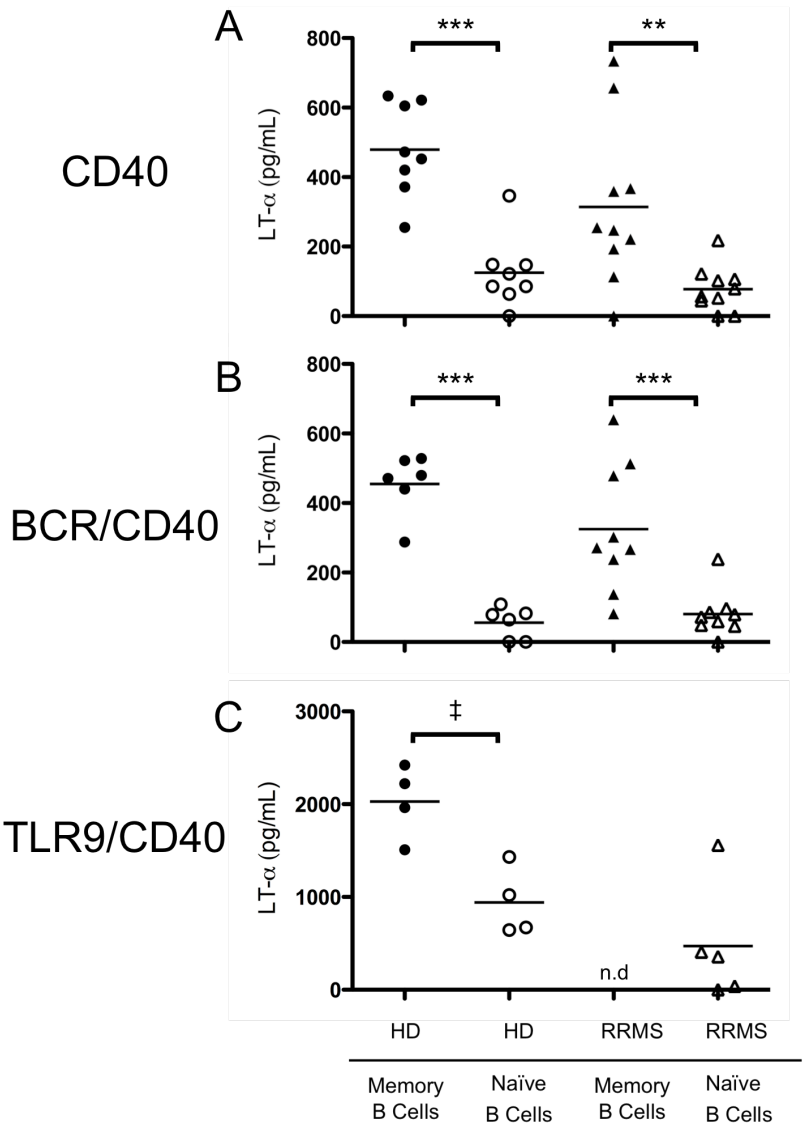


FIGURE 6-03. Memory and naïve B cells from RRMS patients and HDs generate similar LTα profiles in response to polyclonal stimulation. Highly purified, sorted naïve and memory B cell subsets from HDs and RRMS patients were stimulated with CD40L for 48 hours (A), dual-staggered stimulation with BCR crosslinking and CD40L for 72 hours (B), or TLR9 (CpG ODN 10103) and CD40L stimulation for 48 hours (C) as described in materials and methods. LTα (A, B, and C) secretion was measured in cell culture supernatants by ELISA. Individual points represent individual subjects, and black bars represent mean values within group. ** = $p<0.01$, *** = $p<0.001$, multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed-model ANOVA.. ‡ = $p<0.05$ unpaired t-test.

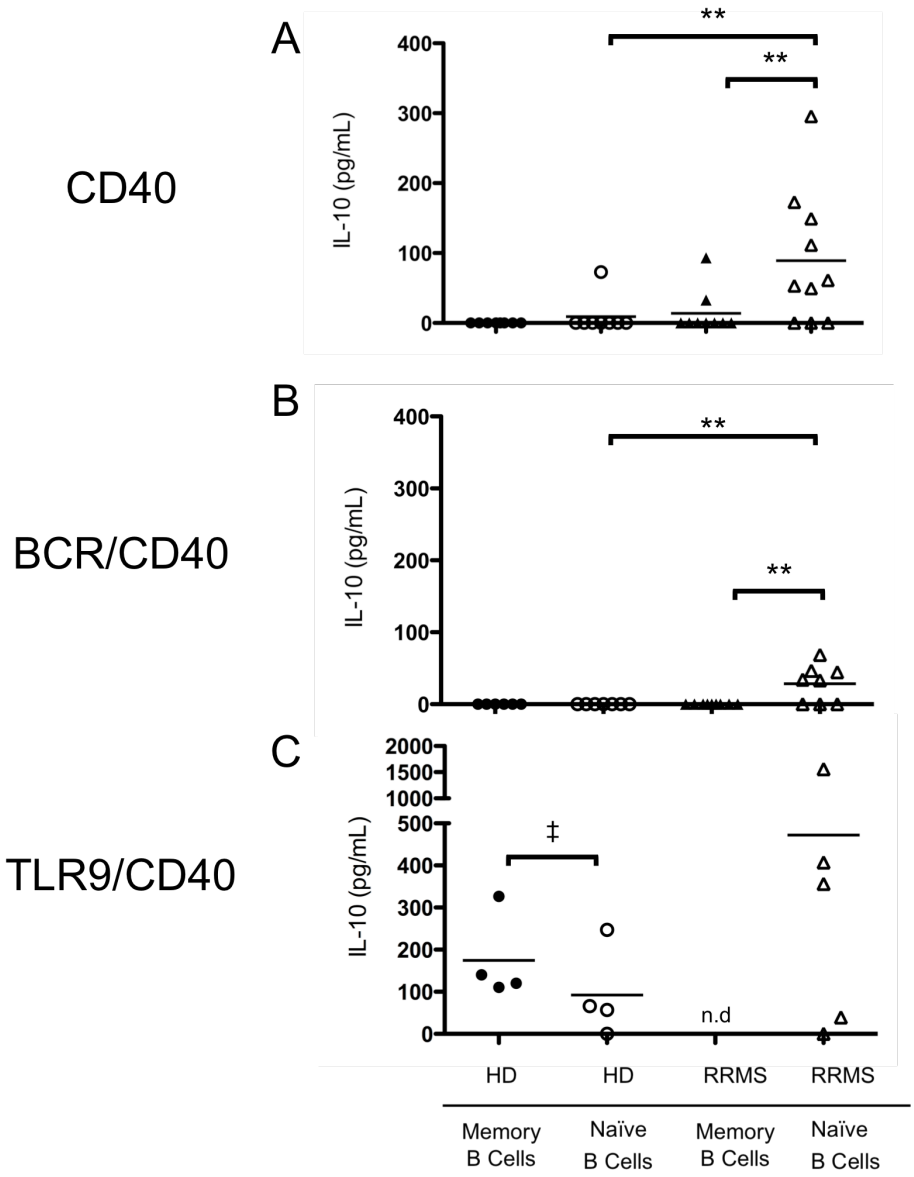


FIGURE 6-04. IL-10 secretion is a prominent feature of RRMS and not HD polyclonally stimulated B cells. Highly purified, sorted naïve and memory B cell subsets from HDs and RRMS patients were stimulated with CD40L for 48 hours (A), dual-staggered stimulation with BCR crosslinking and CD40L for 72 hours (B), or TLR9 (CpG ODN) and CD40L stimulation for 48 hours (C) as described in materials and methods. IL-10 secretion was measured in cell culture supernatants by ELISA. Individual points represent individual subjects, and black bars represent mean values within group. ** = $p<0.01$, multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed-model ANOVA. ‡ = $p<0.05$ unpaired t-test.

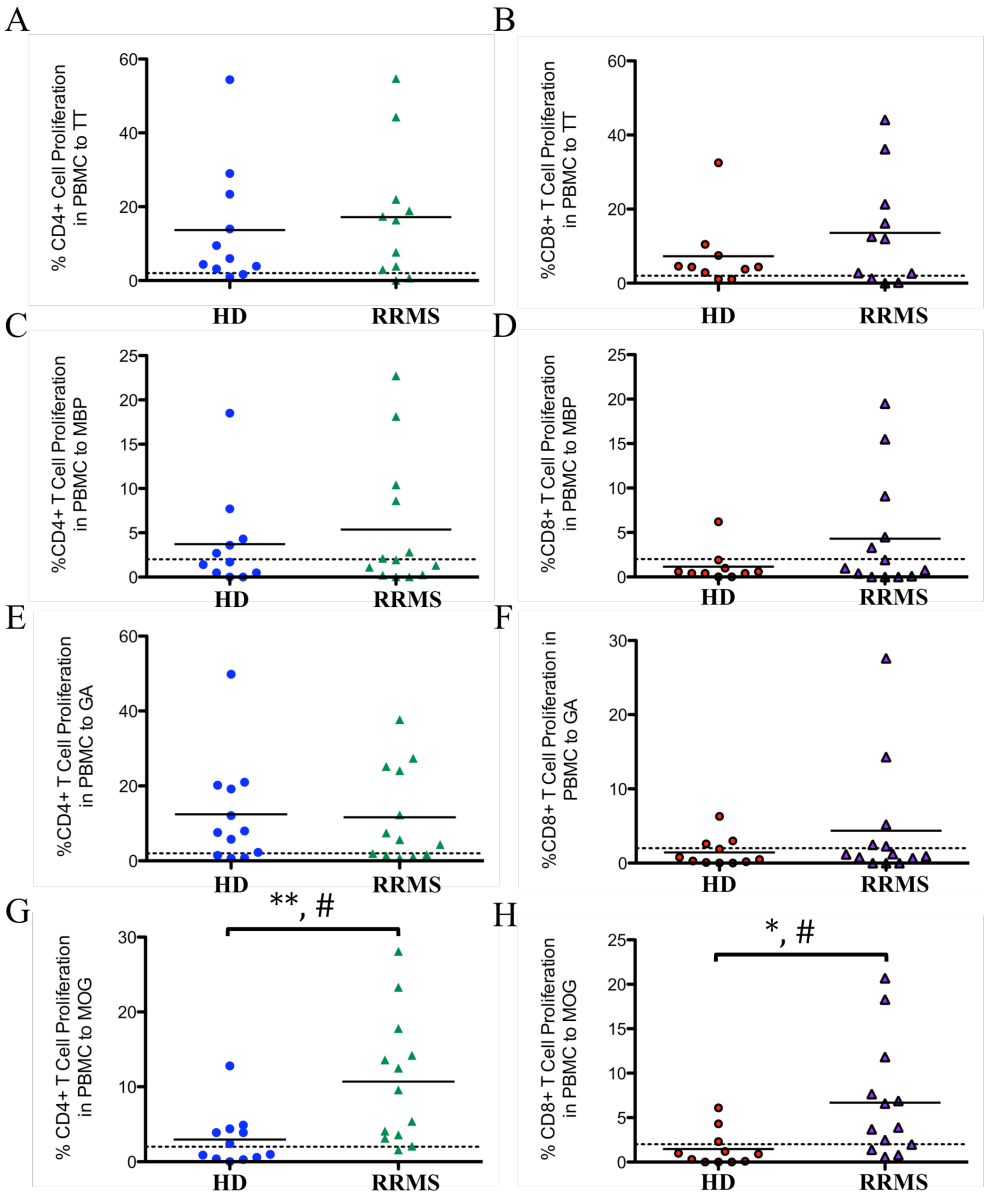


FIGURE 6-05. CD4+ and CD8+ T cell proliferation in response to various control and myelin antigens in HDs and RRMS patient whole PBMC. CD4+ (A, C, E, G), and CD8+ (B, D, F, H) T cell proliferation in response to TT (A, B), MBP (C, D), GA (E, F), and MOG (G, H) in whole PBMCs from donors were measured by CFSE dilution after 5 days in culture. Horizontal bars represent mean proliferation within group * = $p \leq 0.05$, ** = $p \leq 0.01$; un-paired student's t-test comparing mean proliferation. # = $p \leq 0.05$; χ^2-test comparing number of responders (as defined by proliferation greater than 2%).

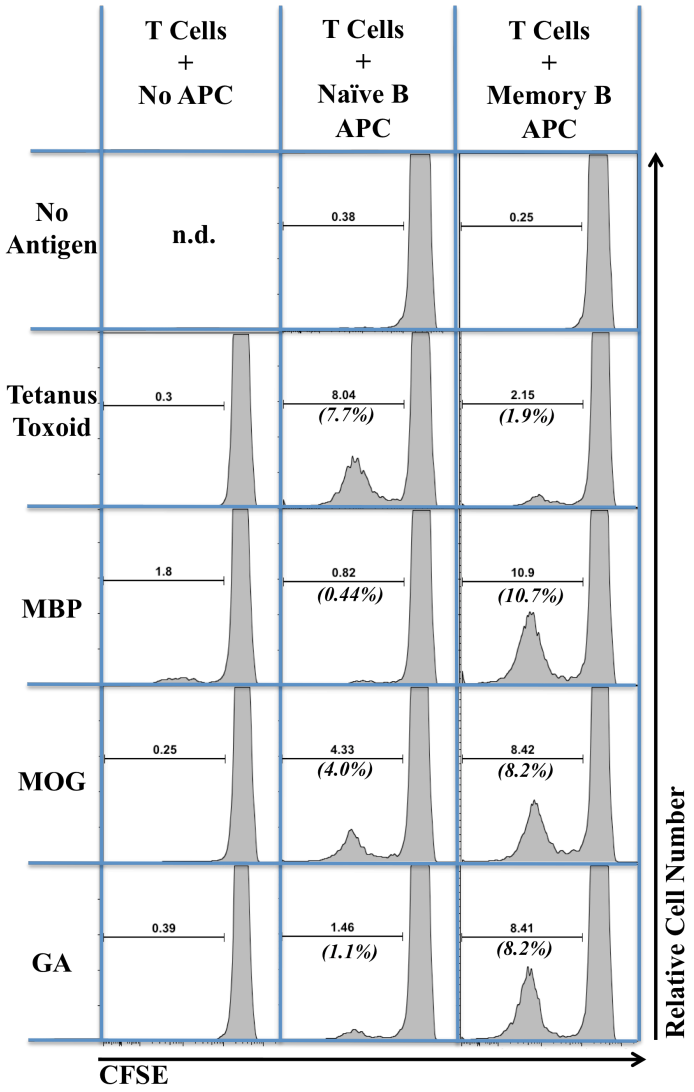


FIGURE 6-06. Example of gating and determination of normalized CD4+ T cell proliferation reported in Figure 6-05 and 6-06. Purified T cells from MS-10 (TP1) were cultured with either no APC, naïve B cell APCs, or memory B cell APCs for 5 days in the presence of exogenous antigen where indicated, and T cell proliferation was determined as described in Section 3.8.2. Histograms represent cells that were gated for live CD3+CD4+ cells. Number above gate represents raw proliferating fraction (% CFSE low) for each antigen. Number below gate in parentheses represents the normalized proliferation (%) for each antigen that was determined using the formula: [antigen specific proliferating fraction (%) – background (No Antigen) proliferating fraction (%)] for each APC type.

