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B Lymphocytes In The Brains Of Multiple Sclerosis Patients Mature In Peripheral Lymph Nodes

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B lymphocytes in the brains of multiple sclerosis patients mature in peripheral lymph nodes

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Abstract

Multiple sclerosis is an autoimmune, demyelinating disorder of the central nervous system that afflicts nearly 2.5 million people worldwide and is among the most common causes of permanent disability in young adults. Although a number of treatments for multiple sclerosis have emerged in recent years, no cure currently exists. From a pathophysiological standpoint, murine models and human genomic studies have established the contribution of T-lymphocytes to multiple sclerosis. However, evidence suggests that B-lymphocytes are also involved in the development of this disease. Immunoglobulins (also known as oligoclonal bands) are routinely measured in the cerebrospinal fluid of patients at the time of diagnosis of multiple sclerosis. In addition, several small-scale clinical trials have demonstrated benefit with B-cell depleting therapies (such as rituximab and ocrelizumab). Prior work has also established the presence of shared B lymphocyte clones in multiple sclerosis lesions as well as in other locations in the CNS, such as meninges. The purpose of this study was to investigate whether shared B lymphocyte clones are also observed in peripheral lymphoid tissues to elucidate the natural history and migration patterns of these cells. Applying conventional DNA sequencing of the B cell receptors (BCRs) to autopsy samples from five patients, we found that B lymphocyte clonal variants are indeed shared between the CNS and periphery. Subsequent work building on this thesis employing high-throughput sequencing (published in Stern et al. [1]) confirmed these findings and found that these cells predominantly mature in the periphery. These results shed light on the role of B cells in multiple sclerosis pathogenesis, clarify the mechanism of current therapeutics that function by modulating lymphocyte migration, and suggest new avenues of investigation for multiple sclerosis treatment.

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List of Abbreviations

BCR	B cell receptor
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity determining region
CNS	central nervous system
CSF	cerebrospinal fluid
CTLA	cytotoxic T-lymphocyte-associated protein
DTT	dithiothreitol
EAE	experimental autoimmune encephalitis
EDTA	ethylenediaminetetraacetic acid
GuSCN	guanidine thiocyanate
Ig	immunoglobulin
IL	interleukin
LB	lysogeny broth
MS	multiple sclerosis
PML	progressive multifocal leukoencephalopathy
SDS	sodium dodecyl sulfate
Th	T helper
TLR	Toll-like receptor
Treg	regulatory T
V_H	variable region, heavy chain
V_L	variable region, light chain

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Introduction

Epidemiology and disease burden of multiple sclerosis

In a series of public lectures in 1868, Jean-Martin Charcot described "sclérose en plaques" in the brain as the defining features of an incapacitating neurological disorder now known as multiple sclerosis (MS) [2]. Multiple sclerosis is an autoimmune demyelinating disorder of the central nervous system that afflicts approximately 400,000 individuals in the U.S. and 2.5 million people worldwide [3]. The economic burden of disease is considerable. The mean annual direct and indirect costs are estimated to be nearly $50,000 per patient (in 2004 U.S dollars), with the total annual cost of drugs and medical care in the U.S. approximately $14 billion [3].

Multiple sclerosis is the most common cause of permanent disability of young adults among the disorders of the central nervous system, with a mean age of onset of approximately thirty years of age [4, 5]. The life expectancy of patients with multiple sclerosis is reduced, with estimates of lifespan shortening ranging from four to twelve years [6, 7]. Multiple sclerosis disproportionately affects women over men, for reasons that are unclear [8]. Moreover, multiple sclerosis most frequently affects northern European populations, and the genetic risk for an individual with a first-degree relative with MS ranges from approximately 2-5% [9]. A number of genetic variants have been linked to multiple sclerosis, including certain alleles of the major histocompatibility complex (MHC), specifically HLA-DRB1, as well as non-MHC genes such as IL-7R [10, 11]. Environmental factors thought to be associated with multiple sclerosis include viral infections (such as Epstein-Barr virus), sunlight exposure and vitamin D levels, among

others [12, 13]. Recent *in vitro* and murine data also suggest that increased salt intake may also be a risk factor for multiple sclerosis, although clinical validation from epidemiological studies is still needed [14, 15].

Clinical features of multiple sclerosis

As noted above, the hallmark of multiple sclerosis is the autoimmune, inflammatory demyelination of the central nervous system [16]. Multiple sclerosis is primarily a clinical diagnosis, with the history and physical examination the most important elements of diagnosis. The test of choice to support diagnosis is magnetic resonance imaging of the brain and spinal cord, and the McDonald diagnostic criteria have been developed to include specific requirements for the dissemination of MS central nervous system lesions in space as well as time [17]. Diagnosis of multiple sclerosis can be supported by the presence of oligoclonal IgG bands in cerebrospinal fluid (but not in serum) and sensory evoked potentials [16]. MS presentations are classified into four clinical subtypes: clinically isolated syndromes, relapsing-remitting MS, secondary progressive MS, and primary progressive MS. A clinically isolated syndrome usually forms the first attack of multiple sclerosis, but does not yet fulfill the requirements for a strict diagnosis of MS (until lesions are separated by both time and space). For patients with a clinically isolated syndrome and MRI lesions at baseline, the risk of developing multiple sclerosis exceeds sixty percent [18].

Most (approximately 85%) of patients have a relapsing-remitting form of the disease that is characterized by clearly demarcated relapses between which no disease progression occurs [19]. These remission phases may either have full recovery or residual

deficits. For most of these patients, relapsing-remitting MS will usually transition to the secondary progressive form characterized by sustained neurological decline, without plateaus or remissions [19]. No clear diagnostic criteria exist to distinguish the transition from relapsing-remitting to MS to secondary progressive disease [20]. Primary progressive MS is a rarer form of the disease (approximately ten percent of patients) in which initial clinical presentation is characterized by progressive neurological decline, without clearly delineated phases of relapse and remission [21].

While no clinical signs are unique to multiple sclerosis, highly characteristic features include a pattern of relapse and remission, onset between the ages of fifteen and fifty years of age, optic neuritis, Lhermitte's sign (a sensation of electric shock radiating inferiorly along the back elicited by flexion of the neck), bilateral internuclear ophthalmoplegia, fatigue, and Uhthoff's phenomenon (a sensitivity of symptoms to heat) [16]. Other common features include variable sensory symptoms, weakness, gait disturbance, bowel and bladder dysfunction, and, eventually, cognitive impairment. [16]. Without treatment, patients with multiple sclerosis exhibit progressive neurological degeneration, with 50% of patients requiring a cane to ambulate and 15% requiring a wheelchair ten years after diagnosis [19].

Pathophysiology of multiple sclerosis

The etiology of multiple sclerosis is complex and incompletely understood. Broadly, the pathogenesis of multiple sclerosis is thought to be mediated by T lymphocytes that react to myelin-specific antigens, which subsequently infiltrate the central nervous system and initiate a chronic inflammatory response [22]. The major animal model of

multiple sclerosis is murine experimental autoimmune encephalomyelitis (EAE), which is generated by immunization with self-antigenic epitopes of myelin [22, 23]. In addition, the application of genome-wide analysis to human patients has opened up a number of new avenues for investigation [24]. Although the putatively pathogenic elements of multiple sclerosis interact with each other, they may be broadly classified into three categories: adaptive T lymphocyte response, adaptive B lymphocyte response, and innate response.

With respect to the T cell response, T lymphocytes are generally rare within an intact nervous system, however they actively infiltrate the central nervous system in multiple sclerosis [22]. Multiple sclerosis has classically been considered to be driven by aberrant Th1-polarized CD4+ lymphocytes on the basis of the EAE model [25]. Clinical evidence, however, has shown that this characterization is only partially complete—trials investigating anti-CD4 and anti-IL12/23 p40 (ustekinumab) antibodies as well as CTLA-4 immunoglobulin have demonstrated mixed results [26-28]. Recent work has uncovered the role of other elements of the T cell response in disease pathogenesis, including Th17-differentiated CD4+ lymphocytes and regulatory T (Treg) cells. For example, these IL-17 secreting Th17 cells have been shown to be essential in driving the disease process in the EAE model [29]. There is evidence that a critical cytokine for Th17 cell differentiation, IL-23, may be the key regulator of autoimmune regulation in the brain and not, as was previously thought, IL-12 (which drives Th1 polarization) [30]. Moreover, Th17 cells have been identified by immunohistochemistry in multiple sclerosis patients and are present (with high levels of IL-17 secretion) in the peripheral blood mononuclear cells of patients suffering from disease exacerbation [31, 32]. Several studies have shown that FoxP3+ regulatory T cells, which modulate the function of Th1, Th2, and Th17 cells, are

dysfunctional in multiple sclerosis [33, 34]. Finally, CD8+ cytotoxic T cells have also been shown to induce an EAE-like disease in mice, and they are also present in CNS lesions of MS patients [35, 36]. Overall, much work remains to be done in fully elucidating the functional interactions between these T cell elements in determining their contributions to disease pathogenesis.

The B lymphocyte component of multiple sclerosis is less well-understood. While oligoclonal IgG bands are present in 95% of patients with multiple sclerosis, the role of these antibodies remains controversial [37]. Although B lymphocytes are known to contribute to demyelination in EAE through the production of anti-myelin antibodies, they have also been shown to play a protective role [22]. In terms of human disease, however, while reactivity of recombinant antibodies derived from clonally expanded plasma cells against multiple sclerosis brain tissue has been shown, attempts to refine the identity of a pathogenic antigen (by testing for reactivity to key candidates myelin basic protein, myelin oligodendrocyte glycoprotein, or proteo-lipid protein), have fallen short [38, 39]. Nevertheless, as described below, several small trials have demonstrated a significant clinical effect with anti-CD20 B-lymphocyte depleting therapies in relapsing-remitting multiple sclerosis, pointing to a role for these cells in disease pathophysiology [40]. More recently, the potassium channel KIR4.1, which is found on the surface of astrocytes and oligodendrocytes, has been identified as a possible target for these for these auto-antibodies [41].

Finally, in terms of the innate response, dendritic cells, microglial cells (macrophages), natural killer cells, and mast cells have all been set forth as possible components of the disease framework [42] In particular, toll-like receptor 9 (TLR-9) and

its adaptor protein MyD88, which together normally function in the recognition of pathogen, activation of the NF-κB transcriptional program, and production of pro-inflammatory molecules, have been shown to function as key mediators of EAE in mice [43]. The EAE model has also shown that TLR-4 ligation, by promoting differentiation of naïve T-cells into Th1 cells and preventing Th17 development, prevents the development of EAE [44]. In addition, microglia/macrophages have been shown in the EAE model to actively ingest and present myelin antigen to auto-reactive T-cells, as well as mediate neuronal damage through the production of myeloperoxidases and reactive oxygen species [42]. One key difficulty in elucidating the microglia/macrophage response, however, is distinguishing between these two cell types [45].

