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Editor's Summary

B Cells Flip the Switch for MS

B cells in multiple sclerosis (MS) patients may mature outside the central nervous system (CNS). Two complementary studies report that B cells found outside the CNS—in peripheral blood and draining cervical lymph nodes (CLNs)—share antigen specificity with intrathecal B cell repertoires. In patients with MS, immune cells attack the CNS; however, it remains unclear whether these cells mature in the CNS or traffic to the CNS as mature cells. Using paired tissues and high-throughput sequencing, Stern *et al.* found that clonally expanded B cells are found in both the CNS and CLNs but that founding members were more often found in the draining CLNs. Palanichamy *et al.* extend these findings by reporting a peripheral blood/CNS axis of mature B cells that have undergone class switch. These data support the therapeutic use of monoclonal antibodies that prevent lymphocytes from crossing the blood-brain barrier or induce peripheral B cell depletion in MS patients.

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MULTIPLE SCLEROSIS

B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes

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Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by autoimmune-mediated demyelination and neurodegeneration. The CNS of patients with MS harbors expanded clones of antigen-experienced B cells that reside in distinct compartments including the meninges, cerebrospinal fluid (CSF), and parenchyma. It is not understood whether this immune infiltrate initiates its development in the CNS or in peripheral tissues. B cells in the CSF can exchange with those in peripheral blood, implying that CNS B cells may have access to lymphoid tissue that may be the specific compartment(s) in which CNS-resident B cells encounter antigen and experience affinity maturation. Paired tissues were used to determine whether the B cells that populate the CNS mature in the draining cervical lymph nodes (CLNs). High-throughput sequencing of the antibody repertoire demonstrated that clonally expanded B cells were present in both compartments. Founding members of clones were more often found in the draining CLNs. More mature clonal members derived from these founders were observed in the draining CLNs and also in the CNS, including lesions. These data provide new evidence that B cells traffic freely across the tissue barrier, with the majority of B cell maturation occurring outside of the CNS in the secondary lymphoid tissue. Our study may aid in further defining the mechanisms of immunomodulatory therapies that either deplete circulating B cells or affect the intrathecal B cell compartment by inhibiting lymphocyte transmigration into the CNS.

INTRODUCTION

Multiple sclerosis (MS) is a multifocal demyelinating disease caused by an autoimmune response in genetically susceptible individuals (*1*). Although animal models of autoimmunity have long suggested a critical role for T cells in disease pathogenesis, it has become clear that the autoimmune response is mediated by a number of cell types. B cells in particular appear to be of fundamental importance in MS (*2*). B cell subsets participate in the production of the hallmark MS cerebrospinal fluid (CSF) oligoclonal bands (OCBs), and more recent seminal findings, which implicate B cells in the disease, include B cell clustering both at the site of central nervous system (CNS) tissue injury (*3*) and the meninges (*4*). Furthermore, several MS autoantibody specificities (*5*, *6*) have recently been reported. B cell depletion has emerged as a beneficial therapeutic approach for MS (*7*). The ENCODE study (*8*) implicated B cells second only to T cells among the cell types affected by MS susceptibility genes. Finally, their role as both effective antigen-presenting cells (APCs) and immune response regulators (*9*) in autoimmunity has been reported.

Within the CNS of patients with MS, B cells can be observed in distinct compartments including white matter lesions, the normal-appearing white matter, the cortex, the CSF, and the meninges (*10*, *11*). B cells found in the meninges often organize into structures resembling those found in lymphoid tissue (*4*, *12*). The B cells that populate these distinct compartments of the CNS form a network of clonally related cells (*10*). Intraclonal variants, which represent steps in the antigen-driven affinity maturation process, are also present in MS CNS compartments (*10*, *13*). OCBs are produced, at least in part, by these resident B cell clones (*14*). Furthermore, it is now appreciated that B cell clones present in the CSF are also represented in the blood (*15*) and that isotype G immunoglobulins (IgGs) representing OCBs are linked to circulating peripheral B cells (*16*).