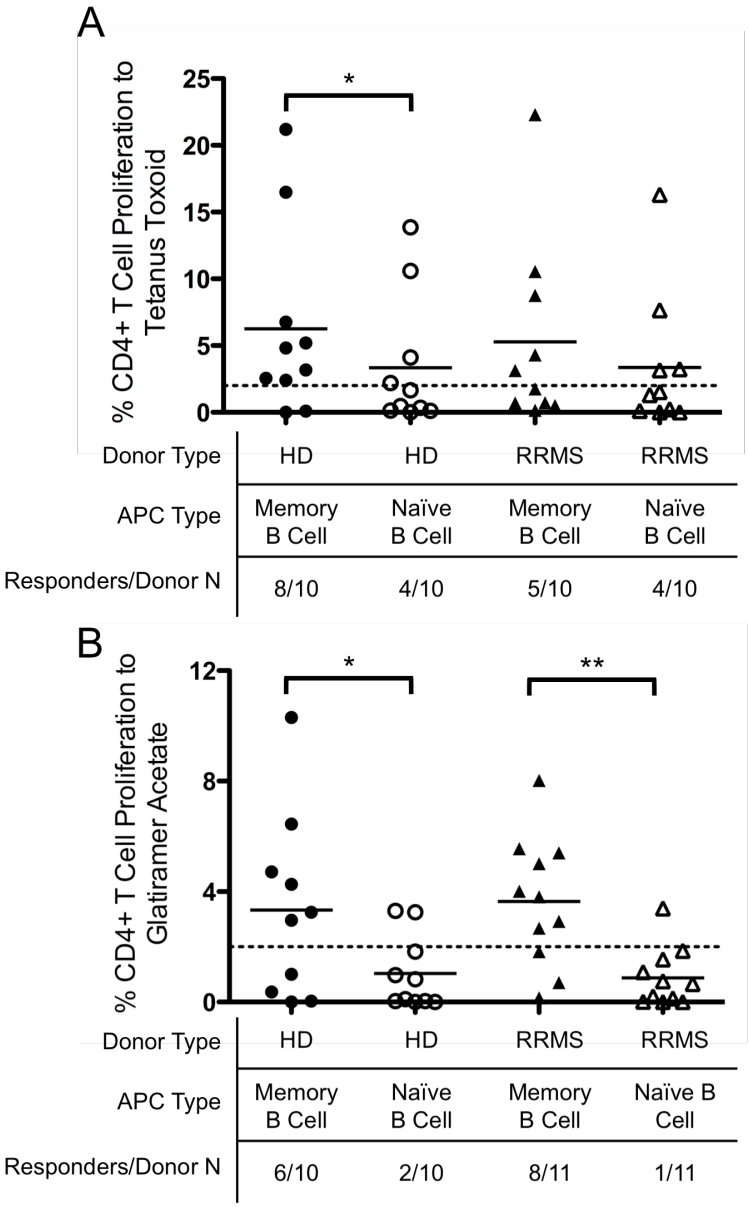


FIGURE 6-07. Memory B cells elicit greater TT and GA specific CD4+ T cell proliferation than naïve B cells in both HDs and RRMS patients. Highly purified, ex vivo naïve and memory B cells from cryopreserved HD and RRMS PBMCs were incubated with purified autologous T cells, and CD4+ T cell proliferation by CFSE dilution was measured in vitro culture after 5 days in response to TT (A) and GA (B). Threshold of CD4+ proliferation considered positive was set at 2% and is depicted by dashed line in all panels. Individual points represent individual responses and black bars represent mean values within group. * = $p<0.05$, ** = $p<0.01$, multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed-model ANOVA.

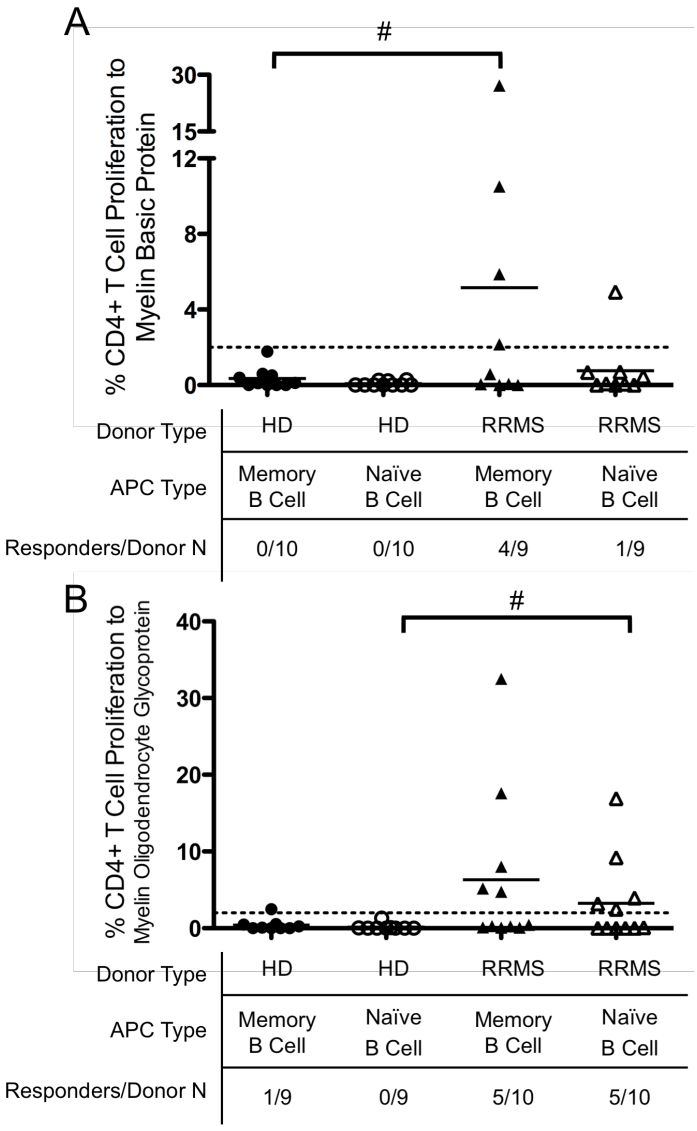


FIGURE 6-08. Memory B cells elicit MBP specific CD4+ T cell proliferation in greater number or RRMS patients than in HDs. Highly purified, ex vivo naïve and memory B cells from cryopreseved HD and RRMS PBMCs were incubated with purified autologous T cells, and CD4+ T cell proliferation by CFSE dilution was measured in vitro culture after 5 days in response to MBP (A) and MOG (B). Threshold of CD4+ proliferation considered positive was set at 2% and is depicted by dashed line in all panels. Individual points represent individual donor responses and black bars represent mean values wihin group. # = $p<0.05$, chi-squared test comparing number of positive responders.

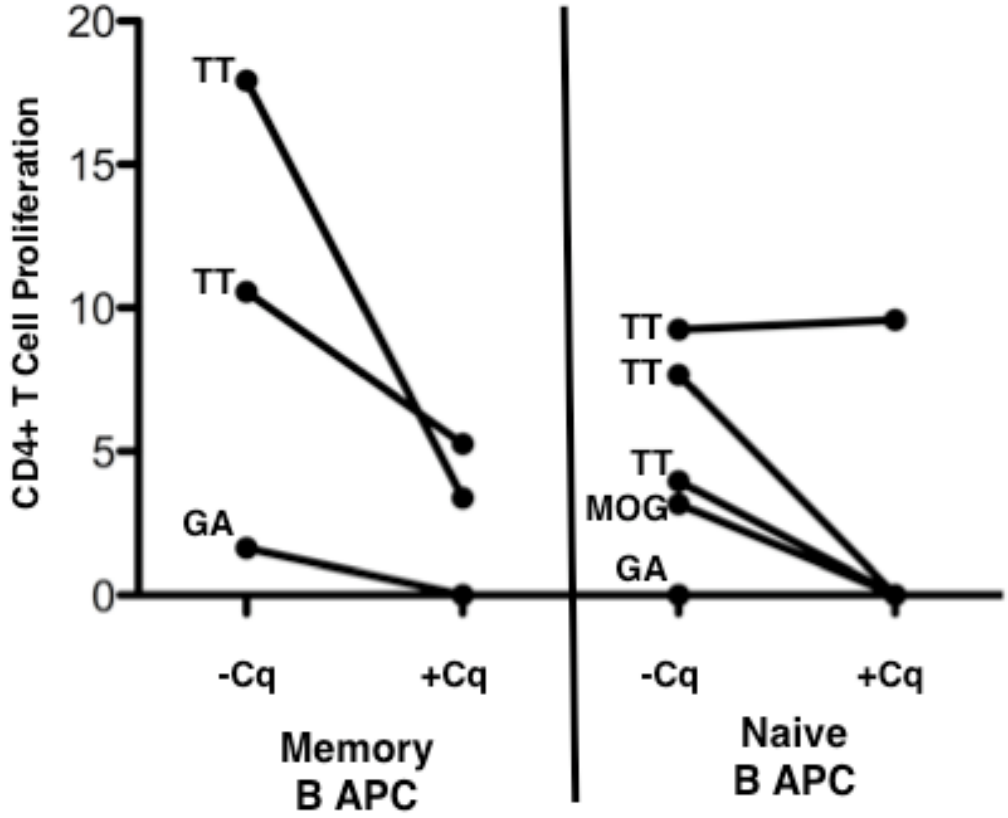


FIGURE 6-09. Chloroquine inhibits both Memory and naïve B cell induced CD4+ T cell proliferation to several antigens. Highly purified, ex vivo naïve and memory B cells from cryopreserved RRMS PBMCs were incubated with purified autologous T cells, and either TT, MOG, or GA, in the presence (+Cq) or absence (-Cq) of Chloroquine (+Cq). CD4+ T cell proliferation by CFSE dilution was measured in vitro culture after 5 days. The type of B APC is listed below the x-axis and the type of antigen added to culture is listed next to each proliferative response. Lines connecting points represent responses from same patient with or without chloroquine.

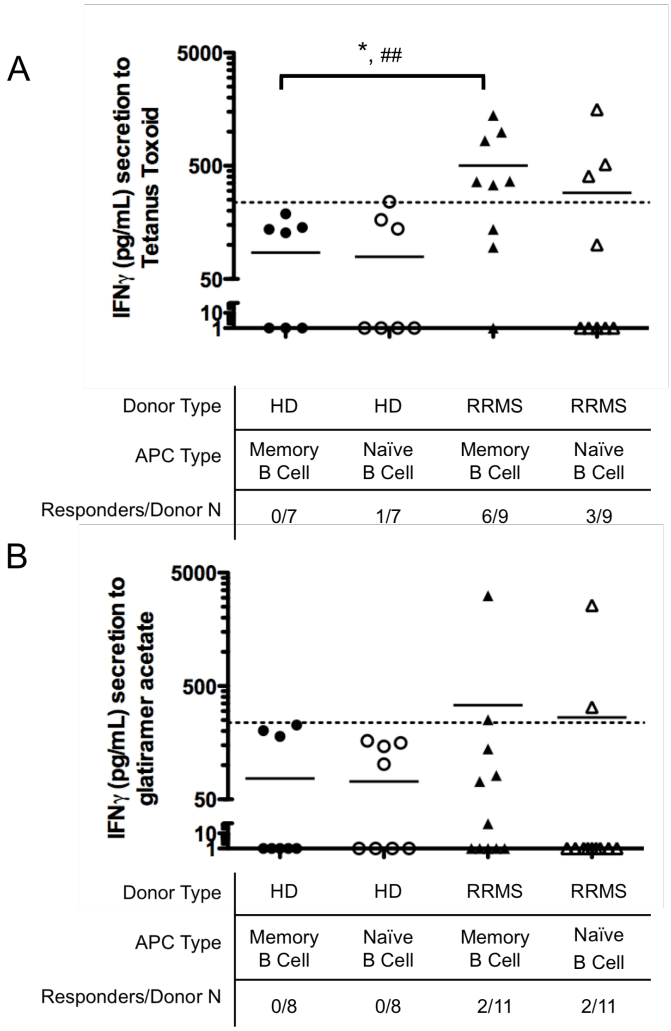


FIGURE 6-10. RRMS memory B cell APCs elicit greater number of antigen-specific TT responders by IFNγ secretion in B-T co-cultures than HD memory B APCs. Highly purified, ex vivo naïve and memory B cell subsets from the HD and RRMS cohorts were incubated with purified autologous T cells, and IFN-γ secretion was measured after 5 days in culture supernatants in response to TT (A) and GA (B). Individual points represent individual donor responses. Threshold of IFNγ production was set at 237 pg/mL and was determined empirically by adding 2 standard deviations to the mean detectible IFNγ production observed in B-T co-cultures when no antigen was present. The threshold is depicted as a dashed line in all panels. Individual points represent individual responses and black bars represent mean values within group. * = $p<0.05$, multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed-model ANOVA. ## = $p<0.01$, chi-squared test comparing number of positive responders.

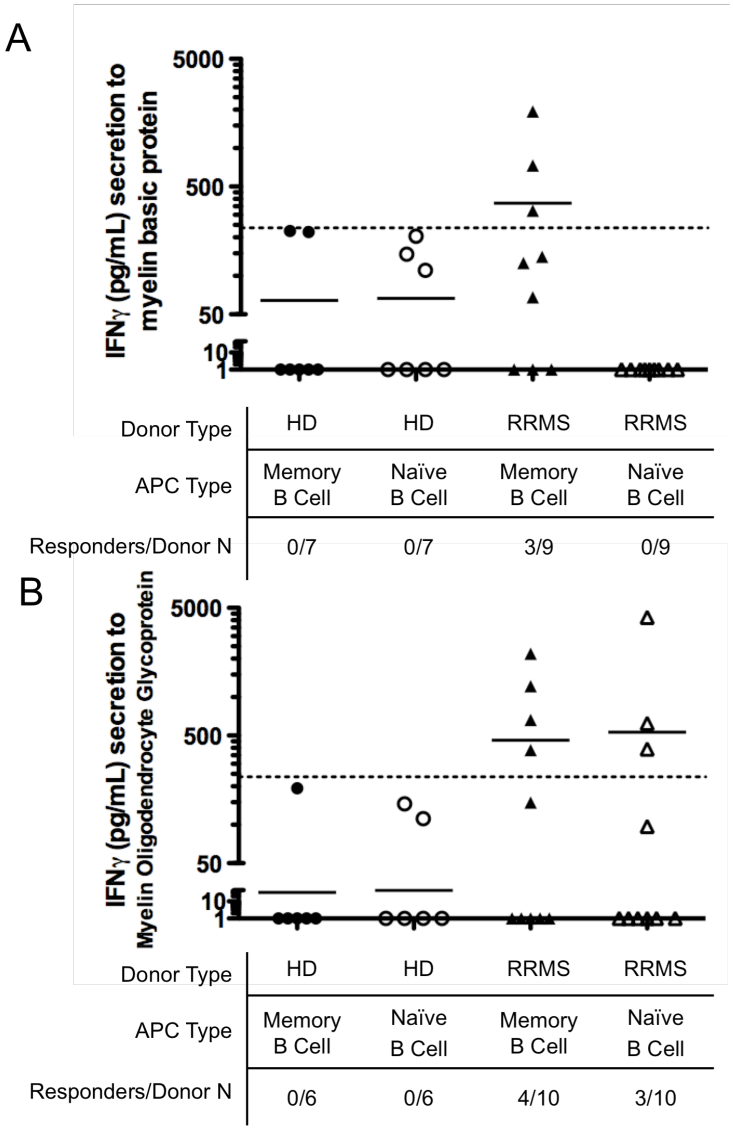


FIGURE 6-11. A subset of RRMS but not HD B cell APCs neuro-antigen-specific IFNγ secretion in B-T co-cultures. Highly purified, ex vivo naïve and memory B cell subsets from the HD and RRMS cohorts were incubated with purified autologous T cells, and IFN-γ secretion was measured after 5 days in culture supernatants in response to MBP (A) and MOG (B). Individual points represent individual donor responses. Threshold of IFNγ production was set at 237 pg/mL and was determined empirically by adding 2 standard deviations to the mean detectible IFNγ production observed in B-T co-cultures when no antigen was present. Individual points represent individual responses and black bars represent mean values within group.