Treatment of multiple sclerosis

Treatment of multiple sclerosis is geared toward the phase of disease, with differing strategies for acute attacks as compared with management of relapsing-remitting MS with disease modifying therapy. As yet, no curative therapy exists for multiple sclerosis. Acute exacerbations of multiple sclerosis are most commonly treated with a three to seven day course of intravenous methylprednisolone with or without an oral taper of prednisone [46]. For patients with acute attacks who do not respond to steroids, plasmapheresis is also an option [47].

A range of disease modifying therapies are available for the treatment of relapsing-remitting multiple sclerosis, with the goal of achieving a diminished relapse rate and slower progression of radiographic lesions. These treatments are primarily immunomodulatory in nature, although the mechanisms of action have not yet been fully established for all of

them. The first disease modifying agent approved for use in multiple sclerosis was recombinant interferon beta, and its mode of action remains unclear, although it is known that it modulates immune responsiveness [48]. Interferon therapy is associated with significant side effects, including injection site reactions, influenza-like symptoms, and asymptomatic transaminitis (with serious hepatotoxicity being rare) [49, 50]. Neutralizing antibodies are known to develop in response to interferon therapy, limiting its effectiveness [51].

Another key immunomodulatory agent is glatiramer acetate (COPAXONE), which is composed of random polymers of four amino acids. Glatiramer is similar to a component of the myelin sheath of nerves, myelin basic protein, and is thought to function by binding to major histocompatibility complex molecules and competing with disease-causing antigens for presentation to T cells [52]. Dimethyl fumarate (Tecfidera) is similarly immunomodulatory [53]. Fingolimod (Gilenya), on the other hand, is an analogue of sphingosine, which binds to the sphingosine-1-phosphate receptor, thereby sequestering lymphocytes in the lymph nodes and preventing their migration into the CNS, as well as exerting a significant effect on astrocytes, as demonstrated *in vitro* and in EAE [54]. Similarly, natalizumab (Tysabri) is a monoclonal antibody against alpha-4 integrins, which prevents adhesion of lymphocytes to vascular endothelium and inhibits access to the brain parenchyma [55]. Its use, however, has been affected by the risk of progressive multifocal leukoencephalopathy (PML), and risk-stratification on the basis of JC virus antibodies in serum is now undertaken prior to initiating treatment with natalizumab [56]. The final therapeutic agents for relapsing-remitting multiple sclerosis include teriflunamide (Aubagio), which inhibits pyrimidine biosynthesis to block T cell interaction with antigen

presenting cells and, rarely, mitoxantrone, which is an anthracycline analogue that causes DNA cross-linking and strand breaks and is now used in a very few cases [57, 58].

The treatment of secondary progressive multiple sclerosis is somewhat different, incorporating monthly pulses of intravenous glucocorticoids, with some evidence to support the use of cyclophosphamide, methotrexate, or interferon therapy [59]. No definitive clinical trial evidence exists for the treatment of primary progressive multiple sclerosis, although the guidelines of the American Academy of Neurology also recommend the use of methylprednisolone, methotrexate, cladribine, and possibly mitoxantrone as well [59].

Investigative therapies for multiple sclerosis

A number of agents are currently under investigation for treatment of relapsing-remitting multiple sclerosis such as the immune suppressant laquinimod [60-63]. The role of glucocorticoids, however, in combination with existing treatment remains unclear [64, 65]. Finally, several monoclonal antibodies are being actively studied, including alemtuzumab (an anti-CD52 antibody), daclizumab (which acts against the IL-2 receptor), and the anti-CD20 antibodies ritixumab and ocrelizumab [66, 67]. Rituximab has been evaluated in a single clinical trial to assess the effects of B cell depletion in multiple sclerosis. That trial, a phase 2, double-blind, randomized placebo-controlled trial enrolling 104 patients demonstrated that treatment with intravenous ritixumab was associated with reduced inflammatory brain lesions and fewer clinical relapses [68]. Ocrelizumab, which binds to a different CD20 epitope than rituximab, was also studied in a phase 2, randomized placebo-controlled trial enrolling 220 patients, which also showed asignificant reduction

in gadolinium enhancing brain lesions (approximately ninety percent) and a reduction in the annualized relapse rate by approximately seventy to eighty percent [69].

Hypothesis and Specific Aims

The focus of this study was the role of B lymphocytes in multiple sclerosis, specifically, their natural history. As noted above, despite the complex and incompletely understood autoimmune etiology of MS, the involvement of B lymphocytes in the disease has been recognized for decades, and oligoclonal bands in cerebrospinal fluid are routinely measured upon diagnosis of multiple sclerosis. Nevertheless, the origin and migration patterns of the plasma cells producing these antibodies remains unclear. These plasma cells are "antigen-experienced" cells that have encountered antigen and subsequently undergone clonal expansion, somatic hypermutation, and selection. Previous work from this laboratory demonstrated, through immunohistochemistry and complementary DNA (cDNA) sequencing of the heavy chains of B-lymphocyte Ig receptors (BCRs), that antigen-experienced B-cell clones were shared between parenchymal lesions and meningeal compartments in multiple sclerosis patients [70]. Having established the presence of antigen-experienced B lymphocytes in the parenchyma and meninges, however, a key unanswered question was whether these clonal variants are also present in the periphery and if they traffic between the central nervous system and the periphery.

This question raises two possibilities: 1) these plasma cells may have experienced the antigen within the central nervous system itself or 2) they may have encountered antigen in the periphery and subsequently migrated to the brain. In the former case, if the antigen resides in the central nervous system, then the brain itself would serve as an

immune compartment that gives rise to potentially disease-causing antibodies. In the latter case, for B-cells to mature peripherally, it may be that the antigen is present peripherally, allowing exclusive maturation outside of the central nervous system and subsequent migration into the brain. Alternatively, the cells may mature centrally but travel back and forth among the different compartments. In either case, elucidating the mechanism of maturation and migration of these cells is particularly relevant to the mechanism of multiple sclerosis treatments such as fingolimod or natalizumab, which function by inhibiting the migration of lymphocytes into the central nervous system. More broadly, understanding the mechanism of B lymphocytes in the pathogenesis of multiple sclerosis may also suggest novel avenues for treatment.

Our work therefore sought to address this question and clarify the migratory path of these B lymphocytes in MS patients. The overall approach involved sequencing the genes for heavy chains of B cell receptors in parenchymal lesions and meningeal tissues and comparing them to peripheral lymphoid tissue to establish whether distinct B-lymphocyte clones are shared among these sites and, further, whether they migrate from peripheral sites to the central nervous system or vice-versa.

Specific Aim #1: Identification of B lymphocytes in MS patient tissues by immunohistochemistry.

We first sought to establish that B lymphocytes were indeed present in our patient samples before attempting to generate B cell receptor sequencing libraries. The tissues studied included multiple sclerosis plaques from brain parenchyma, choroid plexus, cerebral cortex including pia mater, draining cervical lymph node, and in one case, spleen.

This was performed by immunohistochemistry to detect CD20, a marker of B lymphocytes (described in methods below). As described previously, in a study of over twenty patients with progressive multiple sclerosis, antigen-experienced clones of B lymphocytes were demonstrated to be shared between MS lesions and meninges, thereby establishing a relationship between parenchymal infiltrates and extraparenchymal lymphoid tissue [70]. By definition, we would also expect secondary lymphoid tissue to contain B lymphocytes. Owing to the limited scope of the work in this thesis, staining was only performed to identify CD20-positive cells. Subsequent work from this group, however, also performed immunohistochemistry to identify plasma cells, macrophages/monocytes, and T lymphocytes in the same cohort of patients (discussed below). This confirmation step was critical in verifying the presence of these cells in our own samples prior to proceeding with PCR amplification and sequencing.

Specific Aim #2: Comparison of B-cell receptors in CNS and peripheral compartments in MS patients by conventional DNA sequencing.

We next aimed to perform conventional Sanger sequencing of the B cell receptors in the central nervous system (MS lesions, cerebral cortex, meninges, choroid plexus) and periphery (cervical lymph nodes and spleen) and to analyze their mutation patterns. The fully mature B cell receptor is composed of two identical heavy chains and two identical light chains, encoded by variable and constant regions of the BCR gene. While the heavy chain contains three segments, V, D, and J, the light chain is composed solely of V and J segments [71].

During the process of maturation, the B lymphocyte generates a unique receptor through the process of V(D)J recombination, in which heterodimers of randomly selected heavy and light chains are constructed from randomly chosen single “V,” “D,” and “J” protein-coding regions [71]. In addition, further genetic diversity is introduced through the presence of non-germline mutations in the junctions of these coding regions as well as through the process of somatic hypermutation that occurs with expansion when a B lymphocyte experiences antigen [72]. These mutation patterns allow for unique identification of the B cell clone, by counting and comparing mutations of both nucleotides and amino acids in different tissues. We identified B lymphocyte clones as those with identical complementarity determining region (CDR3) sequences, which implied that they had identical antigen-binding regions. Clonal variants were those with the same CDR3, but different mutations in other regions, such as junctional regions, which emerge through the process of somatic hypermutation and affinity maturation. By comparing whether these clonal variants were present in different tissues within the same patient, we were able to investigate the possibility of B cell trafficking between the CNS and the periphery in multiple sclerosis.

Methods

The methods described below are limited to the work performed by the author of this thesis (with the exception of assistance with immunohistochemistry). Subsequent work on this project performed by other members of this group is summarized in the discussion section. That work, as well as the methods and results described below, have been published in Stern et al. [1].

Clinical specimens

Autopsy tissues were obtained from five deceased patients with a median age of 63 years at death with clinically defined multiple sclerosis (MS). No radiographic confirmation was obtained. The original naming schemes from those samples are preserved in this text. Three subjects were female, while two were male. One subject had relapsing-remitting MS, while four had progressive MS. For subject D, tissues were collected from a white matter lesion, choroid plexus, cerebral cortex, cervical lymph node and spleen. For subject 4106, tissues were collected from white matter lesion, choroid plexus, pia mater, and two cervical lymph nodes. For each of the three remaining patients (3928, 4014, and 3931), paired samples of white matter lesion and cervical lymph nodes were obtained. The median post-mortem delay prior to tissue retrieval was 12 hours.

A number of co-morbidities were present in these patients, and in patient D, the cause of death was determined to be massive gastrointestinal hemorrhage secondary to cirrhosis and portal hypertension. Patient 4014 had a significant history of malignancy, including pancreatic (unspecified). The clinical and pathological characteristics of the specimens are summarized in Table 1. The tissue samples were immediately frozen and stored at -80°C. The specimens did not include any personally identifiable information or details about treatment and disease management. The samples were collected under an exempt protocol approved by the Human Research Protection Program at the Yale School of Medicine.