Although many characteristics of the B cells populating the CNS are now understood, it is not known whether these B cells experience maturation outside of the CNS and then traffic within the brain or whether the process is exclusively confined within the CNS. A further understanding of this process would help clarify whether MS is primarily a disease of the CNS or whether lymphocytes activated in the peripheral immune system drive the disease. This is of particular importance considering that some of the most effective MS therapies either deplete circulating B cells (anti-CD20; rituximab, ocrelizumab, and ofatumumab) or affect the intrathecal B cell compartment by inhibiting lymphocyte transmigration into the CNS (anti–VLA-4; natalizumab).

We reasoned that CNS B cells in patients with MS may gain antigen experience and mature in lymph nodes associated with

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the CNS, namely, the cervical lymph nodes (CLNs) that drain the brain tissue. Both neuronal and myelin-derived antigens are present in the draining CLNs (*17*, *18*). This suggests that immune responses in the CNS can be organized and/or initiated in the periphery and implicate the CLNs among the anatomical sites at which such responses materialize. In support of this, surgical removal of cervical and lumbar lymph nodes reduces relapse burden in the commonly used model for MS, experimental autoimmune encephalomyelitis (*17*).

B cell antibody repertoires from a series of paired tissues representing both the CNS and secondary lymphoid organs were constructed so that we could identify and reconstruct B cell lineages across tissues. Founding members of clones were more often found in the draining CLNs, whereas more mature members derived from these founders were observed in both the draining CLNs and the CNS. These data provide new evidence that B cells traffic freely across the tissue barrier, with most B cell maturation occurring outside of the CNS in the draining secondary lymphoid tissue.

RESULTS

Characterization of MS tissue specimens used to build B cell antibody repertoires

We studied the B cell antibody repertoire of both CNS-derived and secondary lymphoid tissue (draining CLNs) in autopsy tissue derived from five subjects with MS (Table 1). The brain parenchyma was evaluated by immunohistochemistry to confirm the presence of demyelinated white matter lesions and immune infiltrates. None of the lesions exhibited active demyelination as characterized by the presence of myelin-phagocytosing macrophages. However, the lesions contained varying degrees of microglial activation ($CD68^+$) and perivascular infiltrations including B cells ($CD20^+$), T cells ($CD3^+$), and monocytes ($CD68^+$) (fig. S3). $CD138^+$ plasma cells, which often accompany B cells in MS lesions (*19*), were not observed.

Related B cells populate the CNS and secondary lymphoid tissue compartments

To explore whether the CNS and peripheral tissues harbored B cells that were related to one another, we initially performed conventional Sanger sequencing of variable region heavy chain and variable region light chain antibody repertoires from serially sectioned tissue specimens from subject M1. We obtained about 1300 sequence reads, which reduced to 754 sequences used for analysis when polymerase chain reaction (PCR) generated repeats were removed. We identified a total of nine clonal lineages populating both the CNS and peripheral secondary lymphoid tissue using this low-throughput approach (fig. S4).

We then applied high-throughput, next-generation sequencing to an additional cohort of specimens to validate our observations, provide a broader examination of the MS B cell repertoire that spans the periphery and CNS, and determine the pattern of B cell traffic across the blood-brain barrier (BBB). Eleven tissue specimens from four subjects (M2 to M5) were serially sectioned, and each was divided into two or three independent heavy chain repertories. About 32 million total raw sequence reads were collected from the 32 separate repertoires. Quality control processing and selection of unique reads that each represent a single mRNA molecule reduced the data to about 550,000 high-fidelity sequences for analysis (table S2). Sequences derived from subject M4 were not included in subsequent analysis because the number of filtered unique sequences derived from the lesion was too low to provide meaningful interpretation.

In subjects M2, M3, and M5, we found that members of clones (and intraclonal variants) resided in the secondary lymphoid tissue and CNS compartments, including lesions, choroid plexus, and pia mater (Fig. 1A). Representative alignments of clones, including intraclonal variants that populated distinct compartments, are shown in fig. S5. The fraction of sequences shared between the CNS tissue and draining CLNs was unambiguous. For example, 12.5% of the individual B cell IgG sequences in the lesion tissue from subject M5 were also represented in one CLN (CLN-A), and 15.3% were represented in the other (CLN-B). Similarly, 6.7 and 7.6% of the individual B cell IgG sequences in the lesion were also represented in the CLNs of subjects M2 and M3, respectively. We also applied this analysis to sequences derived from different subjects. The mean sequence overlap was 0.36% for IgG, 0.06% for IgM, and 0.1% for IgA, further highlighting the significance of the overlap we found between the CNS and CLNs. In subjects M3 and M5, most sequences from clones that included CNS members resided in the CLNs (Fig. 1B). In subject M2, this distribution was not as apparent because the total number of multicompartment clones was lower than that of M3 and M5.