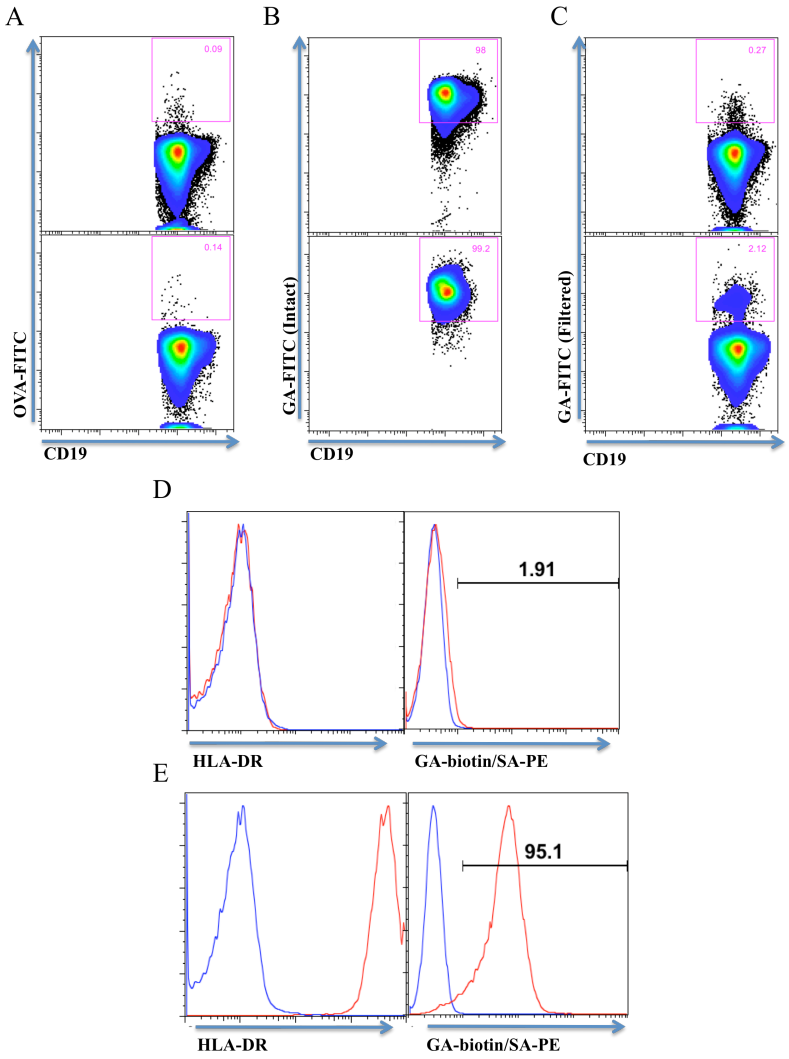


Figure 6-12. Binding of majority of B cells by intact FITC conjugated GA is reduced by significantly by filtering. PBMCs from MS patient (MS-5) were stained with the FITC conjugated proteins ovalbumin (OVA-FITC) as a control (A) or Glatiramer Acetate (GA-FITC) (B,C). GA-FITC was either left intact (B), or filtered and analyzed as described in materials and methods (C). Events were gated for live lymphocytes based on FSC/SSC characteristics. Top row of flow panels are gated live naïve B cells ($CD19^{+}CD27^{-}PI^{-}$), bottom row are gated live memory B cells ($CD19^{+}CD27^{+}PI^{-}$). Intact GA-FITC bound to all B cells (99%), and 11.2% of non-B cells (data not shown). Filtered GA-FITC bound to a small percentage of memory B cells (2.1%) and naïve B cells (0.3%), and less than 0.1% non-B cells (data not shown).

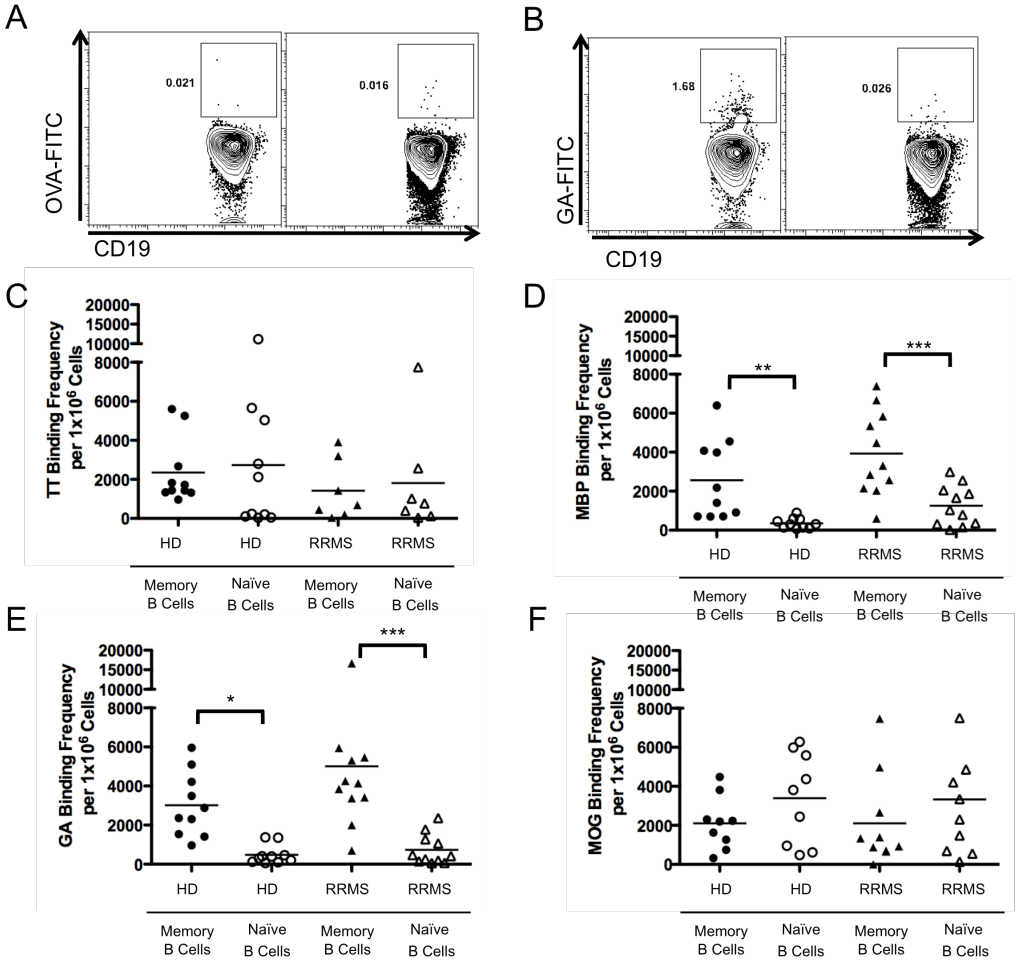


FIGURE 6-13. B cells that bind MBP and GA are more prevalent in the memory B cell pool than the naïve B cell pool in both HD and RRMS patients. PBMCs from HDs and RRMS patients were incubated with FITC conjugated antigens as described in materials and methods. Examples of gating used to enumerate the percentage of memory or naïve B cells that bound OVA (A) and GA (B). The frequency of TT-(C), MBP- (D), MOG- (E), and GA- (F) binding B cells for the RRMS patient and HD cohorts were calculated as described in materials and methods and depicted here as individual points on the vertical scatter plots. The average frequency of binding in each group is shown as a solid black horizontal line in each panel. The mean OVA binding frequency was 93 cells per million for HD memory B cells; 119.3 cells per million for HD naïve B cells; 636.5 cells per million for RRMS memory B cells; and 471.5 cells per million for RRMS naïve B cells. Page 36 of 38 *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$; multiple comparisons post-hoc analysis with Bonferroni correction after two-way mixed model ANOVA.

Table 6-01. Summary of RRMS patient proliferation, cytokine secretion, and protein binding assays.

Antigen	RRMS Patient ID	PBMC % CD4 Proliferation	Memory B-T % CD4 Proliferation	Naïve B-T % CD4 Proliferation	Memory B-T IFN-g (pg/mL)	Naïve B-T IFN-g (pg/mL)	Memory B # Protein Binding	Naïve B # Protein Binding
MBP	MS-1	8.6	0	0	0	0	7397	2988
	MS-5	0	0	0	125.9	0	5844	1032
	MS-10	10.4	10.52	0	0	0	4484	793
	MS-11	0.24	5.87	0	324	0	2030	2548
	MS-14	1.93	0	0	1935.1	0	595	313
	MS-15	22.7	27.13	4.93	731	0	3312	1866
	MS-16	1.3	0	0	0	0	6670	1657
	MS-17	2.8	0	0	140.9	0	2841	27
	MS-20	18.1	2.16	0	68.1	0	2570	160
GA	MS-1	37.7	3.82	0	0	0	5462	1273
	MS-2	25.2	2.67	0	252.4	0	3419	58
	MS-5	5.7	5.01	0	42.9	0	2007	470
	MS-10	7.5	8.03	1.08	81.4	2573.4	697	185
	MS-11	4.36	2.92	0	139.2	0	5306	1774
	MS-14	12.3	5.56	1.55	3133.9	324.3	5952	1064
	MS-15	1.1	4.02	0	0	0	4257	420
	MS-16	27.4	1.82	1.85	0	0	3845	147
	MS-17	1.4	5.4	3.39	72	0	3374	2360
	MS-18	24.1	0	0	0	0	4145	71
	MS-20	2	0	0	0	0	16643	263
TT	MS-1	54.7	17.92	9.24	834.4	403.9	n.d.	n.d.
	MS-2	3.9	3.14	1.53	1397.9	514.2	1433	1018
	MS-5	0.7	0	0	365.9	0	453	771
	MS-10	7.7	1.77	7.66	338.3	1575	196	124
	MS-11	0.09	0	1.3	137.7	100.6	n.d.	n.d.
	MS-14	18.9	8.76	3.23	993.6	0	n.d.	n.d.
	MS-15	n.d.	10.55	3.16	nd	nd	n.d.	n.d.
	MS-16	17.4	0	0	360.9	0	688	398
	MS-18	16.4	4.3	0	0	0	3201	2571
	MS-20	44.3	22.3	16.3	95.2	0	41	45
MOG	MS-1	23.3	0	0	0	0	1341	550
	MS-2	2.1	0	0	385	96.8	7464	8177
	MS-5	1.6	0	0.087	661.2	0	4974	4863
	MS-10	17.8	8.04	3.95	0	4207	925	1488
	MS-11	3.15	5.18	3.19	0	0	665	145
	MS-14	14.2	0	0	2183.9	624	0.11	
	MS-15	9.6	32.5	16.9	1215.7	391.6	705	3351
	MS-16	28.1	0	0	0	0	1395	2302
	MS-17	4.1	4.77	9.19	0	0	0	7506
	MS-18	12.5	0	0	0	0	2660	4209
	MS-20	3.6	17.59	2.5	149.1	0	877	692

nd=not done

CHAPTER SEVEN

Discussion and Conclusions

While CD4+ T helper cells are of central importance in orchestrating immune responses in the development of autoimmune reactions such as multiple sclerosis, the pathways of CD4+ T cell differentiation and effector status depend in large part on the type of priming APC presenting cognate antigen and the specific interactions of cell surface molecules and cytokines secreted by the APC.

The antigen presenting function of B cells has historically been under-appreciated since CD4+ T cell responses can be propagated in the absence of B cell APCs. However, the relatively immediate therapeutic benefit of Rituximab, a B cell depleting antibody, in patients with relapsing remitting MS re-focuses our attention on the potential of B cells to contribute to the pathogenesis of MS by operating as neuro-antigen specific presenting cells.

The work presented in this dissertation culminates in a demonstration that memory B cells from RRMS patients can effectively promote T cell functional responses. I demonstrate that, as a whole the B cell population inhabiting the CSF of MS patients had likely been selected for in the context of a germinal center in contrast to some intrathecally expanded B cell clones which show mutational targeting uncharacteristic of germinal center selection. These findings suggested that the intrathecal B cell population as a whole had undergone affinity maturation to antigen in the periphery, a process that is dependent on antigen presentation to T cells. These findings prompted investigation of peripheral B cell subsets from MS patients for their capacity to elicit neuro-antigen

specific T cell functional responses. However, many model systems had demonstrated that B cells are tolerogenic, or poor initiators of inflammatory T cell responses, so it was necessary to establish that B cells could in fact propagate neuro-antigen specific T cell responses *in vitro*. Work presented in Chapter 4 provides evidence that pre-activated B cells from both HDs and RRMS patients are able elicit neuro-antigen specific CD4+ and CD8+ activation proliferation, in a process that was largely dependent on antigen presentation through HLA-DR. Evidence was also presented that demonstrates that the route of B cell activation drastically modulates the outcome of T cell responses in vitro. These data reinforced the paradigm that the activation status of a B cell greatly influences its capacity to participate in an immune response as a productive antigen-presenting cell. Combining experimental evidence from chapter 3 and chapter 4 led to the hypothesis that neuro-antigen specific memory B cells that had been previously selected in the context of a germinal center to neuro-antigen may potentially be potent neuro-antigen specific antigen presenting cells in instigation of T cell activation. Evidence presented in this dissertation demonstrates that although memory B cells from MS patients phenotypically resemble memory B cells from HDs in many ways, MS memory B cells were overwhelmingly more efficient neuro-antigen specific B cells when compared to HD memory B cells inducing more neuro-antigen specific proliferation and IFNγ secretion than naïve B cells. While these data only begin to address the role of memory B cells in influencing T cell responses in MS, they provide compelling evidence that memory B cells are important effector cells in eliciting neuro-antigen specific T cell responses in MS patients.

MS CSF B cell characteristics; relationship to neuro-antigen specific B cell development in the periphery

With regard to memory B cells, it is interesting to note that a common feature among several compartmental driven autoimmune diseases including MS, is a preferential accumulation of memory B cells at sites of inflammation. For example, the $CD27^+$ memory B cell pool is reduced in the circulating peripheral blood of patients with Sjogren's Syndrome (SS) compared to HDs (189, 219), but dominate the inflamed parotid glands of these patients (189). Similarly, pre-switch $CD27^+$ B cells are decreased in peripheral blood of rheumatoid arthritis (RA) patients compared to HDs, but dominated the inflamed synovium (220). And as others and I have now shown, the CSF B cell compartment is also dominated by $CD27^+$ memory cells.

The demonstration that the majority of B cells that are present in target organs during the course of autoimmunity have a memory phenotype is important as it defines them as important effector cells in the development of autoimmunity. However the tissue site of B cell activation and memory B cell differentiation in MS has remained controversial. The prevailing hypothesis postulates that the initiation of CNS antigen specific lymphocyte activation occurs in the periphery (22). For example foreign antigen injected into regional brain sites of rabbits drain mainly to the cervical lymph node (CLN) (221). In fact mice that have undergone CLN lymphectomization, accrue less severe relapses in a relapsing remitting EAE model (222), and dendritic cells injected into the CSF migrate to B cell areas of the CLN (223). Macrophages aspirated from the CLN of MS patients contained significant concentrations of MBP and PLP, but were rare in CLN of HCs (224). Furthermore, in a spontaneous model of EAE, MBP specific

activated T cells are readily observed in the CLN but at no other site (225). Taken together, these data indicate that secondary peripheral lymphoid organs, rather than the CNS, are sites where the immune system is likely first exposed to CNS auto-antigen. Secondary exposure to CNS auto-antigen within the CNS itself is likely since expansion and activation of B cells within the CNS of MS patients is readily detected in the CSF of MS patients (9, 11-13, 226). In order to determine whether there is a relationship between CNS resident B cells and peripheral B cells, I compared several parameters of variable region gene usage and mutational characteristics to probe this relationship.

I present evidence here that V_H4 and J_H4, and J_H5 expressing B cells are overrepresented in the population of CSF B cells from MS patients in comparison to OND CSF and HCPB. These findings of VH4 overrepresentation in MS CSF B cell repertories were recently substantiated (227, 228) and suggest that CNS antigen is driving the recruitment or selection of B cells harboring CNS antigen specificities that are more frequently encoded by these genes and more importantly, may recognize antigen epitopes important in the development of MS. In order to understand if the B cell population as a whole showed signs of antigen driven selection, which typically require maturation within a germinal center, I examined mutational characteristics associated with this type of selection to HCPB B cells that presumably underwent normal antigen selection.