Table 1 - Clinical characteristics of multiple sclerosis tissue specimens.

Patient	Sex	Age at death (years)	Post-mortem delay (hrs)	Clinical Diagnosis
D	M	39	36	Relapsing-remitting MS
4106	F	63	23	Chronic MS
4014	M	80	12	Chronic progressive MS
3928	F	53	10	Secondary progressive MS
3931	F	74	10	Primary progressive MS

Immunohistochemistry

Frozen blocks of tissue from all five patients (including MS plaque, cortex with pia mater, choroid plexus, cervical lymph node, and spleen) were sectioned at 10 μm thickness and air-dried on glass slides in preparation for immunohistochemistry. Initial staining was performed on a representative sample by quenching and blocking with goat serum and incubating with mouse monoclonal antibody against CD20 (from Dako). Peroxidase-labeled secondary antibodies were applied and visualized with chromogen. Final staining of all samples (including hematoxylin/eosin stains, and all immunohistochemistry presented below) was performed by Yale Pathology Tissue Services. I performed microscopy and image acquisition.

RNA extraction from human tissue samples and cDNA synthesis

Frozen blocks of tissue were sectioned at 14 μm thickness. Serial sections were used to construct the immunoglobulin (Ig) variable region heavy (V_H) and light chain ($V_{L,}$ kappa) libraries. RNA extraction was performed using the *mir*Vana kit manufactured by Life Technologies. All surfaces were cleaned with RNase decontamination solution, and RNase-free pipette tips were used. The tissue samples were washed in cold phosphate-

buffered saline (PBS), and the cells were lysed with the addition of 500 μL of lysis buffer (4 M GuSCN, 0.1 M beta-mercaptoethanol, 0.5% N-lauroyl sarcosine, 25 mM Na-citrate, pH 7.2). The cells were vortexed vigorously to obtain a homogenized lysate. For organic extraction of RNA, 1/10 volume of homogenate additive (comprising 2 M sodium acetate, pH 4) was added, and the samples mixed and left on ice for ten minutes. An equivalent volume of acid-phenol:chloroform was added, the sample was again vortexed, and then centrifuged at maximum speed (10,000 x g) for five minutes to separate the aqueous and organic phases. The aqueous phase was recovered.

For final RNA isolation, 1.25 volumes of room temperature 100% ethanol was added and passed through a filter cartridge (via centrifugation at 10,000 x g for 15 seconds). The RNA was washed once with a solution of 700 μL of 1.6 M GuSCN in 70% ethanol. The RNA was then washed twice with a solution of 500 μL 80% ethanol, 0.1 M NaCl, 4.5 mM EDTA, 10 mM TrisHCl, pH 7.5.The RNA was then eluted with 100 μL of an elution solution containing 0.1 mM EDTA. RNA quality was assessed by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer.

Complementary DNA was then synthesized from the total RNA using the SuperScript kit manufactured by Invitrogen. Denaturation was performed by combining RNA, primer for human constant region C_μ as described previously (5'-GCA GGA GAC GAG GGG GA-3' [73]), and 10mM dNTP, and heating to 65°C for five minutes, followed by placement of the reaction on ice for one minute. A 10 μL total volume solution of RT buffer (containing 200 mM Tris-HCl [pH 8.4] and 500 mM KCl, appropriately diluted), 25 mM $MgCl_2$, 0.1M dithiotreitol (DTT), SuperScript reverse transcriptase, and RNase inhibitor was added to the denatured RNA and primer solution. Synthesis was carried out

at 50°C for 50 minutes, and the reaction was terminated by heating to 85°C for five minutes. RNA was removed from the cDNA solution through the addition of 1 μL RNase H and incubation at 37°C for 20 minutes. An aliquot was then removed for PCR.

Polymerase chain reaction (PCR) amplification

Next, the human Ig variable regions were amplified by polymerase chain reaction (PCR) as previously described by Wang and Stoller [73], with modifications as noted, including the use of TOPO-TA cloning [74]. Two-step PCR was performed to amplify the heavy and light-chain regions with two sets of internal nested primers. The PCR procedure used in this study included the 5' end of the Ig constant region, which allowed isotype to be determined.

For the first PCR step, a set of oligonucleotides corresponding to leader sequences of the heavy chain variable regions was used for the 5' primers (VHL-1: 5'-TCA CCA TGG ACT G(C/G)A CCT GGA-3';VHL-2: 5'-CCA TGG ACA CAC TTT G(C/T)T CCA C-3'; VHL-3: 5'-TCA CCA TGG AGT TTG GGC TGA GC-3'; VHL-4: 5'-AGA ACA TGA AAC A(C/T)C TGT GGT TCT T-3'; VHL-5: 5'-ATG GGG TCA ACC GCC ATC CT-3'; VHL-6: 5'-ACA ATG TCT GTC TCC TTC CTC AT-3'). For the 3' primer, an oligonucleotide complementary to the CH1 region was used (5'-CAG GAG ACG AGG GGG AAA AG-3'). The PCR reaction was set up with DNA template, primers in concentration of 1 μM, 0.5 μL of 50 mM dNTPs, 1 μL of *Taq* polymerase, 5 μL of PCR buffer (containing 600 mM Tris-SO_4 (pH 8.9) and 180 mM $(NH_4)_2SO_4$), and sterile water to a total volume of 50μL. The PCR steps were carried out with three cycles of denaturing (94°C for 45 seconds, 45°C for 45 seconds, and 72°C for 1 minute and 45 seconds),

followed by 30 cycles of amplification (94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute and 45 seconds), concluding with a 10 minute incubation at 72°C.

For the second step of PCR amplification, internally nested primers were used, with 5' primers corresponding to the variable framework-1 heavy chain region (VH-1: 5'-TTG CGG CCG CCA GGT (G/C)CA GCT GGT (G/A)CA GTC-3'; VH-2: 5'-TTG CGG CCG CCA G(A/G)T CAC CTT GAA GGA GTC-3'; VH-3: 5'-TTG CGG CCG C(G/C)A GGT GCA GCT GGT GGA GTC-3'; VH-4: 5'-TTG CGG CCG CCA GGT GCA GCT GCA GGA GTC-3'; VH-5: 5'-TTG CGG CCG CGA (G/A)GT GCA GCT GGT GCA GTC-3'; VH-6: 5'-TTG CGG CCG CCA GGT ACA GCT GCA GCA GTC-3'). The 3' primer was: 5'-GGG AAT TCA AAA GGG TTG GGG CGG ATG CAC T-3'. Thirty cycles of PCR amplification were carried out in the same manner as described above. The products of polymerase chain reaction were directly analyzed using agarose gel electrophoresis. The PCR amplification products are shown in Figure 1.

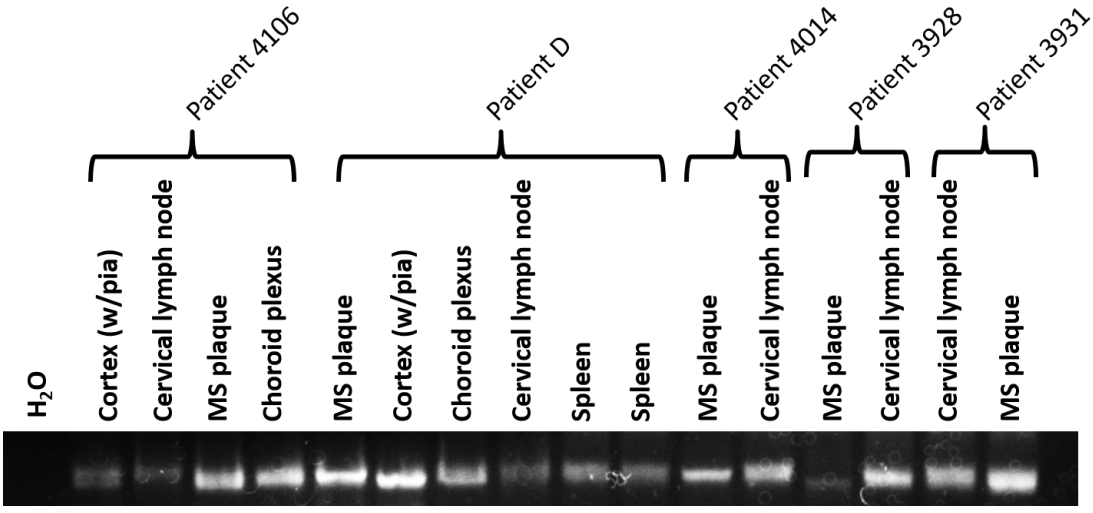

Figure 1 - PCR amplification of Ig variable heavy chain regions from multiple sclerosis patients. The first lane includes a negative control with water. The variable heavy chain regions were successfully amplified from all tissues for all patients.

Purification of PCR products and cloning

For gel extraction of the PCR amplification products, the QIAquick Gel Extraction kit, manufactured by QIAGEN, was used. The DNA fragments were excised from the agarose gel and weighed. The gel fragments were dissolved in three volumes (approximately 300 μL) of buffer containing 5.5 M GuSCN in 20 mM Tris-HCl, pH 6.6 at 50°C for ten minutes. One gel volume (approximately 100 μL) of 100% isopropanol was then added, and the sample was applied to a spin column and centrifuged for 1 minutes to bind the DNA to the spin column. Next, the column was washed with the addition of 500 μL of buffer containing 5.5 M GuSCN in 20 mM Tris-HCl, pH 6.6 and centrifugation for one minute. An additional washing step was performed, with the application of 750 μL of buffer containing 10 mM Tris-HCl, pH 7.5 in 80% ethanol and centrifuged for one minute. The sample was centrifuged again to remove residual ethanol. The DNA was then eluted in 50 μL of buffer containing 10 mM Tris-HCl, pH 8.5.

The PCR products were cloned into the pCR4-TOPO vector from Invitrogen, which included kanamycin and ampicillin resistance genes, as well as a cloning site within a LacZα-ccdB gene fusion for positive selection. The cloning reaction was prepared with 1 μl salt solution containing 200 mM NaCl and 10 mM $MgCl_2$, 1 μL vector, 2 μL of PCR product, and 2 μL of sterile water. The cloning reaction was carried out for five minutes at room temperature and then placed on ice. For transformation of competent *E. coli* cells, 2 μL of the cloning reaction was then added to the cells and incubated on ice for thirty minutes. The cells were then heat-shocked at 42°C for 30 seconds and replaced on ice. Next, 250 μL of S.O.C. medium were added to the cells, which were incubated at 37°C on

a horizontal shaker for one hour. 20 µL of cells were then plated on kanamycin-selective plates and incubated at 37°C overnight.