The CNS B cell repertoire is populated with antigen-experienced cells

In agreement with previous reports (*10*, *13*, *20*, *21*), the MS CNS B cells were characteristic of a post-germinal center reaction in that most had class-switched, acquired somatic mutations and expanded clonally, all of which are representative of antigen-driven selection. The

Table 1. Subject demographics and cellular characteristics of the CNS tissue.

Subject	Disease state	Age	Gender	Histopathologic findings
M1	Relapsing-remitting MS (RRMS)	39	M	Normal-appearing white matter.
M2	Secondary progressive MS	53	F	Small chronic periventricular white matter lesion without both perivascular infiltrations and microglial activation.
M3	Primary progressive MS	74	F	Small chronic white matter lesion with perivascular infiltrates and microglial activation.
M4	Chronic progressive MS	80	M	Small chronic periventricular white matter lesion without both perivascular infiltrations and microglial activation.
M5	Chronic MS	63	F	Large chronic white matter lesion with occasional minimal perivascular infiltrates. No microglial activation.

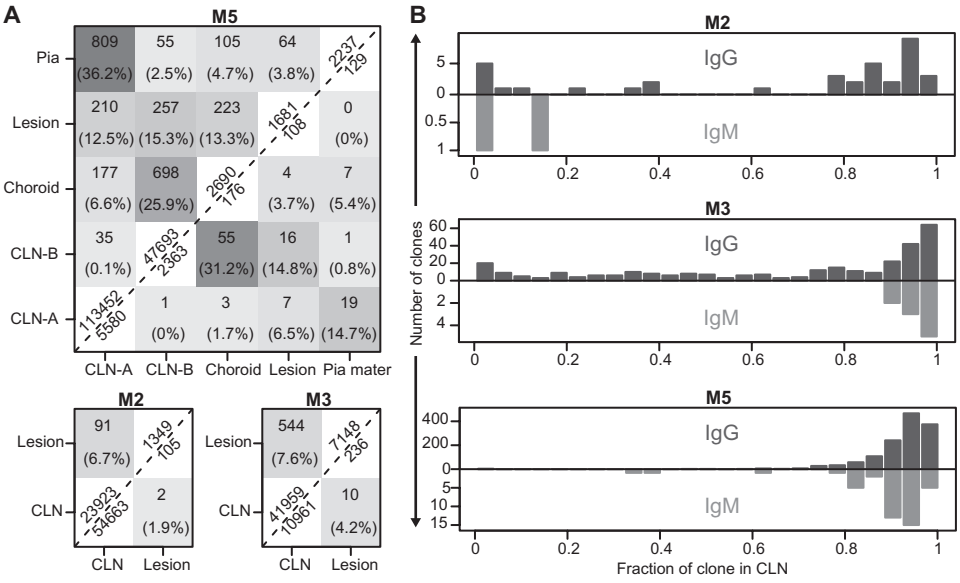

Fig. 1. B cell antibody repertoire sequencing demonstrates that clonally expanding B cells populate the MS CNS and draining CLNs. (**A**) Heat maps showing the sequence overlap between compartments. The number of unique IgG (upper left triangles) and IgM (lower right triangles) sequences that were shared between each pair of tissue compartments in subjects M5, M2, and M3 is displayed. Percentages are calculated as a fraction of sequences from the tissue with the lower number. Values on the transecting axis indicate the total number of distinct sequences collected from each tissue. (**B**) Histograms showing how members of clones were distributed between the CNS and CLN. For each IgG (upper *y* axis) and IgM (lower *y* axis) clone with at least one member found in the CNS, the fraction of distinct mRNA sequences that reside in the CLN is shown.

isotype distribution (table S3) in the CNS from each of the four subjects was predominated by IgG. A fraction belonged to the IgM isotype, and each lesion included a noticeable fraction of IgA ranging from 3 to 13%. The isotype distribution in the choroid plexus specimen from subject M5 was 72, 5, and 23% for IgG, IgM, and IgA, respectively. CLNs expectedly included all three isotypes with IgG or IgM preferentially distributed.