B cells in the CSF of both MS and OND patients exhibited greater mutation frequency than observed in HCPB reflecting the accumulation of memory B cells in the CNS. Targeting of mutations to the CDR, greater R:S ratios in the CDR than FR, and sub-targeting of mutations to RGYW/WRCY motifs within CDRs, were all intact in CSF B cell repertories from MS and OND B cells indicating normal antigen driven selection

in contrast to the clonal CSF B cell population in some MS patients that did not maintain this pattern of mutations indicating perhaps compartmental driven selection in the absence of a germinal center. Although no studies have examined the mutational targeting of BCRs from B cells emanating from ectopic germinal centers found in the meninges of some secondary progressive MS patients (132, 158) the mere presence of these ectopic B cell follicles makes it tempting to hypothesize that clonal expansion and CNS antigen driven selection of B cells are taking place at these locations. Surprisingly, although ectopic lymphoid like structures appear in the inflamed synovium of RA patients, most fail to fully differentiate into germinal centers supportive of B cell clonal expansion and antigen selection (130). These findings may indicate that although the ectopic lymphoid like structures form in the meninges of some MS patients, these may fail to promote normal antigen driven selection of the B cell repertoire. Furthermore it has been demonstrated in mice that although temporary germinal centers can form in the absence of T cells, these quickly involute and do not provide an environment for high mutational selection to specific antigen (229). In addition, these authors provide evidence that extra-follicular plasmablasts/plasma cells undergo low levels of somatic hypermutation (229), perhaps lending an explanation for the observation that some clonally expanded B cells in the CSF of MS patients show hypermutation in the absence of a germinal center. The question then becomes: If at least a subset of memory B cells are preferentially being recruited into the CNS from the periphery I hypothesize that this is due to the specific recruitment of activated, antigen specific B cells in the periphery based on differential expression of chemokine receptors and integrins crucial for recruitment into the CNS.

Role of cytokines and chemokines in the recruitment and modulation of B and T cell responses in the CNS and periphery

One likely hypothesis is that the inflammatory chemokine and cytokine milieu in the CNS influences the recruitment of specific effector B and T cells important in the pathogenesis of MS. In support of this hypothesis, it has been shown that the B cell chemo-attractant, CXCL13, is produced in actively demyelinating lesions but not in inactive lesions in the CNS of secondary progressive patients and 20% of T cells and almost all B cells in the CSF express CXCR5, the receptor for CXCL13 (230). However, naïve and memory B cells express CXCR5 equally, and thus would not explain the specific recruitment of memory B cells into the CNS. CXCR3 is expressed on a subset of memory B cells, but not on naïve B cells (85), and may be a likely candidate for promoting the recruitment of memory B cells into the CNS as at least one of the ligands for CXCR3, CXCL10, is elevated in MS CSF (84, 231) and localized around CNS lesion tissue (231). Additionally, a significantly higher percentage of $CXCR3^+$ B cells and absolute number of $CXCR3^+$ B cell were found in the CSF MS patients undergoing relapse compared to CSF from OND controls (232). Furthermore it was demonstrated that activated memory B cells in the CNS of MS patients and ONDs express much higher levels of CCR1, CCR2, and CCR4 compared to memory B cells from paired donor blood samples suggesting that these chemokine receptors have a role in recruitment and maintenance of these cells into the CNS (139).

In addition, several integrins are differentially regulated between naïve and memory B cells that may be involved in discriminating specific arrest and extravasation

from the peripheral circulation. For example both integrin chains of VLA-4 are expressed by memory B cells but not naïve B cells (82). This could also in part explain efficacy of the monoclonal anti-VLA-4 antibody, Natalizumab, in mediating MS disease amelioration, as MS patients treated with this antibody have a disproportionate increase in circulating B cells as compared to other lymphocyte/monocyte populations (179).

Transcriptional analysis has revealed several additional integrins, chemokine, and cytokine receptors that are differentially regulated between naïve and memory B cells. For example, memory B cells upregulate CD11b (Mac-1), ICAM-2 a ligand for LFA-1, TACI, and Interleukin-2 receptor alpha (IL-2Rα; CD25) in comparison to naïve B cells (233). Taken together these data further indicate that differential regulation of chemokine, integrin, and cytokine receptors on memory and naïve B cells may be responsible for the specific increase in memory B cells in the CNS as compared to naïve B cells. Further study of how these chemokines, integrins, and cytokines influence the migration of memory B cells into the CNS is an area in much need of further investigation. Once activated in the CNS or periphery, B cells can play a significant role in driving inflammatory T cell responses through cytokine secretion.

The results provided here also confirm a lack of IL-10 secretion by memory B cells from both HD and in RRMS patients, consistent with the role of memory B cells in maintaining an inflammatory environment in the CNS following encounter with an activated T cell. While the measurement of LTα and IL-10 provide excellent surrogate markers for inflammatory or anti-inflammatory cytokine secretion capability of B cells respectively, unexplored sub patterns of cytokine secretion differentially modulated in naïve and memory B cells may still exist.

For example, preliminary experiments (data not shown) suggest that IL-2, IL-4, IL-6, IFNγ, and TNFα secretion in HD are not substantially differentially regulated between naïve and memory B cells stimulated with CD40L. BCR crossslinking in addition to CD40L stimulation revealed a substantial increase in IL-6 secretion compared to CD40L stimulation alone in both naïve and memory B cells, with naïve B cells tending to secrete increased IL-6 in comparison to memory B cells. These findings suggest that B cells may be able to provide cytokine microenviroment that supports the differentiation or maintenance of Th17 cells (57).

Transcriptional analysis indicated the presence of RANTES and lymphotactin transcripts by tonsil memory B cells (233), but not naïve B cells, providing further evidence that memory B cells are intrinsically programmed to secrete a pro-inflammatory cytokine profile. Further study of differentially expressed cytokines released by B cell subsets in MS, may compliment findings of the superiority of memory B cells in modulating neuro-antigen specific T cell responses through antigen presentation documented in this study.

Importance of neuro-antigen specificity of memory B cells in antigen presentation

One of the most important features of B cell APCs is their ability to concentrate minute amounts of antigen by means of antigen acquisition through specific BCR recognition (234). Presumably a great deal of the efficiency of memory B cells in regulating T cell responses to TT and GA in HDs and RRMS, and MBP in RRMS, were due to the high affinity of the antigen specific BCR generated through a process of affinity maturation lending to an increase in recognition of antigen. In addition to the

presence of high affinity antigen specific APC, the frequency of antigen specific B cells in the population of B cell is also presumably an important factor. The studies shown are the first to attempt to quantify the frequency of circulating neuro-antigen specific B cells in the context of MS. Many studies have examined the role of circulating neuro-antigen specific antibodies, however, antibody titers do not correlate with the levels of high affinity circulating memory B cells, especially in cases of repeated antigen exposure (235). For the purposes of this study is was important to quantify the frequency of neuro-antigen specific B cells and attempt to correlate this to neuro-antigen specific APC function. I was able to demonstrate several significant trends among binding of antigen to B cells but no direct correlation between antigen binding frequency and CD4+ T cell proliferation. For example, all MS patient with detectible MBP binding memory B cells induced proliferation but others that did not induce proliferation also had detectible MBP binding. MBP and GA bound more preferentially to a small subset of memory B cells as compared to naïve B cells in both HDs and RRMS. In addition, a significantly greater number B cells in the naïve RRMS B cell pool bound MBP as compared to naïve B cells from the HD cohort. Given the high binding of MBP to memory B cells in both HD and RRMS patients, the significance of an increased frequency of MBP binding in RRMS naïve B cells seems inconsequential. Nonetheless this could indicate a lack of central tolerance in eliminating autoreactive MBP specific naïve B cells from the periphery in MS patients. This issue should be explored further.

Few studies have examined the frequency of neuro-antigen specific B cells in the context of MS. Kennel De March et al. demonstrated a significant increase in spontaneous MOG-specific IgA antibody secreting cells detected by ELISPOT in MS

peripheral blood compared to HDs (236). Likewise MOG specific IgG and IgM antibody secreting cells were detected in MS PBMC and reduced by mitoxantrone treatment (237). However none have directly examined neuro-antigen specific B cell frequency in the absence of antibody secretion. The technique I used here does not quantitatively identity B cells of differing affinity for antigen which may be the most useful in correlating binding to APC capacity. In addition it does not take into account the relative differences in BCR expression on the cell surface between naïve and memory B cells.

In order to assess this question directly, MBP binding and non binding ex vivo MS PBBCs, identified and sorted by FACS, would be cultured with CD40L/IL-4 to expand these cells and compared for their ability to stimulate MBP specific T cell proliferation. These experiments would directly assess if the ability of B cells to present MBP was dependent on BCR recognition of antigen. As we hypothesize that MBP specific B cells are enriched in MS CSF B cells (173), it is also of interest to assess CD40L/IL-4 expanded CSF B Cells from OND patients and MS patients, and compare the ability of these populations to efficiently present MBP to autologous peripheral blood MBP specific T cell lines.

My results clearly demonstrate that VH4 expressing B cells are over represented in the overall MS CSF B cell pool compared to both HCPB and OND CSF (Figure 4-03). We hypothesize that this enrichment is due to antigen driven selection either in the CNS or the periphery. Perhaps then, the antigen specificity of peripheral VH4 expressing B cells can be probed by assaying the neuro-antigen specific antigen presentation of these cells. To test the hypothesis, VH4 expressing B cells from the peripheral blood can preferentially prevented from undergoing apoptosis in the presence staphylococcus

enterotoxin D (SED, (238). VH3 expressing B cells can be similarly selected with staphylococcus enterotoxin A (SEA; (239). VH3 and VH4 expressing peripheral B cells would be selected for survival with SEA and SED respectively for 9 days and then cultured with CD40L/IL-4. These cells would then assayed for their ability to stimulate neuro-antigen specific T cell proliferation in vitro using techniques described in chapter 3.

CD27 as a definitive marker for memory B cells in MS PBBCs

The findings demonstrated here, that $CD27^{+}$ memory B cells are capable of eliciting MBP or MOG specific T cell proliferation and IFNγ secretion in a greater frequency of RRMS patients than HDs indicate that a subset of B cells in RRMS but not HDs have been selected for affinity for these antigens. Recently it was demonstrated that in the systemic autoimmune disease systemic lupus erythematosus (SLE), a specific subset of CD19+IgD-CD27- memory B cells is enriched in the peripheral blood (240). In order to exclude the possibility that there was an expansion of memory B cells in the CD19+CD27- compartment that we typically sorted as our naïve B cell pool, I examined 5 out of the 15 RRMS patients included in this study for CD19+IgD-CD27- B cells and did not observe an expansion of this population in our RRMS patient cohort (data not shown). Importantly, plasmablasts also express significantly more CD27 ($CD27^{++}$) than naïve or memory B cells and remain $CD19^{+}$.

Although we identified memory B cells as the major population of B cell subset within the CSF of MS patients, others have identified $CD27^{++}CD138^{+}CD19^{+}$ plasmablasts that persist in MS CSF along with $CD27^{+}CD138^{-}$ memory B cells that is not observed in

infectious neurological disease (138). Short lived plasmablasts from HDs are also typically HLA-DR^{+} unlike long lived plasma cells (241) suggesting that this B cell subset is potentially capable of presenting antigen to autologous T cells. However I would hypothesize that because plasmablasts secreting antibody no longer express membrane bound BCR, this would prevent BCR specific antigen acquisition through receptor mediated endocytosis, rendering plasmablasts capable of present only antigen that was acquired prior to differentiation. There was no indication that a separate CD27^{++} population of B cells was present to any significant degree in either the HD or RRMS PBMC samples, although this was not formally assessed.

Potential role of CD27 in antigen presentation independent T cell activation

There is some evidence that indicates that human CD27+ B cells can participate in the activation of CD8+ T cells in the absence of antigen presentation. For example human B cells added to Flu tetramer+CD8+ T cells that had proliferated to flu peptide in PBMC cultures after 9 days underwent a second round of proliferation after culture addition, suggesting that B cells have a role in the restimulation of CD8+ T cells, separate from antigen presentation (242). However the authors only demonstrate that activated CD8+ T cells can proliferate further in the presence of a CD27+ cell, but fail to show that it must be a B cell expressing CD27. The authors of this study conclude that their results demonstrate a requirement of CD27+ expression on B cells regulating expansion and survival of CD8+ T cells, but did not address this point directly. My results clearly demonstrate that CD27 does not contribute directly to the proliferation of autologous T cells, as significant (> 2% CFSE low) T cell proliferation was never observed when

memory B cell APCs were cultured with autologous T cells in the absence of antigen, however this interaction may have contributed to the magnitude of the antigen specific response. To address the role of CD27/CD70 interactions in antigen presentation in my system, a blocking anti-CD70 monoclonal antibody (BU69) can be added to both naïve and memory B-T cultures and compared to cultures containing non-specific isotype control antibody (murine IgG1). The conclusion that CD27 is major contributor in influencing T cell proliferation is also partially refuted by data presented in Bar-Or et al. where the authors demonstrate that CD80+ memory B cells induce greater antigen specific T cell proliferation than CD80- memory B cells, both of which are CD27+ (77).

Role of differentially expressed cell surface molecules on naïve and memory B cell subsets in mediating T cell activation

Relatively little research has been undertaken to address differences between naïve and memory B cells in regards to cell surface expression of molecules that could potentially impact the potency of T cell activation through antigen presentation. One reason is the relatively recent ability to identify memory or naïve B cells based on CD27 expression (73). Research in this area has also been impeded because of the lack of specific cellular markers of memory B cells in murine models until very recently (243). In fact the observation that CD80 (B7.1) was constitutively expressed on a subset of memory B cells yet absent on naïve B cells, was one of the first indications that memory B cells may have an increased capacity to act as an antigen presenting cell (77).

Costimulatory molecule involvement in B-T collaboration

We confirm here in our cohort that memory B cell subsets from both HDs and RRMS patients express significantly more CD80 on their cell surface than naïve B cells which had been demonstrated previously in HDs (77) and in untreated RRMS patients (187). These results indicate that RRMS naïve and memory B cells in untreated MS patients during remission do no express an overt activation phenotype over their counterparts in HDs. Although I did not examine CD86 expression here, the presence of CD86 (B7.2) on human naive and memory B cell subsets is controversial. One report indicated an absence of CD86 expression both naïve and memory B cells (77), while another indicated that CD86 is expressed on memory B cells (186). While B7 family members have been the most well studied in costimulation of T cells, additional costimulatory molecules that have been demonstrated to be differentially expressed between naïve and memory B cells may also serve an important role in mediating antigen presentation efficiency.

For example, the costimulatory transmembrane molecule OX40L, is a member of the tumor necrosis factor family of proteins, and is expressed on a subset of B cells. In vivo studies in mice suggest that B cell antigen presentation in the absence OX40L, results in CD4+ T cell expansion into predominantly Th1 cells, however Th2 T cell responses can be restored with the addition of $OX40L^{+}$B cells (244). OX40L stimulation of T cells via B cells inhibits IFNγ, and instructs T cells to differentiate into $CXCR5^{+}$ IL-4 secreting T cells (245). OX40L is responsible for B cell differentiation into antibody secreting cells but not into memory or germinal center B cells (246). Transcriptional activity of OX40L is increased in IgM+ human memory B cells compared to naïve and other class switched populations of memory B cells but has not been validated at the

protein level (186). Blocking of OX40L-OX40 interactions ameliorated EAE and reduced activation of neuro-antigen reactive T cells from the CNS (247). Taken together these studies warrant further examination of the involvement of OX40L expression on memory B cells in the promotion of neuro-antigen specific T cell responses in MS. Although the function of these molecules expressed by B cells is not well described, transcriptional profiling analysis revealed an increased expression of receptor-activator-for-nuclear-factor-κ-B ligand (RANKL) (233), as well as CD229, and CD84 (186) to be upregulated in memory B cells but not naïve B cells. The role of these molecules in mediating B cell antigen presentation should be followed.