Conventional DNA sequencing of antibody gene repertoire

For isolation of DNA for sequencing, single colonies were chosen randomly from kanamycin selective plates and inoculated in 4 mL LB medium with kanamycin. The cultures were incubated for 12 hours at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 6800 x g for three minutes at room temperature, and the supernatant drained. Plasmid DNA was purified using the QIAprep kit manufactured by Qiagen. The pelleted cells were resuspended in a volume of 250 µL of a buffer of 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, and100 ug/mL RNase A. The cells were then lysed by adding 250 µL of lysis buffer containing 200 mM NaOH and 1% SDS, mixing, and incubating for five minutes. The lysis reaction was neutralized with the addition of 350 µL of buffer containing 3.0 M potassium acetate, pH 5.5. The DNA was then centrifuged for 10 minutes at 17,900 x g, the supernatant applied to a spin column and centrifuged again for one minute. The column was washed with 500 µL of buffer containing 5 M Gu-HCl in 30% isopropanol and centrifuged for one minute. The final washing step was performed with the addition of 750 µL buffer containing 10 mM Tris-HCl pH 7.5 in 80% ethanol and centrifuged for one minute. The DNA was eluted into 50 µL of elution buffer containing 10 mL Tris-Cl, pH 8.5.

Purified plasmid DNA was prepared for sequencing by combining 500 ng of DNA with 2 µL of the M13 reverse sequencing primer (5´-CAG GAA ACA GCT ATG AC-3´) at a concentration of 4 µM, and sterile water for a total volume of 18 µL. The reactions

were carried out in 96-well plates. Sanger sequencing was performed by a central facility at the Keck DNA Sequencing Lab at Yale University.

Analysis of clonal variants

Evidence of clonal expansion, somatic mutation, and isotype distribution were studied in both the heavy and light chain libraries. The sequences were analyzed using software from the human variable region database (IMGT/HighV-QUEST; http://www.imgt.org) [75], for identification of germline variable [V], diversity [D], and joining [J]) genes, alleles, and functionality. This analysis generated alignments for variable, diversity, and joining regions, as well as translation and nucleotide and amino acid mutation statistics.

The somatic mutations were counted to group clonal variants. Individual clones were identified through unique CDR3 amino acid sequences. Identical sequences from distinct tissue sections were considered to be clonally expanded, as these were B-cell receptors targeting the same antigen in two different tissues. On the other hand, if identical sequences from the same tissue section were identified, these were considered to be the product of polymerase chain reaction (PCR) amplification, as these usually had the same nucleotide and amino acid mutation pattern. Sequences were considered to be derived from clonally related B cells if they possessed identical CDR3 regions and contained different somatic mutations in other regions.

Results

CD20+ B lymphocytes are present in the central nervous system and peripheral lymphoid tissues of multiple sclerosis patients.

B lymphocytes were detected in tissue samples from these five patients in both brain parenchyma and secondary lymphoid tissue by immunohistochemistry for CD20 cell surface markers. Subsequent work beyond the scope of this thesis also identified plasma cells with monoclonal antibody against CD138, macrophages and activated microglia with anti-CD68 antibody, and T lymphocytes with anti-CD3 antibody in these same patient samples as described in Stern et al. [1].

For all patients, high numbers of CD20+ cells were visualized in cervical lymph nodes and spleen, validating the immunohistochemistry methods employed (Figures 2-6). For patient 3928, while greater numbers of CD20+ cells were observed in the cervical lymph nodes, considerably fewer were seen in the MS plaque compared with the other patients, a finding which was borne out by the considerably fewer B cell clones also identified in this patient (described below) (Figure 2). Similarly, many more CD20+ cells were seen in the cervical lymph node in patient 3931, with fewer in the MS plaque (Figure 3).

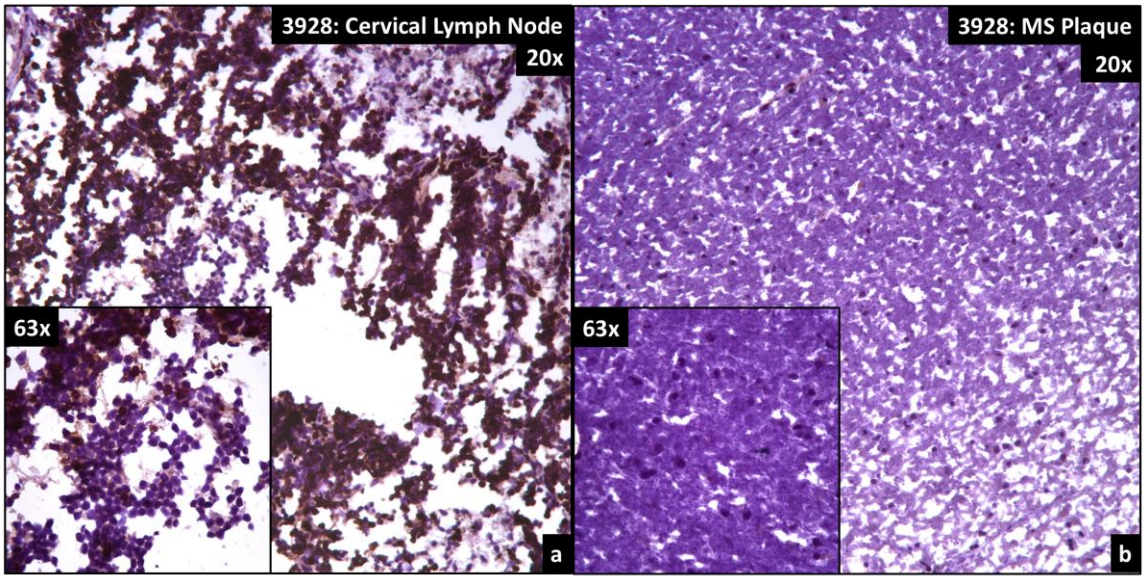

Figure 2 - Immunohistochemical staining for CD20+ B lymphocytes in patient 3928. (a) cervical lymph node; (b) MS plaque. 63x magnification inset. Greater numbers of CD20+ cells were observed in the cervical lymph node, compared to the MS plaque.

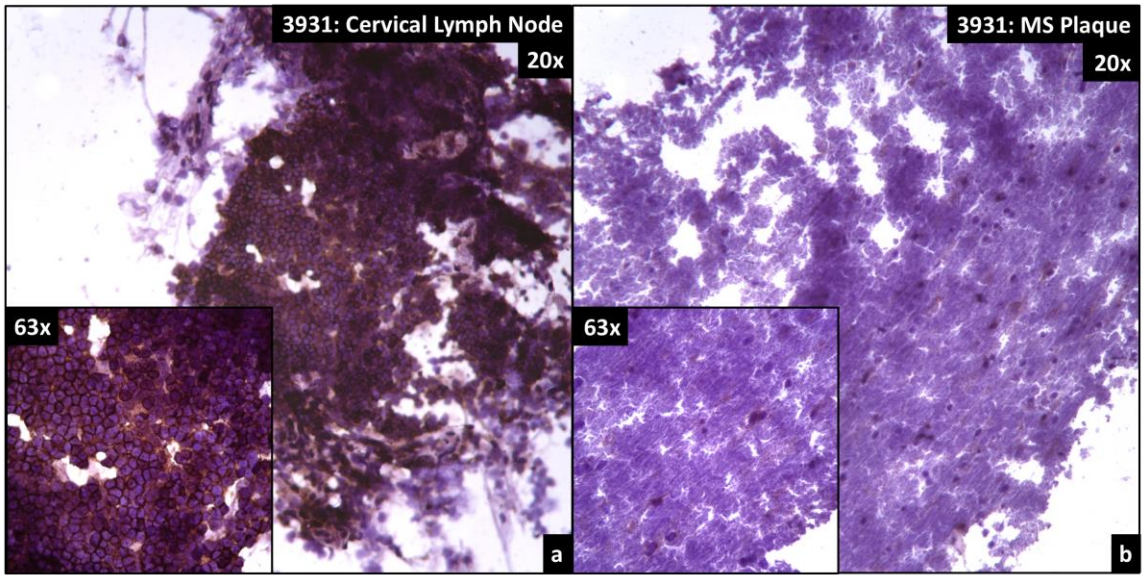

Figure 3 - Immunohistochemical staining for CD20+ B lymphocytes in patient 3931. (a) cervical lymph node; (b) MS plaque**.** 63x magnification inset. Greater numbers of CD20+ cells were observed in the cervical lymph node, compared to the MS plaque.

Patient 4014 was the final patient for which only cervical lymph node and MS plaque were available, and a similar pattern of staining was observed (Figure 4). For patients 4106 and D, greater tissue diversity was available. Again, high numbers of CD20+ cells cervical lymph nodes (and, in the case of patient D, spleen), were seen, with fewer in the MS plaque. Low numbers of CD20+ cells were also detected in the pia mater of patients 4106 and D (Figures 5C, 6D). B lymphocytes were also noted to be present in the choroid plexus of patients 4106 and D (Figures 5B, 6E). The confirmation of the presence of B lymphocytes in these compartments allowed for generation of sequencing libraries of B cell receptors from these tissues.

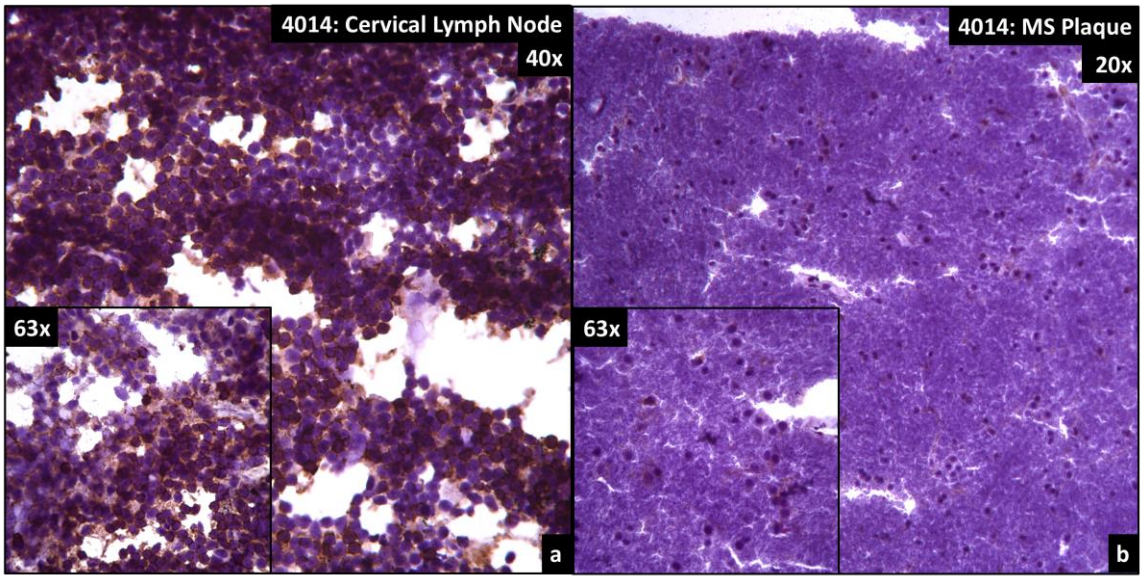

Figure 4 - Immunohistochemical staining for CD20+ B lymphocytes in patient 4014. (a) cervical lymph node; (b) MS plaque. 63x magnification inset. Greater numbers of CD20+ cells were observed in the cervical lymph node, compared to the MS plaque.