The accumulation of somatic mutations was also evident. The mutation frequency in the sequences (number of nucleotide and amino acid mutations accumulated per Ig sequence) was similar in the distinct CNS compartments and the CLNs. This finding was consistent in sequences belonging to clones with multiple members (fig. S6A) and singletons (fig. S6B). The quantity of mutations was consistent with the antigen experience of mature B cell populations observed in other settings (*22*, *23*). To further confirm that the CNS B cells were antigen-driven, we used an algorithm (BASELINe) that quantifies selection by analyzing mutation patterns in experimentally derived Ig sequences (*24*). This analysis showed evidence of negative selection in the framework regions and positive selection in the complementary determining regions (fig. S7), which are both characteristics that are consistent with affinity-matured B cells.

Further highlighting the antigen experience of the CNS repertoire, most B cells were distributed into clones (Table 2). Both the CLNs and CNS compartments included clonally expanded B cells, with many representing more than half of all the B cells. Whereas the class-switched IgG and IgA isotypes most often represent antigen-experienced B cells, the IgM isotype can represent either naïve B cells or a population of nonswitched, experienced memory cells. We found that IgM sequences were often parts of clones with noticeable evidence of expansion (Table 2). We next examined the mutational load that the IgM sequences carried. IgM sequences that were not part of expanded clones had considerably fewer mutations than those belonging to expanded clones (fig. S6), suggesting that both switched and nonswitched memory B cells populate the MS CNS.

The CNS and lymphoid B cell repertoires are distinct

Given that we found considerable overlap between the repertoire in the CNS and the periphery, we sought to determine whether the sequences from each compartment displayed unique features. We first assessed Ig heavy chain variable (*IGHV*) family and Ig heavy chain joining (*IGHJ*) gene usage by calculating risk ratios for each gene segment based on the entire repertoire from each subject. This analysis showed that particular *IGHV* families and *IGHJ* genes were found more often than expected in the CNS and in other instances less frequently than expected (Fig. 2, A and B). Disproportionate *IGHV* family usage has been observed in MS CNS/CSF-derived sequence libraries; the *IGHV4* family is particularly overrepresented (*25*, *26*). We did find *IGHV4* family members enriched in the CNS relative to the CLN of M3 and M5, but not M2 (Fig. 2A and figs. S8 and S9). The usage of *IGHJ* genes (Fig. 2B and fig. S10) was heavily biased toward *IGHJ4* in both the CNS and CLNs. The overuse of *IGHJ4* is consistent with other reports of such bias in the CNS of patients with MS (*21*) and circulation of healthy individuals (*27*), and as such was not a distinguishing characteristic of MS-associated B cells.

Although it is certain that the repertoires of distinct compartments overlap, many clones were present only in a single compartment. The B cell repertoire in the CNS was more focused compared to that in the CLN (Fig. 2C), in terms of both fewer total clones (Hill diversity index with $q = 0$) and less dominance by larger clones (Hill diversity index with $q > 1$). Overall, these results indicate that the B cell repertoire is shared between these distinct compartments but that each compartment has exclusive features.

Mature B cells traffic between the CNS and secondary lymphoid tissue

To gain further insight into how the individual products of affinity maturation were distributed between all of the tissue compartments, lineage trees of the clones were constructed. These trees (Fig. 3) indicate that the members of the different clones trafficked between the CNS and periphery. Both less mature and more experienced offspring were observed in the CNS and secondary lymphoid organs. This suggests that the products of B cell maturation steps are not restricted to a single compartment, but rather circulate during the process, with the possible implication that clonal expansion of B cells occurs in multiple compartments.

Table 2. Distribution of B cells belonging to clone.