Role of toll like receptor (TLR) molecules in regulating naïve and memory B cells

Human memory B cells constitutively express surface toll like receptors TLR6, TLR7, TLR9, and TLR10 while naïve B cells do not (78), although TLR9 and TLR10 can be upregulated by B cell activation (78, 248). This expression pattern suggests that memory B cells would be hyper-responsive to pathogen associated molecular patterns (PAMPs) recognized by these molecules, and indeed memory B cells differentiate rapidly into antibody secreting cells following TLR9 stimulation with CpG ODN (78). I examined the responses of naïve and memory B cells to produce LTα, IL-10 following CD40L stimulation in conjunction with CpG ODN stimulation (Figure 6-03 and 6-05) in HDs. Interestingly CpG ODN and CD40L stimulation acted to significantly increase the expression of both LTα and IL-10 in HD memory and naïve B cells as compared to CD40L stimulation alone. Additionally under these conditions, IL-10 secretion was observed to be greater in the memory B cell compartment in comparison to the naïve B

cell compartment. These data would suggest that treatment of B cells with CpG ODN may suppress the ability of these B cells to act as inflammatory antigen presenting cells. The fact that CpG ODN/CD40L treated B cells secrete significant amounts of IL-10 may indicate why CpG ODN/IL-2 activated B cells were poor antigen presenting cells (Figure 5-02) of a control recall antigen. Indeed it was recently demonstrated that TLR agonist stimulated B cells can suppress EAE through IL-10 mediated suppression of pro-inflammatory dendritic cells (249). Given the pro-inflammatory neuro-antigen specific T cell activation observed following memory B cell antigen presentation of MBP in a subset of MS patients, it is important to determine if these responses can be suppressed by agonist TLR stimulation.

Caveats, Future Directions and concluding remarks

CSF B cell antigen presentation

This study has provided evidence that as a whole, the intrathecal B cell population in MS was likely derived in a germinal center reaction, which allowed for the examination of peripheral B cell subsets as potent neuro-antigen APCs. Regardless of the origin of selection to antigen of these intrathecal B cells, their functional role as antigen presenting cells in recruiting, maintaining, and activating T cells is likely important as Rituximab mediated depletion of intrathecal B cells in RRMS patients is correlated with CSF T cell depletion (16). In addition, because of their recruitment to the CNS, MS CSF B cells are likely enriched for neuro-antigen specificity, a hypothesis with much support based on antibodies recovered from CSF B cells (173, 250) and intrathecal immunoglobulin synthesis (251-253). Although the relative paucity of B cells recovered

from the CSF of MS patients in comparison to the peripheral blood limits the direct assessment of CSF derived B cells in many of the functional assays described here, much knowledge about the interrelationship between neuro-antigen specific B and T cells in the CNS in comparison to the peripheral blood could be gained if a suitable approach were taken to functionally examine these cells. Peripheral naïve B and memory B cell subsets could be examined for their capacity to present neuro-antigen to autologous CSF derived T cells and compared to the functional readouts of antigen presentation to peripheral blood derived T cells. B cells derived from the CSF could also be expanded in vitro using the CD40L/IL-4 in vitro culture system described here or immortalized through in vitro Epstein Barr Virus (EBV) transformation. These B cells could then be assessed for their ability to stimulate peripheral T cells and compared to CD40L/IL-4 activated B cells from the periphery in their capacity to present neuro-antigen.

The role of memory B cells in initiating naïve T cell responses

The findings presented here demonstrate clearly that in a subset of MS patients, B cells are able to present neuro-antigen to autologous T cells to induce CD4+ T cell proliferation in some cases and IFNγ secretion in others. Several previous studies in mice and humans have indicated that B cells are unable to stimulate naïve T cell proliferation, but can induce antigen experienced T cells to proliferate in the absence of additional distinct APCs (102, 182). It has also been demonstrated by several investigators that while the frequency of neuro-antigen specific T cell responses is similar between HDs and MS patients, neuro-antigen specific T cells with an activated /memory phenotype are more prevalent in MS (36, 39, 254). Therefore it is of critical importance

to determine if neuro-antigen specific memory B cell antigen presentation efficiency is a function of B cell APC activity or is a reflection of the presence of activated/memory T cells present in individual patients. In order to dissect these issues, one must first address the question of the ability of neuro-antigen specific B cells to stimulate neuro-antigen specific naïve or memory T cells directly, as no studies to date have attempted to assess the functionality of memory B cells to initiate naïve T cell proliferation, cytokine secretion, or differentiation. Even though I observed a marked contrast between the efficiency of memory and naïve B cell presentation of GA, an antigen which both HDs and RRMS patients were naïve to; my studies did not specifically address the ability of B cells to stimulate subpopulations of T cells, specifically naïve or memory. One way to specifically address this issue would be to sort naïve CD4+ T cells (based on the expression of $CD45RA^{+}CD4^{+}CD3^{+}$) and memory CD4+ T cells (based on the expression of $CD45RO^{+}CD4^{+}CD3^{+}$) and test the ability of naïve or memory B cells to stimulate these populations to proliferate, and secrete polarizing cytokines such as IFNγ, IL-17, and IL-5 in the presence of processing dependent (whole) antigen. The protocols presented in Chapter 2, could be readily adapted to assess these issues. These studies would allow us to begin to dissect the role that B cells play in initiation of neuro-antigen specific T cell expansion, or if their role is relegated to the reactivation of neuro-antigen specific memory T cell clones previously activated by a non-B cell APC.

One additional experimental protocol that could be used to exclude the possible influence of T cell precursor frequency on the magnitude of B cell antigen presentation, would be the use of T cell lines. In this way, MBP or MOG reactive T cell lines can be generated from PBMC as described (255). Naïve and memory B cells can then be

isolated as described in chapter 3, and culture with rested T cell lines and either cognizant or irrelevant antigen as a control.

Correlation with response to B cell depletion with Rituximab

The efficacy of B cell depleting agents such as Rituximab in multiple sclerosis has lead to renewed interest in the function of B cells in pathogenesis of RRMS. Most likely however, the function of B cells in RRMS is heterogeneous and not all patients will respond favorably both clinically or diagnostically to this type of treatment. Certainly, such heterogeneity is evident when simply comparing subtypes of brain pathology by assessing the involvement of antibody and complement deposition in the development of inflammatory lesions (21). Currently however, no specific diagnostic assessments exist to determine the extent of B cell involvement in specific RRMS cases. Furthermore, prediction of a favorable clinical response to B cell depletion therapy is currently not optimal. Yet this would be of great diagnostic value for both the clinician and the patient to determine if this type of treatment would be efficacious, as B cell depletion therapy is currently very expensive, and carries considerable adverse risk as evidenced the development of progressive multifocal leukoencephalopathy (PML) in a small minority of treated patients (256).

Therefore determining both the extent of B cell involvement in disease and potential response to B cell depletion therapy could be accomplished by assessing if a positive correlation exists between the antigen presenting capacity of particular neuro-antigen memory B cell antigen presentation to clinical response to Rituximab. Prior to B cell depletion therapy, RRMS patients would provide blood or leukopheresis sample.

Memory and naïve B cells would be isolated and examined for their ability to present a panel of neuro-antigens to autologous purified T cells. RRMS patients would then be examined for clinical improvement following Rituximab treatment and this would be correlated with neuro-antigen specific B cell APC function. Of course it would also be useful to identify the individual B cell receptor genes that are mediating neuro-antigen specific T cell proliferation. It is hypothesized that antigen specific B cells presenting cognate antigen to T cells in culture may in turn proliferate in culture. Indeed I have observed $CD19^+$ B cell proliferation in HD PBMCs cultures with TT (data not shown) as have others (126). However the antigen specificity of these proliferating B cells has not been formally documented. In order to determine if proliferating B cells are indeed antigen specific, PBMCs labeled with CFSE as described in materials and methods would be cultured with MBP or MOG for 5 days. Proliferating B cells (CFSE low) would then be isolated by FACS and used in a classical MBP or MOG specific ELISPOT assay to determine if these B cells contain a population of neuro-antigen specific antibody secreting cells. Proliferating B cells from these cultures can also be isolated by FACS, expanded with CD40L/IL-4 and tested for their ability to present specific antigen to autologous T cells. If proliferating B cells in PBMC cultures are found to be antigen specific then these B cells can be isolated through single cell sorting, lysed, V genes amplified, and sequenced to determine the V gene usage and mutational characteristics associated with neuro-antigen specific B cells.

Conclusions

These studies provide a framework for the potential role of B cells in antigen presentation and specifically provide evidence that activated and memory B cells support neuro-antigen specific T cell activation, proliferation, and cytokine secretion in a subset of MS patients. Importantly, memory B cells do not support neuro- antigen specific T cell activation and proliferation in HDs even though non-specific antigen presenting cells could, revealing that neuro-antigen specific memory B cells had not developed in these donors. These results are some of the first to identify potential difference in neuro-antigen specific reactivity between HDs an MS patients, and therefore could potentially be used to determine the extent of an immune response that has developed against neuro-antigens, unlike a typical mixed lymphocyte reaction. These assays more specifically document for the first time, a functional role in B cell antigen presentation in a human autoimmune disease process, a finding that likely can be used to evaluate the role of B cell antigen presentation in autoimmune disorders where B cell involvement is suspected. These results together with previous findings by others, allow for the development of a hypothetical model of B cell activation in MS, where B cells undergo somatic hypermutation in the periphery in the context of a germinal center, migrate to the sites of CNS inflammation, and undergo further clonal expansion intrathecally. In the CNS as well as in the periphery. My findings indicate that neuro-antigen selected B cells can contribute most significantly to T cell activation through antigen presentation and pro-inflammatory cytokine secretion. Targeting these neuro-antigen specific B cells and their interactions with T cells will be an important pursuit in the treatment of MS.

REFERENCES

1. Hirtz, D., D.J. Thurman, K. Gwinn-Hardy, M. Mohamed, A.R. Chaudhuri, and R. Zalutsky. 2007. How common are the "common" neurologic disorders? *Neurology* 68:326-337.

2. Frohman, E.M., M.K. Racke, and C.S. Raine. 2006. Multiple sclerosis--the plaque and its pathogenesis. *N Engl J Med* 354:942-955.

3. Wolf, S.D., B.N. Dittel, F. Hardardottir, and C.A. Janeway, Jr. 1996. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* 184:2271-2278.

4. Link, H., and M.A. Laurenzi. 1979. Immunoglobulin class and light chain type of oligoclonal bands in CSF in multiple sclerosis determined by agarose gel electrophoresis and immunofixation. *Ann Neurol* 6:107-110.

5. Link, H., and R. Muller. 1971. Immunoglobulins in multiple sclerosis and infections of the nervous system. *Arch Neurol* 25:326-344.

6. Hauser, S.L., H.L. Weiner, and K.A. Ault. 1982. Clonally restricted B cells in peripheral blood of multiple sclerosis patients: kappa/lambda staining patterns. *Ann Neurol* 11:408-412.

7. Olsson, J.E., and H. Link. 1973. Immunoglobulin abnormalities in multiple sclerosis. Relation to clinical parameters: exacerbations and remissions. *Arch Neurol* 28:392-399.

8. Perez, L., J.C. Alvarez-Cermeno, C. Rodriguez, E. Roldan, and J.A. Brieva. 1995. B cells capable of spontaneous IgG secretion in cerebrospinal fluid from patients with multiple sclerosis: dependency on local IL-6 production. *Clin Exp Immunol* 101:449-452.

9. Colombo, M., M. Dono, P. Gazzola, S. Roncella, A. Valetto, N. Chiorazzi, G.L. Mancardi, and M. Ferrarini. 2000. Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. *J Immunol* 164:2782-2789.

10. Owens, G.P., J.L. Bennett, H. Lassmann, K.C. O'Connor, A.M. Ritchie, A. Shearer, C. Lam, X. Yu, M. Birlea, C. DuPree, R.A. Williamson, D.A. Hafler, M.P. Burgoon, and D. Gilden. 2009. Antibodies produced by

clonally expanded plasma cells in multiple sclerosis cerebrospinal fluid. *Ann Neurol* 65:639-649.

11. Qin, Y., P. Duquette, Y. Zhang, P. Talbot, R. Poole, and J. Antel. 1998. Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J Clin Invest* 102:1045-1050.

12. Owens, G.P., A.M. Ritchie, M.P. Burgoon, R.A. Williamson, J.R. Corboy, and D.H. Gilden. 2003. Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid. *J Immunol* 171:2725-2733.

13. Monson, N.L., H.P. Brezinschek, R.I. Brezinschek, A. Mobley, G.K. Vaughan, E.M. Frohman, M.K. Racke, and P.E. Lipsky. 2005. Receptor revision and atypical mutational characteristics in clonally expanded B cells from the cerebrospinal fluid of recently diagnosed multiple sclerosis patients. *J Neuroimmunol* 158:170-181.

14. Hauser, S.L., E. Waubant, D.L. Arnold, T. Vollmer, J. Antel, R.J. Fox, A. Bar-Or, M. Panzara, N. Sarkar, S. Agarwal, A. Langer-Gould, and C.H. Smith. 2008. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 358:676-688.

15. Stuve, O., V.I. Leussink, R. Frohlich, B. Hemmer, H.P. Hartung, T. Menge, and B.C. Kieseier. 2009. Long-term B-lymphocyte depletion with rituximab in patients with relapsing-remitting multiple sclerosis. *Arch Neurol* 66:259-261.

16. Cross, A.H., J.L. Stark, J. Lauber, M.J. Ramsbottom, and J.A. Lyons. 2006. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol* 180:63-70.

17. Ransohoff, R.M. 2007. Natalizumab for multiple sclerosis. *N Engl J Med* 356:2622-2629.

18. Noseworthy, J.H., C. Lucchinetti, M. Rodriguez, and B.G. Weinshenker. 2000. Multiple sclerosis. *N Engl J Med* 343:938-952.

19. Miller, D.H., R.I. Grossman, S.C. Reingold, and H.F. McFarland. 1998. The role of magnetic resonance techniques in understanding and managing multiple sclerosis. *Brain* 121 (Pt 1):3-24.

20. Tallantyre, E.C., L. Bo, O. Al-Rawashdeh, T. Owens, C.H. Polman, J. Lowe, and N. Evangelou. 2009. Greater loss of axons in primary progressive multiple sclerosis plaques compared to secondary progressive disease. *Brain* 132:1190-1199.

21. Lucchinetti, C., W. Bruck, J. Parisi, B. Scheithauer, M. Rodriguez, and H. Lassmann. 2000. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 47:707-717.

22. Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* 9:393-407.

23. Conly, J.M., and A.R. Ronald. 1983. Cerebrospinal fluid as a diagnostic body fluid. *Am J Med* 75:102-108.

24. Kappos, L. 2004. Effect of drugs in secondary disease progression in patients with multiple sclerosis. *Mult Scler* 10 Suppl 1:S46-54; discussion S54-45.

25. Miller, D.H., and S.M. Leary. 2007. Primary-progressive multiple sclerosis. *Lancet Neurol* 6:903-912.

26. Ebers, G.C., D.E. Bulman, A.D. Sadovnick, D.W. Paty, S. Warren, W. Hader, T.J. Murray, T.P. Seland, P. Duquette, T. Grey, and et al. 1986. A population-based study of multiple sclerosis in twins. *N Engl J Med* 315:1638-1642.

27. Sadovnick, A.D., G.C. Ebers, D.A. Dyment, and N.J. Risch. 1996. Evidence for genetic basis of multiple sclerosis. The Canadian Collaborative Study Group. *Lancet* 347:1728-1730.

28. Hafler, D.A., A. Compston, S. Sawcer, E.S. Lander, M.J. Daly, P.L. De Jager, P.I. de Bakker, S.B. Gabriel, D.B. Mirel, A.J. Ivinson, M.A. Pericak-Vance, S.G. Gregory, J.D. Rioux, J.L. McCauley, J.L. Haines, L.F. Barcellos, B. Cree, J.R. Oksenberg, and S.L. Hauser. 2007. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 357:851-862.

29. Kurtzke, J.F. 1993. Epidemiologic evidence for multiple sclerosis as an infection. *Clin Microbiol Rev* 6:382-427.

30. Racke, M.K. 2001. Experimental autoimmune encephalomyelitis (EAE). *Curr Protoc Neurosci* Chapter 9:Unit9 7.

31. Cserr, H.F., and P.M. Knopf. 1992. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol Today* 13:507-512.

32. Raine, C.S. 1994. The Dale E. McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion. *Ann Neurol* 36 Suppl:S61-72.

33. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23:683-747.