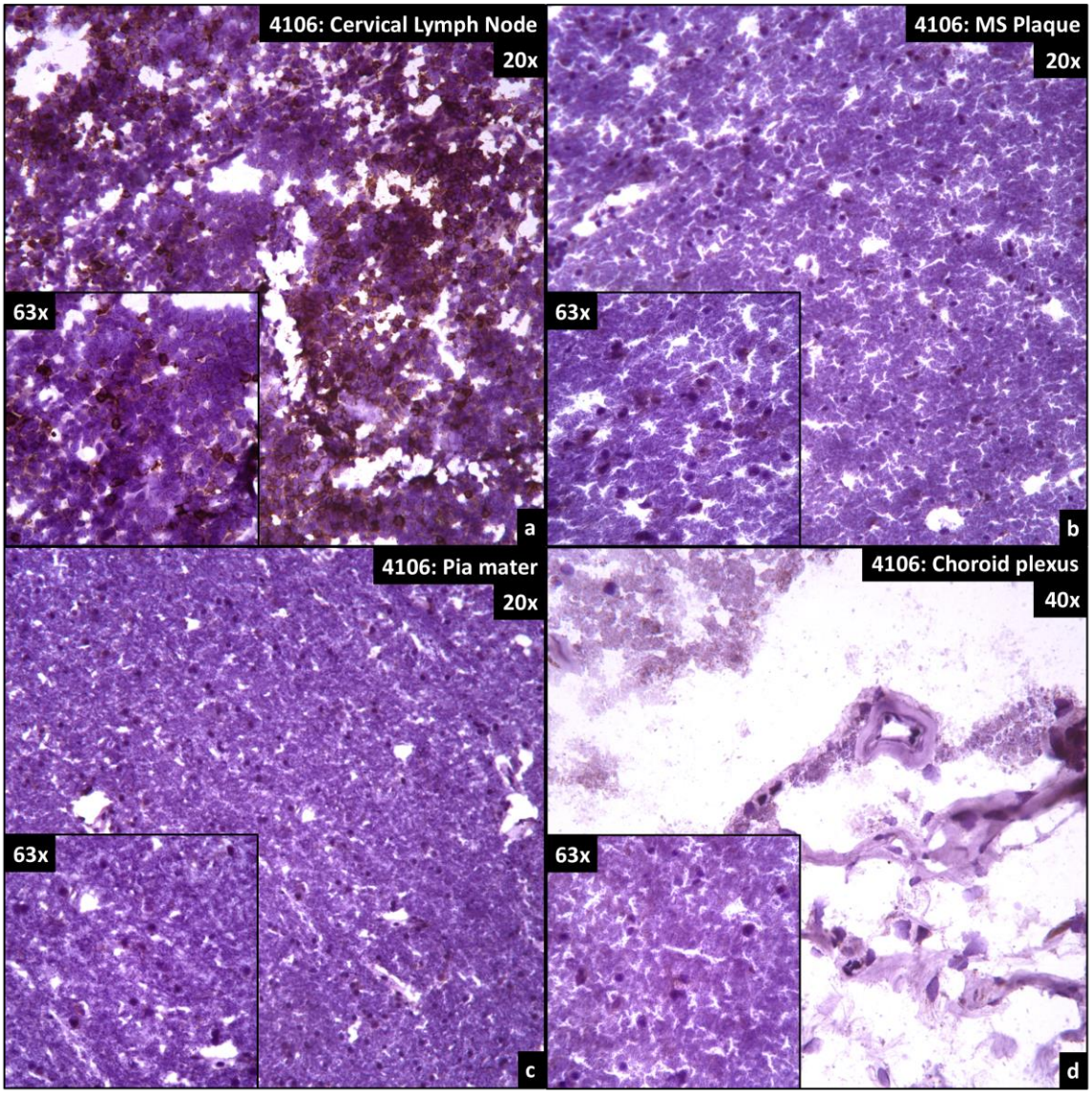

Figure 5 - Immunohistochemical staining for CD20+ B lymphocytes in patient 4106. (a) cervical lymph node; (b) MS plaque; (c) pia mater; (d) choroid plexus**.** 63x magnification inset. Greater numbers of CD20+ cells were observed in the cervical lymph node, with fewer noted in the MS plaque, pia mater, and choroid plexus.

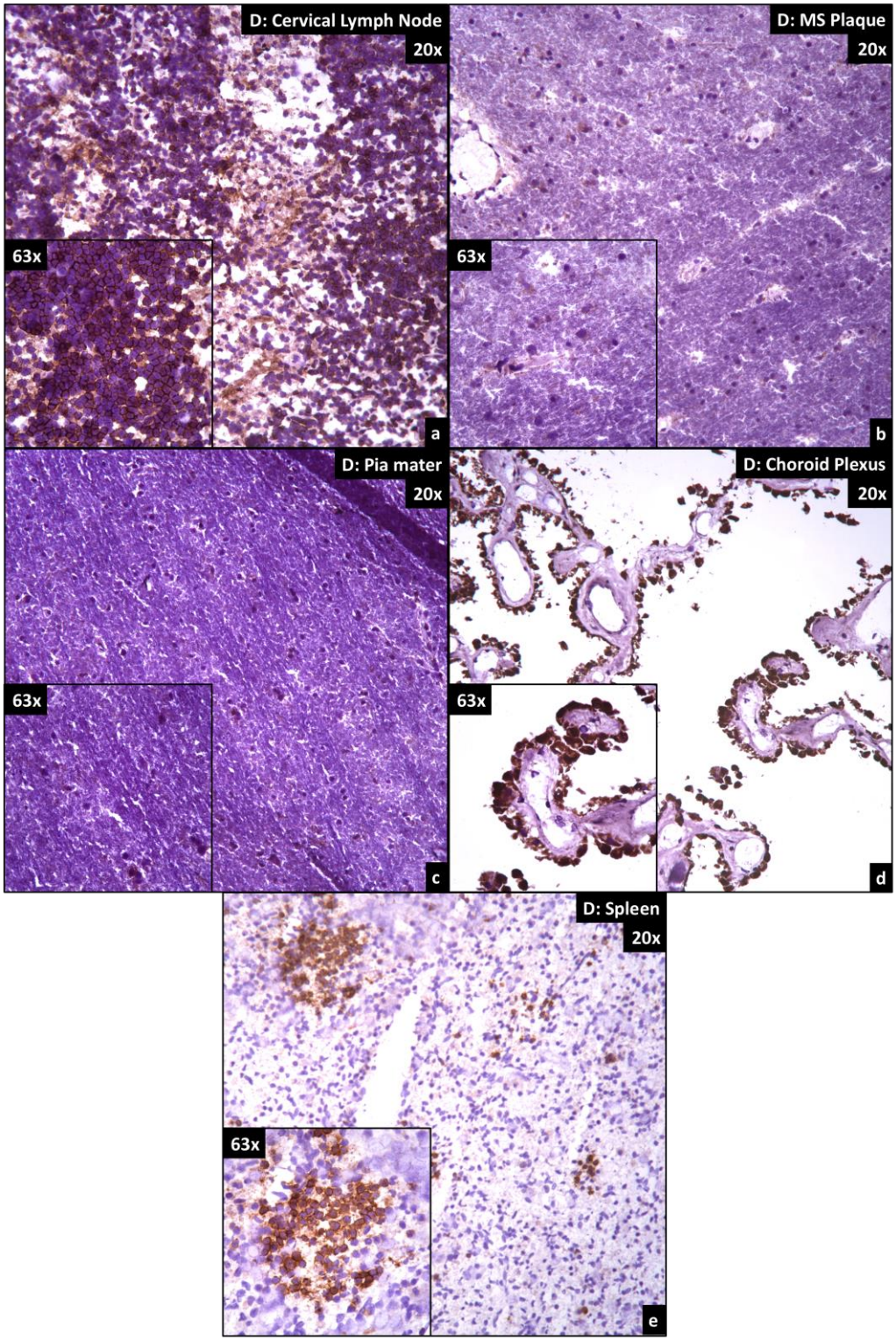

Figure 6 - Immunohistochemical staining for CD20+ B lymphocytes in patient D. (a) cervical lymph node; (b) MS plaque; (c) pia mater; (d) choroid plexus; (e) spleen. 63x magnification inset. High numbers of CD20+ cells were observed in the cervical lymph node, spleen, and choroid plexus, with fewer in pia mater and MS plaque.

B lymphocyte clonal variants are shared among the central nervous system and secondary lymphoid tissue in multiple sclerosis patients.

From traditional Sanger sequencing, 1,336 sequence reads of Ig heavy chains were generated from the five patients. Of these, 670 were identified as unique, productive sequences (666 reads were excluded as null reads, duplicates [e.g. polymerase chain reaction amplification-generated repeats], or unproductive gene rearrangements). The results of the Sanger sequencing, including the total numbers of unique sequences for each patient and tissue type, are summarized in Table 2. The B lymphocytes identified in the CNS were predominantly class switched, had acquired somatic mutations, and had undergone clonal expansion, which serves as evidence of antigen experience. With respect to somatic mutations, a similar number of mutations (in both nucleotides and amino acids) were observed in both the CNS compartments as well as the cervical lymph nodes. Most of the B-cell receptors identified were IgG, which served as further evidence that these cells had experienced antigen. Clonal variants were initially defined strictly, as sequence reads possessing identical CDR3 translated regions. Nucleotide and amino acid mutations were also computed for each clonal variant, and the V, D, and J genotypes were identified. A representative overlapping clonal variant identified through this analysis is depicted in Figure 7.

Table 2 - Results of conventional Sanger sequencing for heavy chain isoforms. Total number of raw sequence reads as well as final numbers of unique sequences after elimination of null reads and PCR amplification duplicates are shown.