Subject	Isotype	Tissue compartment	Total sequences	B cells belonging to a clone (%)
M2	IgA	CLN	15,235	49
	IgA	CNS	159	40
	IgG	CLN	24,478	60
	IgG	CNS	1,434	54
	IgM	CLN	54,896	13
	IgM	CNS	108	45
M3	IgA	CLN	9,605	82
	IgA	CNS	285	32
	IgG	CLN	48,023	94
	IgG	CNS	7,803	95
	IgM	CLN	11,704	61
	IgM	CNS	237	7
M4	IgA	CLN	11,598	75
	IgA	CNS	1	0
	IgG	CLN	71,709	89
	IgG	CNS	28	21
	IgM	CLN	20,125	48
	IgM	CNS	9	22
M5	IgA	CLN	17,072	74
	IgA	CNS	1,534	77
	IgG	CLN	190,980	89
	IgG	CNS	7,219	71
	IgM	CLN	8,896	55
	IgM	CNS	435	52

Ancestral clonal members reside in multiple compartments

Given that we found clones carrying evidence of affinity maturation in both the periphery and CNS, we next examined whether we could determine the direction of the traffic flow by examining the steps of the maturation process in distinct compartments. The lineage trees from expanded clones (>10 unique sequences) that included sequences from the CNS compartment (39 trees for M2, 335 trees for M3, and 1516 trees for M5) were collectively examined to determine the mutation profiles of B cells that populated multiple or single compartments. By analyzing the topology of the lineage trees, we found that B cells that resided in a single compartment tended to have maturation profiles with fewer downstream mutations than those that were found in multiple tissues (Fig. 4A), whereas B cells found in both the CNS and CLN demonstrated a propensity to give rise to multiple immediate daughter cells. That is, when considering B cell clonal lineages that span multiple compartments, B cells that resided in both the CNS and CLN behaved as ancestors and tended to produce progeny that underwent further rounds of somatic hypermutation. Conversely, their counterparts residing in a single tissue compartment often represent terminal nodes of the lineage tree. Figure 4B further illustrates the ancestral character of multicompartment B cells by showing that these B cells are more likely to be observed as ancestors than expected by chance. Together, this implies that local expansion of B cell populations regularly occurs after trafficking between compartments.

Clonal lineages that span multiple compartments likely originate in lymphoid tissue

To determine the origin site of multicompartment B cell clones, we analyzed the distribution of founder compartments. For each lineage tree that contained both CNS and CLN sequences, the founder compartment was defined by the node(s) with the fewest number of mutational events (that is, appearing closest to the germline sequence). We found that multicompartment B cells, despite their low abundance (2.7 to 3.7% of sequences), were commonly founders (18.7 to 45.0% of founders), with statistically significant enrichment of founder occurrence at $P < 3.7 \times 10^{-5}$ for all three subjects combined (Table 3). Because the origin of multicompartment B cells is ambiguous, we then looked at only the trees with founders that could be unambiguously assigned to a single compartment. We observed that most such founders were from the CLN (87.6 to 93.9% of single-compartment founders) and that the observed number of CLN founders was greater than expected by chance (taking into consideration the fact that most sequences are CLN) with statistically significant enrichment at $P < 4.3 \times 10^{-5}$ for all three subjects combined (Table 3). Although many founders were highly mutated, 18 lineage trees contained single-compartment founders that carried unmutated *IGHV/J* segments (all from subject M5, shown in fig. S11). In every such lineage tree, the unmutated founder was derived from the CLN (Table 3). Overall, these data provide evidence for a peripheral origin of the expanded clones found on both sides of the BBB.

Model proposal: B cells primarily mature in the periphery and then migrate to the CNS

Antigen-experienced, clonally expanded B cells reside in both the CNS and secondary lymphoid organs (Fig. 5 and fig. S12). The products of B cell antigen-driven affinity maturation traffic between the CNS and draining secondary lymphoid tissue through an ongoing dynamic process. B cells actively experiencing affinity maturation traffic between the draining CLNs and CNS compartments. More evolved clonal members, which represent the products of maturation, are more stationary because their residence is restricted to single compartments. However, less mature products (founders or ancestors) are shared between multiple compartments and more often originate in the peripheral lymphoid tissue. This model proposes that B cell maturation is not confined to the MS CNS but occurs in both the periphery and CNS and further proposes that antigen-driven maturation originates in the periphery.