34. Benveniste, E.N., V.T. Nguyen, and D.R. Wesemann. 2004. Molecular regulation of CD40 gene expression in macrophages and microglia. *Brain Behav Immun* 18:7-12.

35. Pouly, S., and J.P. Antel. 1999. Multiple sclerosis and central nervous system demyelination. *J Autoimmun* 13:297-306.

36. Lovett-Racke, A.E., J.L. Trotter, J. Lauber, P.J. Perrin, C.H. June, and M.K. Racke. 1998. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest* 101:725-730.

37. Crawford, M.P., S.X. Yan, S.B. Ortega, R.S. Mehta, R.E. Hewitt, D.A. Price, P. Stastny, D.C. Douek, R.A. Koup, M.K. Racke, and N.J. Karandikar. 2004. High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood* 103:4222-4231.

38. Markovic-Plese, S., I. Cortese, K.P. Wandinger, H.F. McFarland, and R. Martin. 2001. CD4+CD28- costimulation-independent T cells in multiple sclerosis. *J Clin Invest* 108:1185-1194.

39. Scholz, C., K.T. Patton, D.E. Anderson, G.J. Freeman, and D.A. Hafler. 1998. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J Immunol* 160:1532-1538.

40. Croft, M., L.M. Bradley, and S.L. Swain. 1994. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol* 152:2675-2685.

41. Eylar, E.H., S. Brostoff, G. Hashim, J. Caccam, and P. Burnett. 1971. Basic A1 protein of the myelin membrane. The complete amino acid sequence. *J Biol Chem* 246:5770-5784.

42. Vandenheede, J.R., J. Van Lint, C. Vanden Abeele, and W. Merlevede. 1987. Interaction of myelin basic protein with the different components of the ATP,Mg-dependent protein phosphatase system. *FEBS Lett* 211:190-194.

43. Husted, C. 2006. Structural insight into the role of myelin basic protein in multiple sclerosis. *Proc Natl Acad Sci U S A* 103:4339-4340.

44. Carson, J.H., E. Barbarese, P.E. Braun, and T.A. McPherson. 1978. Components in multiple sclerosis cerebrospinal fluid that are detected by radioimmunoassay for myelin basic protein. *Proc Natl Acad Sci U S A* 75:1976-1978.

45. Mokhtarian, F., D.E. McFarlin, and C.S. Raine. 1984. Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature* 309:356-358.

46. Wucherpfennig, K.W., and J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80:695-705.

47. Dhib-Jalbut, S. 2003. Glatiramer acetate (Copaxone) therapy for multiple sclerosis. *Pharmacol Ther* 98:245-255.

48. Teitelbaum, D., C. Webb, M. Bree, A. Meshorer, R. Arnon, and M. Sela. 1974. Suppression of experimental allergic encephalomyelitis in Rhesus monkeys by a synthetic basic copolymer. *Clin Immunol Immunopathol* 3:256-262.

49. Amiguet, P., M.V. Gardinier, J.P. Zanetta, and J.M. Matthieu. 1992. Purification and partial structural and functional characterization of mouse myelin/oligodendrocyte glycoprotein. *J Neurochem* 58:1676-1682.

50. Brunner, C., H. Lassmann, T.V. Waehneldt, J.M. Matthieu, and C. Linington. 1989. Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J Neurochem* 52:296-304.

51. Linington, C., M. Bradl, H. Lassmann, C. Brunner, and K. Vass. 1988. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am J Pathol* 130:443-454.

52. Martin Mdel, P., and N.L. Monson. 2007. Potential role of humoral immunity in the pathogenesis of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). *Front Biosci* 12:2735-2749.

53. Kerlero de Rosbo, N., R. Milo, M.B. Lees, D. Burger, C.C. Bernard, and A. Ben-Nun. 1993. Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein. *J Clin Invest* 92:2602-2608.

54. Kerlero de Rosbo, N., M. Hoffman, I. Mendel, I. Yust, J. Kaye, R. Bakimer, S. Flechter, O. Abramsky, R. Milo, A. Karni, and A. Ben-Nun. 1997. Predominance of the autoimmune response to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis: reactivity to the extracellular domain of MOG is directed against three main regions. *Eur J Immunol* 27:3059-3069.

55. Sun, J., H. Link, T. Olsson, B.G. Xiao, G. Andersson, H.P. Ekre, C. Linington, and P. Diener. 1991. T and B cell responses to myelin-oligodendrocyte glycoprotein in multiple sclerosis. *J Immunol* 146:1490-1495.

56. Lindert, R.B., C.G. Haase, U. Brehm, C. Linington, H. Wekerle, and R. Hohlfeld. 1999. Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein. *Brain* 122 (Pt 11):2089-2100.

57. Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485-517.

58. Abbas, A.K., K.M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787-793.

59. Ramos, H.J., A.M. Davis, T.C. George, and J.D. Farrar. 2007. IFN-alpha is not sufficient to drive Th1 development due to lack of stable T-bet expression. *J Immunol* 179:3792-3803.

60. Sornasse, T., P.V. Larenas, K.A. Davis, J.E. de Vries, and H. Yssel. 1996. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4+ T cells, analyzed at the single-cell level. *J Exp Med* 184:473-483.

61. Miossec, P., T. Korn, and V.K. Kuchroo. 2009. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361:888-898.

62. Lafaille, J.J., F.V. Keere, A.L. Hsu, J.L. Baron, W. Haas, C.S. Raine, and S. Tonegawa. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J Exp Med* 186:307-312.

63. Wilson, C.B., E. Rowell, and M. Sekimata. 2009. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* 9:91-105.

64. Duda, P.W., M.C. Schmied, S.L. Cook, J.I. Krieger, and D.A. Hafler. 2000. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest* 105:967-976.

65. Tennakoon, D.K., R.S. Mehta, S.B. Ortega, V. Bhoj, M.K. Racke, and N.J. Karandikar. 2006. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J Immunol* 176:7119-7129.

66. Matsuda, F., K. Ishii, P. Bourvagnet, K. Kuma, H. Hayashida, T. Miyata, and T. Honjo. 1998. The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. *J Exp Med* 188:2151-2162.

67. Corbett, S.J., I.M. Tomlinson, E.L. Sonnhammer, D. Buck, and G. Winter. 1997. Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol* 270:587-597.

68. Croce, C.M., M. Shander, J. Martinis, L. Cicurel, G.G. D'Ancona, T.W. Dolby, and H. Koprowski. 1979. Chromosomal location of the genes for human immunoglobulin heavy chains. *Proc Natl Acad Sci U S A* 76:3416-3419.

69. Hood, L., J.H. Campbell, and S.C. Elgin. 1975. The organization, expression, and evolution of antibody genes and other multigene families. *Annu Rev Genet* 9:305-353.

70. Hieter, P.A., S.J. Korsmeyer, T.A. Waldmann, and P. Leder. 1981. Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. *Nature* 290:368-372.

71. van Ham, S.M., E.P. Tjin, B.F. Lillemeier, U. Gruneberg, K.E. van Meijgaarden, L. Pastoors, D. Verwoerd, A. Tulp, B. Canas, D. Rahman, T.H. Ottenhoff, D.J. Pappin, J. Trowsdale, and J. Neefjes. 1997. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* 7:950-957.

72. Rodriguez-Pinto, D. 2005. B cells as antigen presenting cells. *Cell Immunol* 238:67-75.

73. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 188:1679-1689.

74. Agematsu, K., H. Nagumo, Y. Oguchi, T. Nakazawa, K. Fukushima, K. Yasui, S. Ito, T. Kobata, C. Morimoto, and A. Komiyama. 1998. Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. *Blood* 91:173-180.

75. Nagumo, H., and K. Agematsu. 1998. Synergistic augmentative effect of interleukin-10 and CD27/CD70 interactions on B-cell immunoglobulin synthesis. *Immunology* 94:388-394.

76. Lens, S.M., P.A. Baars, B. Hooibrink, M.H. van Oers, and R.A. van Lier. 1997. Antigen-presenting cell-derived signals determine expression levels of CD70 on primed T cells. *Immunology* 90:38-45.

77. Bar-Or, A., E.M. Oliveira, D.E. Anderson, J.I. Krieger, M. Duddy, K.C. O'Connor, and D.A. Hafler. 2001. Immunological memory: contribution of memory B cells expressing costimulatory molecules in the resting state. *J Immunol* 167:5669-5677.

78. Bernasconi, N.L., N. Onai, and A. Lanzavecchia. 2003. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 101:4500-4504.

79. Benson, M.J., R. Elgueta, W. Schpero, M. Molloy, W. Zhang, E. Usherwood, and R.J. Noelle. 2009. Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals. *J Exp Med* 206:2013-2025.

80. Bernasconi, N.L., E. Traggiai, and A. Lanzavecchia. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 298:2199-2202.

81. Alter, A., M. Duddy, S. Hebert, K. Biernacki, A. Prat, J.P. Antel, V.W. Yong, R.K. Nuttall, C.J. Pennington, D.R. Edwards, and A. Bar-Or. 2003. Determinants of human B cell migration across brain endothelial cells. *J Immunol* 170:4497-4505.

82. Silvy, A., P. Altevogt, P. Mondiere, C. Bella, and T. Defrance. 1997. A role for the VLA-4 integrin in the activation of human memory B cells. *Eur J Immunol* 27:2757-2764.

83. Liu, Y.J., J. Zhang, P.J. Lane, E.Y. Chan, and I.C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur J Immunol* 21:2951-2962.

84. Sorensen, T.L., M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V.A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R.M. Strieter, J.L. Frederiksen, and R.M. Ransohoff. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* 103:807-815.

85. Muehlinghaus, G., L. Cigliano, S. Huehn, A. Peddinghaus, H. Leyendeckers, A.E. Hauser, F. Hiepe, A. Radbruch, S. Arce, and R.A. Manz. 2005. Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* 105:3965-3971.

86. Bleul, C.C., J.L. Schultze, and T.A. Springer. 1998. B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. *J Exp Med* 187:753-762.

87. Amu, S., A. Tarkowski, T. Dorner, M. Bokarewa, and M. Brisslert. 2007. The human immunomodulatory CD25+ B cell population belongs to the memory B cell pool. *Scand J Immunol* 66:77-86.

88. Corinti, S., D. Medaglini, C. Prezzi, A. Cavani, G. Pozzi, and G. Girolomoni. 2000. Human dendritic cells are superior to B cells at presenting a major histocompatibility complex class II-restricted heterologous antigen expressed on recombinant Streptococcus gordonii. *Infect Immun* 68:1879-1883.

89. Milich, D.R., M. Chen, F. Schodel, D.L. Peterson, J.E. Jones, and J.L. Hughes. 1997. Role of B cells in antigen presentation of the hepatitis B core. *Proc Natl Acad Sci U S A* 94:14648-14653.

90. Mauri, C., and M.R. Ehrenstein. 2008. The 'short' history of regulatory B cells. *Trends Immunol* 29:34-40.

91. Fillatreau, S., C.H. Sweenie, M.J. McGeachy, D. Gray, and S.M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* 3:944-950.

92. Matsushita, T., K. Yanaba, J.D. Bouaziz, M. Fujimoto, and T.F. Tedder. 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest* 118:3420-3430.

93. Tsitoura, D.C., V.P. Yeung, R.H. DeKruyff, and D.T. Umetsu. 2002. Critical role of B cells in the development of T cell tolerance to aeroallergens. *Int Immunol* 14:659-667.

94. Seamons, A., A. Perchellet, and J. Goverman. 2006. Endogenous myelin basic protein is presented in the periphery by both dendritic cells and resting B cells with different functional consequences. *J Immunol* 177:2097-2106.

95. Evans, D.E., M.W. Munks, J.M. Purkerson, and D.C. Parker. 2000. Resting B lymphocytes as APC for naive T lymphocytes: dependence on CD40 ligand/CD40. *J Immunol* 164:688-697.

96. Reichardt, P., B. Dornbach, S. Rong, S. Beissert, F. Gueler, K. Loser, and M. Gunzer. 2007. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood* 110:1519-1529.

97. Genc, K., D.L. Dona, and A.T. Reder. 1997. Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. *J Clin Invest* 99:2664-2671.

98. Clatza, A., L.C. Bonifaz, D.A. Vignali, and J. Moreno. 2003. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J Immunol* 171:6478-6487.

99. Cepok, S., M. Jacobsen, S. Schock, B. Omer, S. Jaekel, I. Boddeker, W.H. Oertel, N. Sommer, and B. Hemmer. 2001. Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis. *Brain* 124:2169-2176.

100. Sarwal, M., M.S. Chua, N. Kambham, S.C. Hsieh, T. Satterwhite, M. Masek, and O. Salvatierra, Jr. 2003. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 349:125-138.

101. Constant, S., N. Schweitzer, J. West, P. Ranney, and K. Bottomly. 1995. B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. *J Immunol* 155:3734-3741.

102. Ronchese, F., and B. Hausmann. 1993. B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J Exp Med* 177:679-690.

103. Phillips, J.A., C.G. Romball, M.V. Hobbs, D.N. Ernst, L. Shultz, and W.O. Weigle. 1996. CD4+ T cell activation and tolerance induction in B cell knockout mice. *J Exp Med* 183:1339-1344.

104. Rivera, A., C.C. Chen, N. Ron, J.P. Dougherty, and Y. Ron. 2001. Role of B cells as antigen-presenting cells in vivo revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. *Int Immunol* 13:1583-1593.

105. Whitmire, J.K., M.S. Asano, S.M. Kaech, S. Sarkar, L.G. Hannum, M.J. Shlomchik, and R. Ahmed. 2009. Requirement of B cells for generating CD4+ T cell memory. *J Immunol* 182:1868-1876.

106. Linton, P.J., J. Harbertson, and L.M. Bradley. 2000. A critical role for B cells in the development of memory CD4 cells. *J Immunol* 165:5558-5565.

107. Shen, H., J.K. Whitmire, X. Fan, D.J. Shedlock, S.M. Kaech, and R. Ahmed. 2003. A specific role for B cells in the generation of CD8 T cell memory by recombinant Listeria monocytogenes. *J Immunol* 170:1443-1451.

108. Lund, F.E., M. Hollifield, K. Schuer, J.L. Lines, T.D. Randall, and B.A. Garvy. 2006. B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection. *J Immunol* 176:6147-6154.

109. Menard, L.C., L.A. Minns, S. Darche, D.W. Mielcarz, D.M. Foureau, D. Roos, F. Dzierszinski, L.H. Kasper, and D. Buzoni-Gatel. 2007. B cells amplify IFN-gamma production by T cells via a TNF-alpha-mediated mechanism. *J Immunol* 179:4857-4866.

110. Kuppers, R. 2003. B cells under influence: transformation of B cells by Epstein-Barr virus. *Nat Rev Immunol* 3:801-812.

111. Monson, N.L., T. Dorner, and P.E. Lipsky. 2000. Targeting and selection of mutations in human Vlambda rearrangements. *Eur J Immunol* 30:1597-1605.

112. Dorner, T., H.P. Brezinschek, S.J. Foster, R.I. Brezinschek, N.L. Farner, and P.E. Lipsky. 1998. Comparable impact of mutational and selective influences in shaping the expressed repertoire of peripheral IgM+/CD5- and IgM+/CD5+ B cells. *Eur J Immunol* 28:657-668.

113. Foster, S.J., T. Dorner, and P.E. Lipsky. 1999. Somatic hypermutation of VkappaJkappa rearrangements: targeting of RGYW motifs on both DNA strands and preferential selection of mutated codons within RGYW motifs. *Eur J Immunol* 29:4011-4021.

114. Beck, R.W., D.L. Chandler, S.R. Cole, J.H. Simon, L.D. Jacobs, R.P. Kinkel, J.B. Selhorst, J.W. Rose, J.A. Cooper, G. Rice, T.J. Murray, and A.W. Sandrock. 2002. Interferon beta-1a for early multiple sclerosis: CHAMPS trial subgroup analyses. *Ann Neurol* 51:481-490.

115. Funderud, S., B. Erikstein, H.C. Asheim, K. Nustad, T. Stokke, H.K. Blomhoff, H. Holte, and E.B. Smeland. 1990. Functional properties of CD19+ B lymphocytes positively selected from buffy coats by immunomagnetic separation. *Eur J Immunol* 20:201-206.