Patient ID	Isoform	Anatomical Location	Number of Unique Sequences	Number of Sequence Reads (Total)
Case D	heavy	cervical lymph node	48	72
		choroid plexus	86	212
		cortex (with pia mater)	24	57
		MS plaque	32	102
		spleen	75	94
4106	heavy	cervical lymph node	62	76
		choroid plexus	28	50
		cortex (with pia mater)	32	56
		MS plaque	50	80
4014	heavy	cervical lymph node	46	57
		MS plaque	8	62
3928	heavy	cervical lymph node	100	112
		MS plaque	0	156
3931	heavy	cervical lymph node	35	59
		MS plaque	44	91

V-GENE	D-GENE	J-GENE	CDR3	Isoform	AA mutations	Nucleotide mutations	Anatomical Location
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	21	40	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	5	6	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	4	5	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	6	9	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	5	7	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	4	5	choroid plexus
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	4	5	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	2	3	MS plaque
IGHV4-4*02, or IGHV4-4*03 or IGHV4-4*04 or IGHV4-4*05	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	3	4	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	4	6	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	5	7	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	5	6	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	8	11	MS plaque
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	C A A D T R A M T V F Y W	heavy	3	4	MS plaque

Figure 7 - Representative alignment of clonal variants. V, D, and J gene alleles, translated CDR3 alignment, amino acid and nucleotide mutational analysis, and anatomical tissue locations are shown. The overlapping clonal variants are defined as those with identical CDR3 regions, but different amino acid and nucleotide mutations found in multiple compartments.

Using this strict definition, from patient D, which included the greatest tissue diversity, 265 unique heavy chain sequences were identified, of which 48 sequences were derived from cervical lymph node, 86 from choroid plexus, 24 from the cortex, 32 from MS plaques, and 75 from the spleen. Four clones of identical CDR3 regions were discovered in the cervical lymph node, sixteen clones in the choroid plexus, seven in the cortex, and five in MS plaques. Of these, two CDR3 clones were shared between cervical lymph node and choroid plexus, and three clones were shared between choroid plexus and MS plaque (Figure 8).

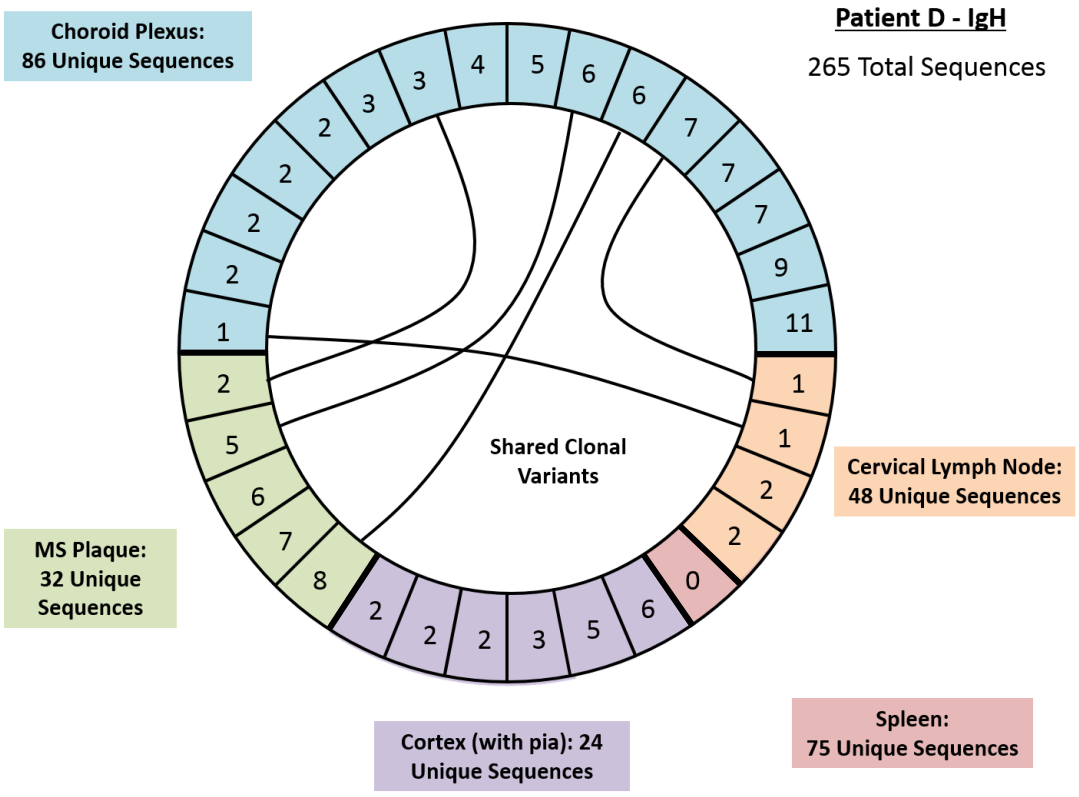

Figure 8 - Overlapping clonal variants were observed in multiple anatomical compartments for patient D. A clone was defined as a cluster of sequences with identical CDR3 amino acid sequences. Clonal variants were members of each clone with identical CDR3 translation but differing mutation patterns in other regions. Each clone is shown as one block in the wheel diagram, with the number of clonal variants detected in each cluster shown inside the block. Blue = choroid plexus, green = MS plaque, purple = pia mater, red = spleen, and orange = cervical lymph node.

From patient 4106, 172 unique sequences were identified, of which 62 were derived from cervical lymph node, 28 from choroid plexus, 32 from cortex, and 50 from MS plaque. Two clones of identical CDR3 regions were discovered in the cervical lymph node, seven in the cortex, five in the choroid plexus, and seven in the MS plaque. Of these, one clone was shared between the MS plaque and cervical lymph node, one was shared between the MS plaque and choroid plexus, one was shared between the MS plaque and cortex, and one was shared between choroid plexus and cortex (Figure 9).

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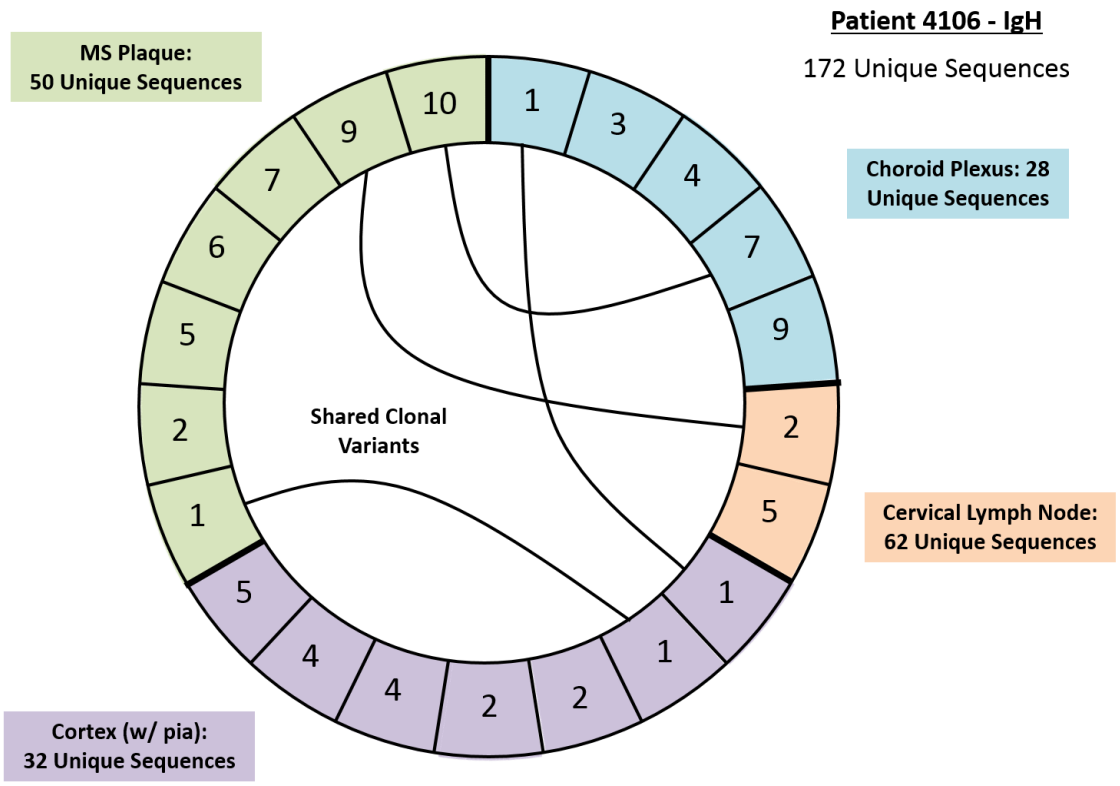

Figure 9 - Overlapping clonal variants were observed in multiple anatomical compartments for patient 4106. Definitions of clonal variants are provided above. Blue = choroid plexus, green = MS plaque, purple = pia mater, and orange = cervical lymph node.

For the remaining patients, only tissue from MS plaques and cervical lymph nodes was available. From patient 3931, 79 unique sequences were identified, of which 35 were derived from the cervical lymph node and 44 from MS plaques. Two clones of identical CDR3 regions were discovered in the cervical lymph node and nine in MS plaque. Of these, one CDR3 clone was shared between the two regions (Figure 10).

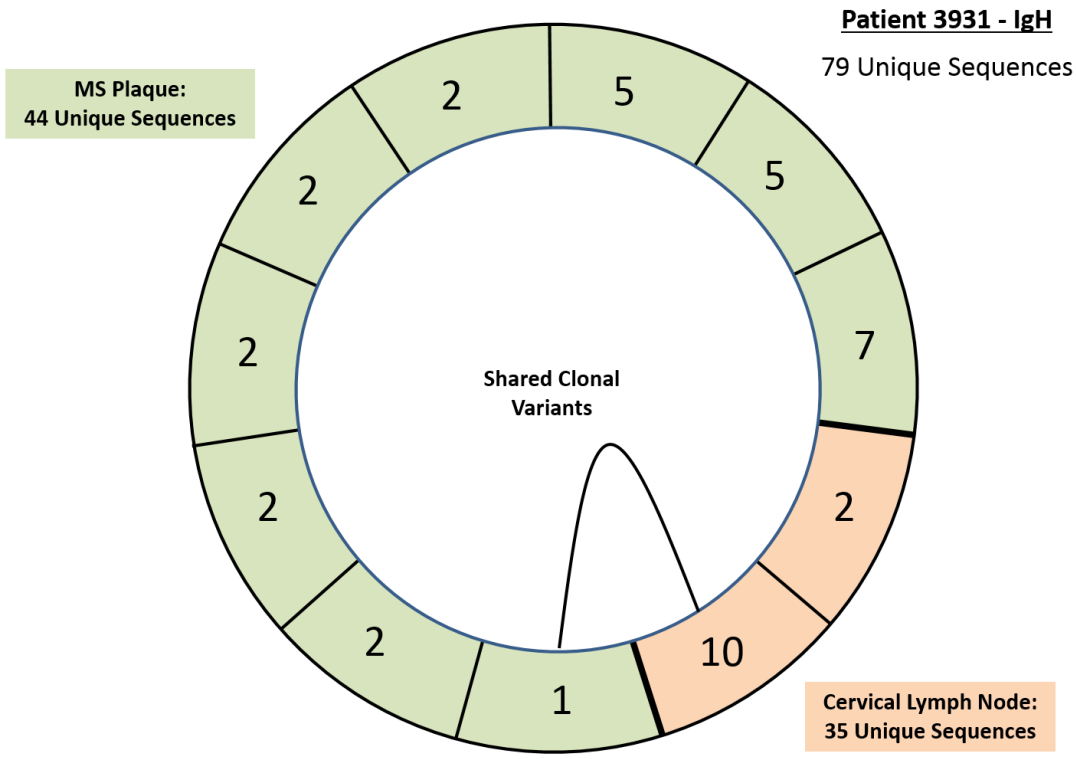

Figure 10 - Overlapping clonal variants were observed in the CNS and periphery for patient 3931. Definitions of clonal variants are provided above. Green = MS plaque and orange = cervical lymph node.

From patient 3928, 100 unique sequences were identified, of which all were derived from the cervical lymph node and none from the MS plaque. Six clones of identical CDR3 regions were discovered in the cervical lymph node. Therefore, no overlaps were observed between the two anatomical compartments, which also correlated with minimal CD20 staining for B lymphocytes by immunohistochemistry. From patient 4014, 54 unique sequences were, of which 46 were derived from the cervical lymph node and 8 from the MS plaque. Eight clones of identical CDR3 regions were observed in the cervical lymph node and two were observed in the MS plaque. No overlaps were observed between the

two anatomical compartments (Figure 11). Most importantly, none of the overlapping sequences were detected in multiple patients.

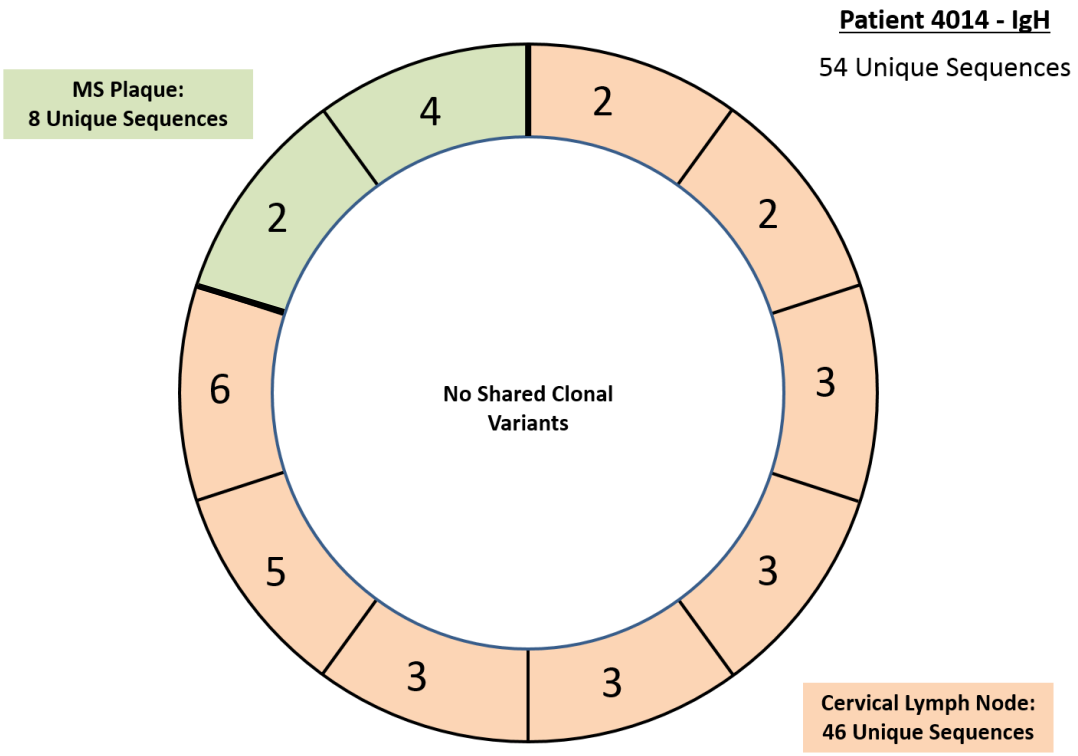

Figure 11 - No overlapping clonal variants were shared for patient 4014. Definitions of clonal variants provided above. No shared clonal variants were identified between the CNS and the periphery of this patient. Green = MS plaque; orange = cervical lymph node.

Traditional Sanger sequencing was also performed for the light chain regions (kappa) of tissue from patient D, however, it was not possible to construct a receptor by pairing heavy and light chains. Sequencing of light chains was also attempted for the remaining patients, however insufficient clones were present for analysis, and the Ig heavy chains alone can uniquely identify B lymphocytes. The light chain clones from patient D are summarized in Table 3. From 317 sequence reads of patient D, 193 unique sequences were identified. 59 were derived from cervical lymph nodes, 38 from choroid plexus, 13 from pia mater, 28 from MS plaque, and 55 from spleen. Of these, one was shared between cervical lymph node and spleen, one between choroid plexus and MS plaque, one between spleen and MS plaque, and one between spleen and pia mater (Figure 12).

Table 3 - Results of conventional Sanger sequencing for light chain (kappa) isoforms. Total number of raw sequence reads as well as final numbers of unique sequences after elimination of null reads and PCR amplification duplicates are shown.

Patient ID	Isoform	Anatomical Location	Number of Unique Sequences	Number of Sequence Reads (Total)
Case D	light (κ)	cervical lymph node	59	72
		choroid plexus	38	97
		cortex (with pia mater)	13	41
		MS plaque	28	43
		spleen	55	64

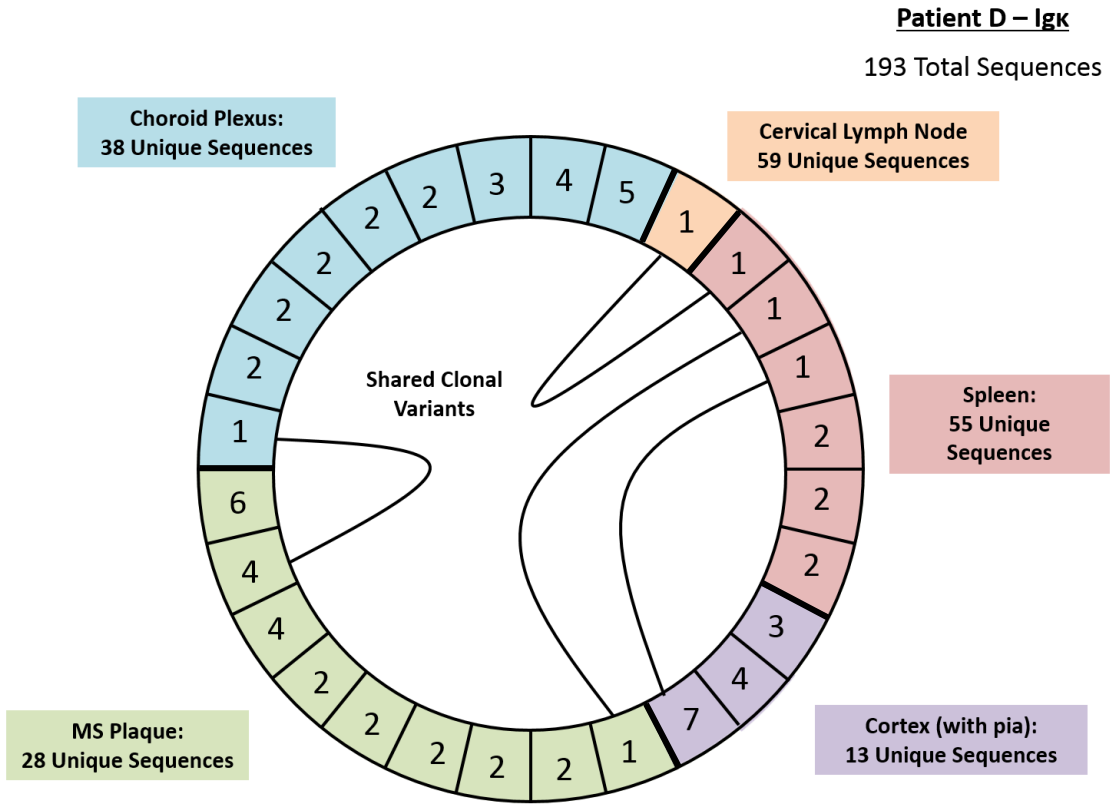

Figure 12 - Overlapping clonal variants for light chain isoforms were observed in multiple anatomical compartments in patient D. Definitions of clonal variants are provided above. Blue = choroid plexus, red = spleen, green = MS plaque, purple = pia mater, and orange = cervical lymph node.

These data were then re-analyzed for three of the patients using a more liberal definition of a clonal variant, in which exact CDR3 identity was not required (e.g. sequence homology was tolerated). Here, for patient D, 14 overlapping clonal variants were identified. Of these, one was shared among the three compartments of spleen, cervical lymph node, and cortex (with pia mater). Five were shared between MS plaque and choroid plexus, one was shared between MS plaque and spleen, four were shared between cervical lymph node and spleen, two were shared between cervical lymph node and choroid plexus, and one was shared between spleen and choroid plexus (Figure 13).