116. Parish, C.R., M.H. Glidden, B.J. Quah, and H.S. Warren. 2009. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. *Curr Protoc Immunol* Chapter 4:Unit4 9.

117. Brezinschek, H.P., R.I. Brezinschek, and P.E. Lipsky. 1995. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J Immunol* 155:190-202.

118. Foster, S.J., H.P. Brezinschek, R.I. Brezinschek, and P.E. Lipsky. 1997. Molecular mechanisms and selective influences that shape the kappa gene repertoire of IgM+ B cells. *J Clin Invest* 99:1614-1627.

119. Farner, N.L., T. Dorner, and P.E. Lipsky. 1999. Molecular mechanisms and selection influence the generation of the human V lambda J lambda repertoire. *J Immunol* 162:2137-2145.

120. Lefranc, M.P., V. Giudicelli, C. Ginestoux, J. Bodmer, W. Muller, R. Bontrop, M. Lemaitre, A. Malik, V. Barbie, and D. Chaume. 1999. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res* 27:209-212.

121. Yousfi Monod, M., V. Giudicelli, D. Chaume, and M.P. Lefranc. 2004. IMGT/JunctionAnalysis: the first tool for the analysis of the immunoglobulin and T cell receptor complex V-J and V-D-J JUNCTIONs. *Bioinformatics* 20 Suppl 1:i379-385.

122. Urashima, M., D. Chauhan, H. Uchiyama, G.J. Freeman, and K.C. Anderson. 1995. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. *Blood* 85:1903-1912.

123. Schultze, J.L., A.A. Cardoso, G.J. Freeman, M.J. Seamon, J. Daley, G.S. Pinkus, J.G. Gribben, and L.M. Nadler. 1995. Follicular lymphomas can be induced to present alloantigen efficiently: a conceptual model to improve their tumor immunogenicity. *Proc Natl Acad Sci U S A* 92:8200-8204.

124. Amanna, I.J., and M.K. Slifka. 2006. Quantitation of rare memory B cell populations by two independent and complementary approaches. *J Immunol Methods* 317:175-185.

125. Duddy, M., M. Niino, F. Adatia, S. Hebert, M. Freedman, H. Atkins, H.J. Kim, and A. Bar-Or. 2007. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol* 178:6092-6099.

126. Mannering, S.I., J.S. Morris, K.P. Jensen, A.W. Purcell, M.C. Honeyman, P.M. van Endert, and L.C. Harrison. 2003. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. *J Immunol Methods* 283:173-183.

127. Moody, M.A., and B.F. Haynes. 2008. Antigen-specific B cell detection reagents: use and quality control. *Cytometry A* 73:1086-1092.

128. Stuve, O., C.M. Marra, K.R. Jerome, L. Cook, P.D. Cravens, S. Cepok, E.M. Frohman, J.T. Phillips, G. Arendt, B. Hemmer, N.L. Monson, and M.K. Racke. 2006. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol* 59:743-747.

129. Kurtzke, J.F. 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 33:1444-1452.

130. Cantaert, T., J. Kolln, T. Timmer, T.C. van der Pouw Kraan, B. Vandooren, R.M. Thurlings, J.D. Canete, A.I. Catrina, T. Out, C.L. Verweij, Y. Zhang, P.P. Tak, and D. Baeten. 2008. B lymphocyte autoimmunity in rheumatoid synovitis is independent of ectopic lymphoid neogenesis. *J Immunol* 181:785-794.

131. Hansen, A., P.E. Lipsky, and T. Dorner. 2003. New concepts in the pathogenesis of Sjogren syndrome: many questions, fewer answers. *Curr Opin Rheumatol* 15:563-570.

132. Magliozzi, R., O. Howell, A. Vora, B. Serafini, R. Nicholas, M. Puopolo, R. Reynolds, and F. Aloisi. 2007. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 130:1089-1104.

133. Jolly, C.J., S.D. Wagner, C. Rada, N. Klix, C. Milstein, and M.S. Neuberger. 1996. The targeting of somatic hypermutation. *Semin Immunol* 8:159-168.

134. Monson, N.L., S.J. Foster, H.P. Brezinschek, R.I. Brezinschek, T. Dorner, and P.E. Lipsky. 2001. The role of CD40-CD40 ligand (CD154) interactions in immunoglobulin light chain repertoire generation and somatic mutation. *Clin Immunol* 100:71-81.

135. Neuberger, M.S., M.R. Ehrenstein, N. Klix, C.J. Jolly, J. Yelamos, C. Rada, and C. Milstein. 1998. Monitoring and interpreting the intrinsic features of somatic hypermutation. *Immunol Rev* 162:107-116.

136. Neuberger, M.S., and C. Milstein. 1995. Somatic hypermutation. *Curr Opin Immunol* 7:248-254.

137. Rada, C., M.R. Ehrenstein, M.S. Neuberger, and C. Milstein. 1998. Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting. *Immunity* 9:135-141.

138. Cepok, S., B. Rosche, V. Grummel, F. Vogel, D. Zhou, J. Sayn, N. Sommer, H.P. Hartung, and B. Hemmer. 2005. Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis. *Brain* 128:1667-1676.

139. Corcione, A., S. Casazza, E. Ferretti, D. Giunti, E. Zappia, A. Pistorio, C. Gambini, G.L. Mancardi, A. Uccelli, and V. Pistoia. 2004. Recapitulation of B cell differentiation in the central nervous system of patients with multiple sclerosis. *Proc Natl Acad Sci U S A* 101:11064-11069.

140. Nemazee, D. 2000. Receptor editing in B cells. *Adv Immunol* 74:89-126.

141. Dorner, T., H.P. Brezinschek, R.I. Brezinschek, S.J. Foster, R. Domiati-Saad, and P.E. Lipsky. 1997. Analysis of the frequency and pattern of somatic mutations within nonproductively rearranged human variable heavy chain genes. *J Immunol* 158:2779-2789.

142. Dorner, T., H.P. Brezinschek, S.J. Foster, R.I. Brezinschek, N.L. Farner, and P.E. Lipsky. 1998. Delineation of selective influences shaping the mutated expressed human Ig heavy chain repertoire. *J Immunol* 160:2831-2841.

143. Dorner, T., S.J. Foster, N.L. Farner, and P.E. Lipsky. 1998. Somatic hypermutation of human immunoglobulin heavy chain genes: targeting of RGYW motifs on both DNA strands. *Eur J Immunol* 28:3384-3396.

144. Dorner, T., N.L. Farner, and P.E. Lipsky. 1999. Two mechanisms of somatic hypermutation of Ig V genes. *The Immunologist* 7:153-162.

145. Shlomchik, M.J., A. Marshak-Rothstein, C.B. Wolfowicz, T.L. Rothstein, and M.G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature* 328:805-811.

146. Yurasov, S., H. Wardemann, J. Hammersen, M. Tsuiji, E. Meffre, V. Pascual, and M.C. Nussenzweig. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* 201:703-711.

147. Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301:1374-1377.

148. Haubold, K., G.P. Owens, P. Kaur, A.M. Ritchie, D.H. Gilden, and J.L. Bennett. 2004. B-lymphocyte and plasma cell clonal expansion in monosymptomatic optic neuritis cerebrospinal fluid. *Ann Neurol* 56:97-107.

149. Milstein, C., M.S. Neuberger, and R. Staden. 1998. Both DNA strands of antibody genes are hypermutation targets. *Proc Natl Acad Sci U S A* 95:8791-8794.

150. Odegard, V.H., and D.G. Schatz. 2006. Targeting of somatic hypermutation. *Nat Rev Immunol* 6:573-583.

151. Oprea, M., L.G. Cowell, and T.B. Kepler. 2001. The targeting of somatic hypermutation closely resembles that of meiotic mutation. *J Immunol* 166:892-899.

152. Rogozin, I.B., and M. Diaz. 2004. Cutting edge: DGYW/WRCH is a better predictor of mutability at G:C bases in Ig hypermutation than the widely accepted RGYW/WRCY motif and probably reflects a two-step activation-induced cytidine deaminase-triggered process. *J Immunol* 172:3382-3384.

153. Rogozin, I.B., and N.A. Kolchanov. 1992. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim Biophys Acta* 1171:11-18.

154. Rogozin, I.B., Y.I. Pavlov, K. Bebenek, T. Matsuda, and T.A. Kunkel. 2001. Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. *Nat Immunol* 2:530-536.

155. William, J., C. Euler, S. Christensen, and M.J. Shlomchik. 2002. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297:2066-2070.

156. Corcione, A., F. Aloisi, B. Serafini, E. Capello, G.L. Mancardi, V. Pistoia, and A. Uccelli. 2005. B-cell differentiation in the CNS of patients with multiple sclerosis. *Autoimmun Rev* 4:549-554.

157. McFarland, H.F. 2008. The B cell--old player, new position on the team. *N Engl J Med* 358:664-665.

158. Serafini, B., B. Rosicarelli, R. Magliozzi, E. Stigliano, and F. Aloisi. 2004. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 14:164-174.

159. Gray, D. 2002. A role for antigen in the maintenance of immunological memory. *Nat Rev Immunol* 2:60-65.

160. Schultze, J.L., S. Michalak, M.J. Seamon, G. Dranoff, K. Jung, J. Daley, J.C. Delgado, J.G. Gribben, and L.M. Nadler. 1997. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J Clin Invest* 100:2757-2765.

161. Fecteau, J.F., and S. Neron. 2003. CD40 stimulation of human peripheral B lymphocytes: distinct response from naive and memory cells. *J Immunol* 171:4621-4629.

162. Mardiney, M., 3rd, M.R. Brown, and T.A. Fleisher. 1996. Measurement of T-cell CD69 expression: a rapid and efficient means to assess mitogen- or antigen-induced proliferative capacity in normals. *Cytometry* 26:305-310.

163. Jingwu, Z., R. Medaer, G.A. Hashim, Y. Chin, E. van den Berg-Loonen, and J.C. Raus. 1992. Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: precursor frequency, fine specificity, and cytotoxicity. *Ann Neurol* 32:330-338.

164. Teitelbaum, D., A. Meshorer, T. Hirshfeld, R. Arnon, and M. Sela. 1971. Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur J Immunol* 1:242-248.

165. Karandikar, N.J., M.P. Crawford, X. Yan, R.B. Ratts, J.M. Brenchley, D.R. Ambrozak, A.E. Lovett-Racke, E.M. Frohman, P. Stastny, D.C. Douek, R.A. Koup, and M.K. Racke. 2002. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest* 109:641-649.

166. Banchereau, J., P. de Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science* 251:70-72.

167. Lapointe, R., A. Bellemare-Pelletier, F. Housseau, J. Thibodeau, and P. Hwu. 2003. CD40-stimulated B lymphocytes pulsed with tumor antigens are effective antigen-presenting cells that can generate specific T cells. *Cancer Res* 63:2836-2843.

168. Jiang, W., M.M. Lederman, C.V. Harding, B. Rodriguez, R.J. Mohner, and S.F. Sieg. 2007. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. *Eur J Immunol* 37:2205-2213.

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

170. Hawker K, e.a. 2009. Efficacy and safety of rituximab in patients with primary progressive multiple sclerosis (PPMS): Results of a randomized double-blind placebo-controlled multicenter trial. *Neurology* 72:A254.

171. Arbour, N., R. Lapointe, P. Saikali, E. McCrea, T. Regen, and J.P. Antel. 2006. A new clinically relevant approach to expand myelin specific T cells. *J Immunol Methods* 310:53-61.

172. Duda, P.W., J.I. Krieger, M.C. Schmied, C. Balentine, and D.A. Hafler. 2000. Human and murine CD4 T cell reactivity to a complex antigen: recognition of the synthetic random polypeptide glatiramer acetate. *J Immunol* 165:7300-7307.

173. Lambracht-Washington, D., K.C. O'Connor, E.M. Cameron, A. Jowdry, E.S. Ward, E. Frohman, M.K. Racke, and N.L. Monson. 2007. Antigen specificity of clonally expanded and receptor edited cerebrospinal fluid B cells from patients with relapsing remitting MS. *J Neuroimmunol* 186:164-176.

174. Bar-Or, A., P.A. Calabresi, D. Arnold, C. Markowitz, S. Shafer, L.H. Kasper, E. Waubant, S. Gazda, R.J. Fox, M. Panzara, N. Sarkar, S. Agarwal, and C.H. Smith. 2008. Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. *Ann Neurol* 63:395-400.

175. Mizoguchi, A., and A.K. Bhan. 2006. A case for regulatory B cells. *J Immunol* 176:705-710.

176. Mauri, C., D. Gray, N. Mushtaq, and M. Londei. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* 197:489-501.

177. Biagi, E., E. Yvon, G. Dotti, P.J. Amrolia, S. Takahashi, U. Popat, F. Marini, M. Andreeff, M.K. Brenner, and R.F. Rousseau. 2003. Bystander transfer of functional human CD40 ligand from gene-modified fibroblasts to B-chronic lymphocytic leukemia cells. *Hum Gene Ther* 14:545-559.

178. Biagi, E., G. Dotti, E. Yvon, E. Lee, M. Pule, S. Vigouroux, S. Gottschalk, U. Popat, R. Rousseau, and M. Brenner. 2005. Molecular transfer of CD40 and OX40 ligands to leukemic human B cells induces expansion of autologous tumor-reactive cytotoxic T lymphocytes. *Blood* 105:2436-2442.

179. Krumbholz, M., I. Meinl, T. Kumpfel, R. Hohlfeld, and E. Meinl. 2008. Natalizumab disproportionately increases circulating pre-B and B cells in multiple sclerosis. *Neurology* 71:1350-1354.

180. Martin Mdel, P., P.D. Cravens, R. Winger, B.C. Kieseier, S. Cepok, T.N. Eagar, S.S. Zamvil, M.S. Weber, E.M. Frohman, B.K. Kleinschmidt-Demasters, T.J. Montine, B. Hemmer, C.M. Marra, and O. Stuve. 2009. Depletion of B lymphocytes from cerebral perivascular spaces by rituximab. *Arch Neurol* 66:1016-1020.

181. Pranzatelli, M.R., E.D. Tate, A.L. Travelstead, and J.A. Colliver. 2009. Long-Term Cerebrospinal Fluid and Blood Lymphocyte Dynamics After Rituximab for Pediatric Opsoclonus-Myoclonus. *J Clin Immunol*

182. Fuchs, E.J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science* 258:1156-1159.

183. Chen, L.C., J.C. Delgado, P.E. Jensen, and X. Chen. 2009. Direct expansion of human allospecific FoxP3+CD4+ regulatory T cells with allogeneic B cells for therapeutic application. *J Immunol* 183:4094-4102.

184. Eynon, E.E., and D.C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J Exp Med* 175:131-138.

185. Attanavanich, K., and J.F. Kearney. 2004. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J Immunol* 172:803-811.

186. Good, K.L., D.T. Avery, and S.G. Tangye. 2009. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol* 182:890-901.

187. Niino, M., M. Hirotani, Y. Miyazaki, and H. Sasaki. 2009. Memory and naive B-cell subsets in patients with multiple sclerosis. *Neurosci Lett* 464:74-78.

188. Harp, C., J. Lee, D. Lambracht-Washington, E. Cameron, G. Olsen, E. Frohman, M. Racke, and N. Monson. 2007. Cerebrospinal fluid B cells from multiple sclerosis patients are subject to normal germinal center selection. *J Neuroimmunol* 183:189-199.

189. Hansen, A., M. Odendahl, K. Reiter, A.M. Jacobi, E. Feist, J. Scholze, G.R. Burmester, P.E. Lipsky, and T. Dorner. 2002. Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjogren's syndrome. *Arthritis Rheum* 46:2160-2171.

190. Harp, C.T., A.E. Lovett-Racke, M.K. Racke, E.M. Frohman, and N.L. Monson. 2008. Impact of myelin-specific antigen presenting B cells on T cell activation in multiple sclerosis. *Clin Immunol* 128:382-391.