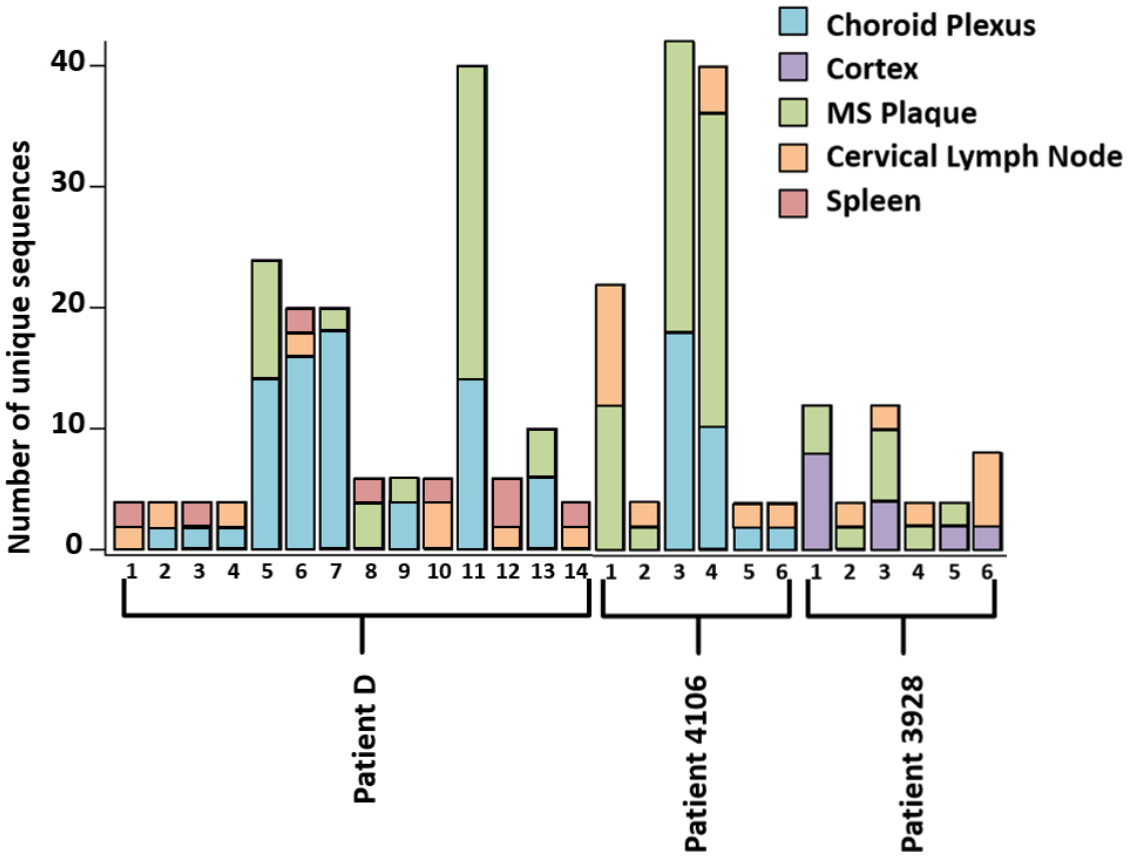

Figure 13 – Broadening the definition of clonal variants reveals greater overlap in multiple anatomical compartments for patients D, 4106, and 3928. Each bar on the graph represents an individual clone (after defining a clone more liberally). The number of unique sequences is depicted on the vertical axis. Ig heavy chain sequences only are shown here. Blue = choroid plexus, red = spleen, green = MS plaque, purple = pia mater, and orange = cervical lymph node.

For patient 4106, six overlapping clonal variants were identified. Of these, one clone was shared among the three compartment of MS plaque, choroid plexus, and cervical lymph node. One clonal variant was present in both MS plaque and choroid plexus, two were shared between MS plaque and cervical lymph node, and two between cervical lymph node and choroid plexus (Figure 13). For patient 3928, six overlapping clonal variants were also identified. Of these, one was shared among MS plaque, cortex, and cervical lymph node. Two were shared between MS plaque and cortex (including pia mater), two between

MS plaque and cervical lymph node, and one between cortex and cervical lymph node (Figure 13).

Overall, therefore, the results of the traditional Sanger sequencing in five patients with multiple sclerosis demonstrated the presence of shared class-switched, clonally-expanded, antigen-experienced B lymphocytes in both the central nervous system (including multiple sclerosis lesions, pia mater, and choroid plexus) and secondary lymphoid tissue (including cervical lymph node and spleen). These results suggested the possibility of free migration of mature B lymphocytes between the CNS and periphery in multiple sclerosis patients.

Discussion

The underlying hypothesis of our work was based on the observation that clonally expanded, antigen-experienced B lymphocytes are present in distinct compartments of the central nervous system of patients with multiple sclerosis, including parenchymal lesions and meninges. We sought to clarify where these B cells encounter antigen and better understand the mechanism of maturation and trafficking. The work presented here demonstrates that mature, clonally expanded B cell variants are indeed shared between CNS and peripheral lymphoid compartments, which lent support to the hypothesis that these cells encountered antigen in peripheral tissues and migrated into the central nervous system, although it did not exclude the possibility that these cells also encounter antigen in the CNS and migrated outward into the periphery.

Limitations of this study

Several limitations to this study exist. For instance, our study drew upon only five patients (although our samples were well-represented with respect to clinical subtypes of multiple sclerosis, including relapsing-remitting, secondary progressive, and primary progressive). Since brain biopsy from living patients is extremely rare for multiple sclerosis, relying on autopsy tissue is a necessity, which by itself limits the number of available samples. In doing so, however, it was necessary to use fixed tissue (as fresh tissue was not available on site), which prevented us from assigning sequence data to particular B lymphocyte phenotypes, as would have been possible by flow cytometric sorting, for example. Direct proof of B lymphocyte trafficking in living patients would require *in vivo* tracking, which is not possible with currently available techniques.

Most notably among the limitations of this study, however, traditional Sanger sequencing represents a low-throughput approach to investigating this question. Each BCR sequence was individually vector cloned, transformed into bacterial cells, cultured, and purified manually for sequencing, to generate a total of approximately 1300 sequences. This method identified, on average, approximately fifty unique sequences per compartment, with the result that we were only able to identify a handful of clonal variants with this approach, particularly when stringent criteria for clonal variants were applied. Indeed, using the strict criteria, no shared clonal variants were identified in one of the patients. We were similarly limited in analysis of migration patterns by this small number. Moreover, in two cases, notably patients 4106 and D, PCR amplification yielded only a minor signal in the lymph node and spleen compared to the MS tissue, which may be the result of variability in processing the tissues. Therefore, while this work was useful in

corroborating our hypothesis, higher-throughput approaches were necessary to fully clarify the role of B lymphocytes, which were subsequently performed by other members of this group and are described below.

Subsequent findings

The methods and results presented above have been recently published by Stern et al. and are readily available [1]. Those findings provided initial corroboration of the hypothesis and foundation for subsequent work performed by others in our group, which I briefly summarize below. The tissue specimens were further characterized beyond the CD20 staining performed by us and identified varying degrees of CD68+ microglial activation and perivascular infiltration with CD20+ B lymphocytes, CD3+ T lymphocytes, and monocytes, without the presence of CD138+ plasma cells [1]. Next, building off of the low-throughput approach presented above, high-throughput next generation sequencing was applied to an additional cohort of specimens in a different geographic location, which generated 32 million raw sequence reads, which in turn reduced to 550,000 high-fidelity sequence reads [1].

As observed using the low-throughput approach, clonal variants were present in both the central nervous system (including MS plaque, meninges, and choroid plexus) as well as peripheral compartments, including draining cervical lymph node [1]. The B cell repertoire generated was also found to be antigen-experienced, populated predominantly by class-switched IgG isotypes. Using the BASELINe algorithm to quantify selection through analysis of mutation patterns, evidence of negative selection in framework regions

and positive selection in complementarity determining regions (CDRs) was identified, demonstrating affinity maturation of these B lymphocytes [1].

Using diversity analysis, it was demonstrated that while the B cell repertoire is shared between the distinct compartments, each compartment had distinguishing features [1]. Lineage trees were then constructed, which showed that both experienced and less mature B cells were observed in both the CNS and the periphery, which suggested that clonal expansion of these B lymphocytes may occur in multiple compartments [1]. In order to determine the origin of these multi-compartment B lymphocyte clones, the distribution of founder compartments was studied, and statistically significant enrichment was observed in the cervical lymph nodes, which suggested a peripheral origin for these expanded B cell clones [1]. The model that arose from this data posits that maturation of B cell lymphocytes occurs in both the central nervous system and the periphery, and also proposes that antigen experience and maturation occurs in secondary lymphoid tissue, with free trafficking among these compartments.

A similar study contemporaneously published by a different group performed deep sequencing of B lymphocytes in peripheral blood and cerebrospinal fluid of multiple sclerosis patients and also observed the presence of class-switched B lymphocytes in the periphery connected to the central nervous system compartment, although it did not examine draining cervical lymph node tissues [76]. Taken together, these two studies and the data presented above strongly suggest the relevance of B lymphocytes to multiple sclerosis pathogenesis.

B lymphocyte trafficking and treatment of multiple sclerosis

As noted above, these findings provide a possible mechanism for the benefit observed in small trials studying the effects of B-cell depleting therapies (rituximab, ocrelizumab, and ofatumumab), as these agents act peripherally and are not known to penetrate the CNS to a significant degree. If antigen-experienced B lymphocytes are indeed freely trafficking between the periphery and central nervous system, it is reasonable that depletion of B cells in the periphery should result in a reduction of the autoimmune response in the central nervous system in these patients.

These findings are also relevant with respect to the therapies that block lymphocyte migration into the central nervous system, notably fingolimod and natalizumab. As described above, fingolimod binds to the sphingosine-1-phosphate receptor and sequesters lymphocytes in the lymphoid organs, preventing migration into the CNS, while natalizumab binds to alpha-4-integrins to prevent lymphocyte adhesion to vasculature and similarly block transmigration. In both of these cases, clinical benefit is tied to inhibition of lymphocyte entry into the CNS, and their efficacy is at least partially explained by the proposed model of free B lymphocyte trafficking between the CNS and the periphery (assuming that B cells are indeed pathogenically involved).

Future directions

Having demonstrated that B cell clonal variants are present in both the central nervous system and secondary lymphoid tissue of multiple sclerosis patients, a critical question remains in establishing precisely how these B lymphocytes contribute to pathogenesis of the disease. Of note, even in the high throughput sequencing experiments

described, the number of patients was still limited to five. In order to clarify these migratory and maturation pathways across different clinical subtypes, much larger studies in many more patients will need to be carried out. In particular, the evidence base for B cell-depleting therapies in multiple sclerosis remains highly limited, and large, randomized trials would provide the gold standard for the clinical relevance of the mechanisms described here.

Moreover, as described above, antibodies in the form of cerebrospinal fluid oligoclonal bands continue to be routinely measured to support the clinical diagnosis of multiple sclerosis, however the antigen specificity of these antibodies has never been conclusively determined. It is possible that these antibodies target multiple antigens that are released in the course of tissue damage in the development of multiple sclerosis [1]. In addition, the chronic nature of the disease may mean that a persistent B cell infiltrate is present, representing ongoing affinity maturation instead of single, well-targeted antibodies [1]. The presence of this chronic autoimmune state may even indicate a chronic infection [1]. Future work will therefore require the use of recombinant antibodies from the B cell clones identified here in order to screen for potential antigens. Candidate antigens include a diverse population extending far beyond small peptides, such as post-translationally modified proteins, lipids, and possibly other components of microbes.

In summary, the data presented here and subsequent findings published in Stern et al. [1] demonstrate that clonal variants of B lymphocytes are shared between the central nervous system and peripheral lymphoid compartments of multiple sclerosis patients across several clinical subtypes, may predominantly mature in the periphery, and freely traffic back and forth between these compartments. These findings shed light on the

mechanism of current therapeutics for multiple sclerosis and may open up new avenues of treatment for this debilitating neurological illness.

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