191. Suen, W.E., C.M. Bergman, P. Hjelmstrom, and N.H. Ruddle. 1997. A critical role for lymphotoxin in experimental allergic encephalomyelitis. *J Exp Med* 186:1233-1240.

192. Taga, K., and G. Tosato. 1992. IL-10 inhibits human T cell proliferation and IL-2 production. *J Immunol* 148:1143-1148.

193. Wagner, M., H. Poeck, B. Jahrsdoerfer, S. Rothenfusser, D. Prell, B. Bohle, E. Tuma, T. Giese, J.W. Ellwart, S. Endres, and G. Hartmann. 2004. IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. *J Immunol* 172:954-963.

194. Prinz, M., F. Garbe, H. Schmidt, A. Mildner, I. Gutcher, K. Wolter, M. Piesche, R. Schroers, E. Weiss, C.J. Kirschning, C.D. Rochford, W. Bruck, and B. Becher. 2006. Innate immunity mediated by TLR9

modulates pathogenicity in an animal model of multiple sclerosis. *J Clin Invest* 116:456-464.

195. Barth, H., P.A. Berg, and R. Klein. 2003. Methods for the in vitro determination of an individual disposition towards TH1- or TH2-reactivity by the application of appropriate stimulatory antigens. *Clin Exp Immunol* 134:78-85.

196. Lenarczyk, A., J. Helsloot, K. Farmer, L. Peters, A. Sturgess, and B. Kirkham. 2000. Antigen-induced IL-17 response in the peripheral blood mononuclear cells (PBMC) of healthy controls. *Clin Exp Immunol* 122:41-48.

197. Julius, M.H., T. Masuda, and L.A. Herzenberg. 1972. Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter. *Proc Natl Acad Sci U S A* 69:1934-1938.

198. Julius, M.H., C.A. Janeway, Jr., and L.A. Herzenberg. 1976. Isolation of antigen-binding cells from unprimed mice. II. Evidence for monospecificity of antigen-binding cells. *Eur J Immunol* 6:288-292.

199. Doucett, V.P., W. Gerhard, K. Owler, D. Curry, L. Brown, and N. Baumgarth. 2005. Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. *J Immunol Methods* 303:40-52.

200. Newman, J., J.S. Rice, C. Wang, S.L. Harris, and B. Diamond. 2003. Identification of an antigen-specific B cell population. *J Immunol Methods* 272:177-187.

201. Fridkis-Hareli, M., D. Teitelbaum, E. Gurevich, I. Pecht, C. Brautbar, O.J. Kwon, T. Brenner, R. Arnon, and M. Sela. 1994. Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells--specificity and promiscuity. *Proc Natl Acad Sci U S A* 91:4872-4876.

202. Putheti, P., M. Soderstrom, H. Link, and Y.M. Huang. 2003. Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis. *J Neuroimmunol* 144:125-131.

203. Haas, J., M. Korporal, B. Balint, B. Fritzsching, A. Schwarz, and B. Wildemann. 2009. Glatiramer acetate improves regulatory T-cell function by expansion of naive CD4(+)CD25(+)FOXP3(+)CD31(+) T-cells in patients with multiple sclerosis. *J Neuroimmunol* 216:113-117.

204. Barth, H., R. Klein, P.A. Berg, B. Wiedenmann, U. Hopf, and T. Berg. 2003. Analysis of the effect of IL-12 therapy on immunoregulatory T-cell subsets in patients with chronic hepatitis C infection. *Hepatogastroenterology* 50:201-206.

205. Davis, L.S., H. Schulze-Koops, and P.E. Lipsky. 1999. Human CD4+ T cell differentiation and effector function: implications for autoimmunity. *Immunol Res* 19:25-34.

206. Rivino, L., M. Messi, D. Jarrossay, A. Lanzavecchia, F. Sallusto, and J. Geginat. 2004. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. *J Exp Med* 200:725-735.

207. Moldovan, I.R., A.C. Cotleur, N. Zamor, R.S. Butler, and C.M. Pelfrey. 2008. Multiple sclerosis patients show sexual dimorphism in cytokine responses to myelin antigens. *J Neuroimmunol* 193:161-169.

208. Neuhaus, O., C. Farina, A. Yassouridis, H. Wiendl, F. Then Bergh, T. Dose, H. Wekerle, and R. Hohlfeld. 2000. Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *Proc Natl Acad Sci U S A* 97:7452-7457.

209. Ruprecht, C.R., and A. Lanzavecchia. 2006. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur J Immunol* 36:810-816.

210. Reindl, M., C. Linington, U. Brehm, R. Egg, E. Dilitz, F. Deisenhammer, W. Poewe, and T. Berger. 1999. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 122 (Pt 11):2047-2056.

211. Guggenmos, J., A.S. Schubart, S. Ogg, M. Andersson, T. Olsson, I.H. Mather, and C. Linington. 2004. Antibody cross-reactivity between myelin

oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis. *J Immunol* 172:661-668.

212. Litzenburger, T., H. Bluthmann, P. Morales, D. Pham-Dinh, A. Dautigny, H. Wekerle, and A. Iglesias. 2000. Development of myelin oligodendrocyte glycoprotein autoreactive transgenic B lymphocytes: receptor editing in vivo after encounter of a self-antigen distinct from myelin oligodendrocyte glycoprotein. *J Immunol* 165:5360-5366.

213. Vollmer, J., S. Tluk, C. Schmitz, S. Hamm, M. Jurk, A. Forsbach, S. Akira, K.M. Kelly, W.H. Reeves, S. Bauer, and A.M. Krieg. 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J Exp Med* 202:1575-1585.

214. Lee, J., T.H. Chuang, V. Redecke, L. She, P.M. Pitha, D.A. Carson, E. Raz, and H.B. Cottam. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc Natl Acad Sci U S A* 100:6646-6651.

215. Rutz, M., J. Metzger, T. Gellert, P. Luppa, G.B. Lipford, H. Wagner, and S. Bauer. 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur J Immunol* 34:2541-2550.

216. Cappione, A., 3rd, J.H. Anolik, A. Pugh-Bernard, J. Barnard, P. Dutcher, G. Silverman, and I. Sanz. 2005. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J Clin Invest* 115:3205-3216.

217. Guay, H.M., J. Larkin, 3rd, C.C. Picca, L. Panarey, and A.J. Caton. 2007. Spontaneous autoreactive memory B cell formation driven by a high frequency of autoreactive CD4+ T cells. *J Immunol* 178:4793-4802.

218. Pugh-Bernard, A.E., G.J. Silverman, A.J. Cappione, M.E. Villano, D.H. Ryan, R.A. Insel, and I. Sanz. 2001. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. *J Clin Invest* 108:1061-1070.

219. Bohnhorst, J.O., J.E. Thoen, J.B. Natvig, and K.M. Thompson. 2001. Significantly depressed percentage of CD27+ (memory) B cells among

peripheral blood B cells in patients with primary Sjogren's syndrome. *Scand J Immunol* 54:421-427.

220. Souto-Carneiro, M.M., V. Mahadevan, K. Takada, R. Fritsch-Stork, T. Nanki, M. Brown, T.A. Fleisher, M. Wilson, R. Goldbach-Mansky, and P.E. Lipsky. 2009. Alterations in peripheral blood memory B cells in patients with active rheumatoid arthritis are dependent on the action of tumour necrosis factor. *Arthritis Res Ther* 11:R84.

221. Yamada, S., M. DePasquale, C.S. Patlak, and H.F. Cserr. 1991. Albumin outflow into deep cervical lymph from different regions of rabbit brain. *Am J Physiol* 261:H1197-1204.

222. van Zwam, M., R. Huizinga, N. Heijmans, M. van Meurs, A.F. Wierenga-Wolf, M.J. Melief, R.Q. Hintzen, B.A. t Hart, S. Amor, L.A. Boven, and J.D. Laman. 2009. Surgical excision of CNS-draining lymph nodes reduces relapse severity in chronic-relapsing experimental autoimmune encephalomyelitis. *J Pathol* 217:543-551.

223. Hatterer, E., N. Davoust, M. Didier-Bazes, C. Vuaillat, C. Malcus, M.F. Belin, and S. Nataf. 2006. How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes. *Blood* 107:806-812.

224. Fabriek, B.O., J.N. Zwemmer, C.E. Teunissen, C.D. Dijkstra, C.H. Polman, J.D. Laman, and J.A. Castelijns. 2005. In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology. *J Neuroimmunol* 161:190-194.

225. Furtado, G.C., M.C. Marcondes, J.A. Latkowski, J. Tsai, A. Wensky, and J.J. Lafaille. 2008. Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis. *J Immunol* 181:4648-4655.

226. Baranzini, S.E., M.C. Jeong, C. Butunoi, R.S. Murray, C.C. Bernard, and J.R. Oksenberg. 1999. B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J Immunol* 163:5133-5144.

227. Cameron, E.M., S. Spencer, J. Lazarini, C.T. Harp, E.S. Ward, M. Burgoon, G.P. Owens, M.K. Racke, J.L. Bennett, E.M. Frohman, and N.L. Monson. 2009. Potential of a unique antibody gene signature to predict

conversion to clinically definite multiple sclerosis. *J Neuroimmunol* 213:123-130.

228. Owens, G.P., K.M. Winges, A.M. Ritchie, S. Edwards, M.P. Burgoon, L. Lehnhoff, K. Nielsen, J. Corboy, D.H. Gilden, and J.L. Bennett. 2007. VH4 gene segments dominate the intrathecal humoral immune response in multiple sclerosis. *J Immunol* 179:6343-6351.

229. Toellner, K.M., W.E. Jenkinson, D.R. Taylor, M. Khan, D.M. Sze, D.M. Sansom, C.G. Vinuesa, and I.C. MacLennan. 2002. Low-level hypermutation in T cell-independent germinal centers compared with high mutation rates associated with T cell-dependent germinal centers. *J Exp Med* 195:383-389.

230. Krumbholz, M., D. Theil, S. Cepok, B. Hemmer, P. Kivisakk, R.M. Ransohoff, M. Hofbauer, C. Farina, T. Derfuss, C. Hartle, J. Newcombe, R. Hohlfeld, and E. Meinl. 2006. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. *Brain* 129:200-211.

231. Sorensen, T.L., C. Trebst, P. Kivisakk, K.L. Klaege, A. Majmudar, R. Ravid, H. Lassmann, D.B. Olsen, R.M. Strieter, R.M. Ransohoff, and F. Sellebjerg. 2002. Multiple sclerosis: a study of CXCL10 and CXCR3 co-localization in the inflamed central nervous system. *J Neuroimmunol* 127:59-68.

232. Sorensen, T.L., H. Roed, and F. Sellebjerg. 2002. Chemokine receptor expression on B cells and effect of interferon-beta in multiple sclerosis. *J Neuroimmunol* 122:125-131.

233. Klein, U., Y. Tu, G.A. Stolovitzky, J.L. Keller, J. Haddad, Jr., V. Miljkovic, G. Cattoretti, A. Califano, and R. Dalla-Favera. 2003. Transcriptional analysis of the B cell germinal center reaction. *Proc Natl Acad Sci U S A* 100:2639-2644.

234. Chesnut, R.W., and H.M. Grey. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J Immunol* 126:1075-1079.

235. Amanna, I.J., N.E. Carlson, and M.K. Slifka. 2007. Duration of humoral immunity to common viral and vaccine antigens. *N Engl J Med* 357:1903-1915.

236. Kennel De March, A., M. De Bouwerie, M.N. Kolopp-Sarda, G.C. Faure, M.C. Bene, and C.C. Bernard. 2003. Anti-myelin oligodendrocyte glycoprotein B-cell responses in multiple sclerosis. *J Neuroimmunol* 135:117-125.

237. Correale, J., C. Rush, A. Amengual, and M.T. Goicochea. 2005. Mitoxantrone as rescue therapy in worsening relapsing-remitting MS patients receiving IFN-beta. *J Neuroimmunol* 162:173-183.

238. Domiati-Saad, R., J.F. Attrep, H.P. Brezinschek, A.H. Cherrie, D.R. Karp, and P.E. Lipsky. 1996. Staphylococcal enterotoxin D functions as a human B cell superantigen by rescuing VH4-expressing B cells from apoptosis. *J Immunol* 156:3608-3620.

239. Domiati-Saad, R., and P.E. Lipsky. 1998. Staphylococcal enterotoxin A induces survival of VH3-expressing human B cells by binding to the VH region with low affinity. *J Immunol* 161:1257-1266.

240. Sanz, I., C. Wei, F.E. Lee, and J. Anolik. 2008. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 20:67-82.

241. Medina, F., C. Segundo, A. Campos-Caro, I. Gonzalez-Garcia, and J.A. Brieva. 2002. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. *Blood* 99:2154-2161.

242. Deola, S., M.C. Panelli, D. Maric, S. Selleri, N.I. Dmitrieva, C.Y. Voss, H. Klein, D. Stroncek, E. Wang, and F.M. Marincola. 2008. Helper B cells promote cytotoxic T cell survival and proliferation independently of antigen presentation through CD27/CD70 interactions. *J Immunol* 180:1362-1372.

243. Anderson, S.M., M.M. Tomayko, A. Ahuja, A.M. Haberman, and M.J. Shlomchik. 2007. New markers for murine memory B cells that define mutated and unmutated subsets. *J Exp Med* 204:2103-2114.

244. Linton, P.J., B. Bautista, E. Biederman, E.S. Bradley, J. Harbertson, R.M. Kondrack, R.C. Padrick, and L.M. Bradley. 2003. Costimulation via OX40L expressed by B cells is sufficient to determine the extent of

primary CD4 cell expansion and Th2 cytokine secretion in vivo. *J Exp Med* 197:875-883.

245. Flynn, S., K.M. Toellner, C. Raykundalia, M. Goodall, and P. Lane. 1998. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J Exp Med* 188:297-304.

246. Stuber, E., and W. Strober. 1996. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J Exp Med* 183:979-989.

247. Weinberg, A.D., K.W. Wegmann, C. Funatake, and R.H. Whitham. 1999. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol* 162:1818-1826.

248. Bourke, E., D. Bosisio, J. Golay, N. Polentarutti, and A. Mantovani. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. *Blood* 102:956-963.

249. Lampropoulou, V., K. Hoehlig, T. Roch, P. Neves, E. Calderon Gomez, C.H. Sweenie, Y. Hao, A.A. Freitas, U. Steinhoff, S.M. Anderton, and S. Fillatreau. 2008. TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol* 180:4763-4773.

250. Cash, E., S. Weerth, R. Voltz, and M. Kornhuber. 1992. Cells of cerebrospinal fluid of multiple sclerosis patients secrete antibodies to myelin basic protein in vitro. *Scand J Immunol* 35:695-701.

251. Vogt, M.H., C.E. Teunissen, E. Iacobaeus, D.A. Heijnen, E.C. Breij, T. Olsson, L. Brundin, J. Killestein, and C.D. Dijkstra. 2009. Cerebrospinal fluid anti-myelin antibodies are related to magnetic resonance measures of disease activity in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 80:1110-1115.

252. Markovic, M., V. Trajkovic, J. Drulovic, S. Mesaros, N. Stojsavljevic, I. Dujmovic, and M. Mostarica Stojkovic. 2003. Antibodies against myelin oligodendrocyte glycoprotein in the cerebrospinal fluid of multiple sclerosis patients. *J Neurol Sci* 211:67-73.

253. Garcia-Merino, A., M.A. Persson, J. Ernerudh, J.J. Diaz-Gil, and T. Olsson. 1986. Serum and cerebrospinal fluid antibodies against myelin basic protein and their IgG subclass distribution in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 49:1066-1070.

254. Bieganowska, K.D., L.J. Ausubel, Y. Modabber, E. Slovik, W. Messersmith, and D.A. Hafler. 1997. Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. *J Exp Med* 185:1585-1594.

255. Yssel, H., and H. Spits. 2002. Generation and maintenance of cloned human T cell lines. *Curr Protoc Immunol* Chapter 7:Unit 7 19.

256. Major, E.O. 2009. Progressive Multifocal Leukoencephalopathy in Patients on Immunomodulatory Therapies. *Annu Rev Med*