DISCUSSION

The CNS of patients with MS harbors expanded clones of antigen-experienced B cells that reside in distinct compartments including the meninges, CSF, and parenchyma. It remains unclear where MS CNS B cells encounter antigen and develop the characteristics that accompany post-germinal center experience. We address this fundamental question regarding the mechanism of B cell maturation and

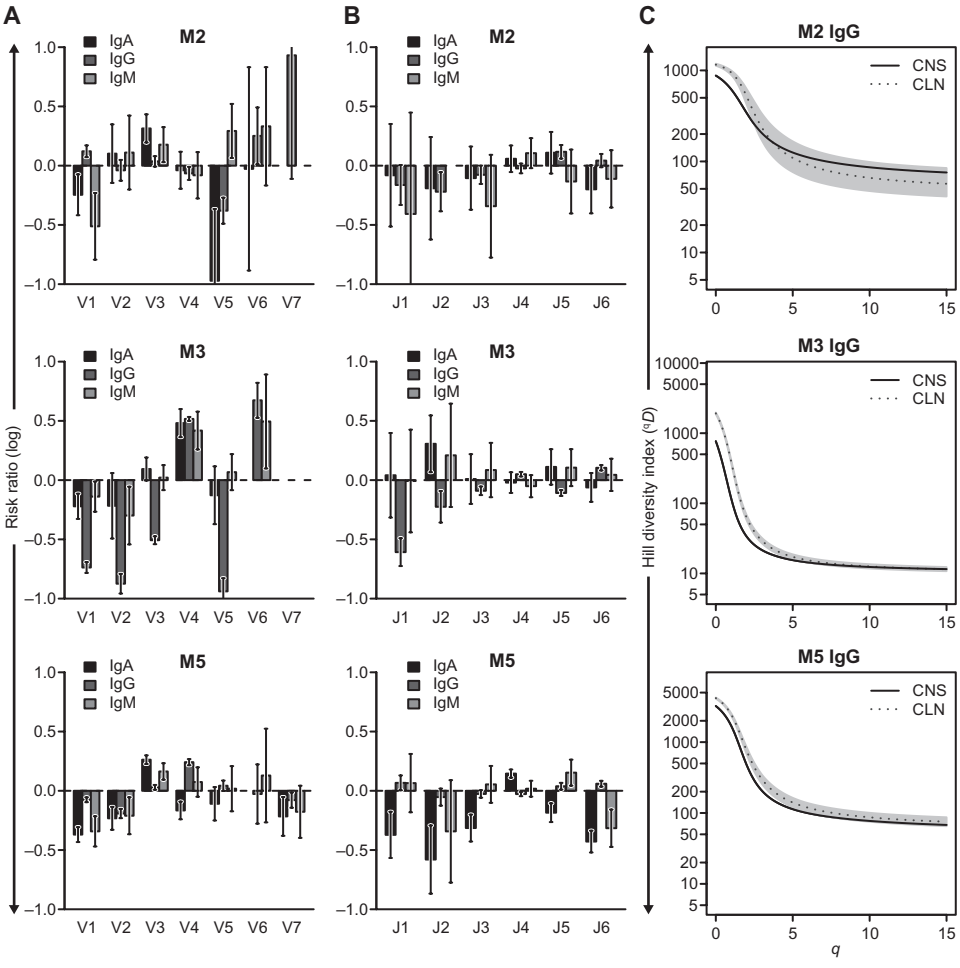

Fig. 2. The antibody repertoire in the CNS and CLN share features. (**A** and **B**) Usage of both *IGHV* families (A) and *IGHJ* genes (B) was compared between the CNS and CLN. For each subject, the risk ratio (log) was calculated by measuring the distribution of the *IGHV* families in the CNS and normalizing it by the distribution in the periphery for the same subject. Positive values indicate that the observed segment usage is greater in the CNS, and negative values indicate greater usage in the periphery. (**C**) The Hill diversity index (^qD), which measures diversity in a population, was calculated for the set of IgG clones present in the CNS (solid black line curves) and CLN (dotted curves) to determine whether the diversity in the repertoires differed between the compartments. For each subject (shown in different panels), the repertoire was subsampled to the number of sequences in the smallest sample, and the Hill diversity index was calculated independently in 1000 equally spaced q values between 0 and 15. Because q varies from 0 to infinity, the diversity (^qD) depends less on rare species and more on common ones as q increases, thus encompassing a range of definitions that can be visualized as a single curve. For $q = 0$, the diversity is defined as the total number of clones. As q approaches infinity, the diversity is given by one over the frequency of the largest clone. At a given value of q (x axis), lower values of ^qD (y axis) indicate lower diversity. The gray bands indicate the middle 95% percentiles of the sampled distribution; thus, when both lines are separated and fall outside of the gray band, the difference between the two is significant. The analysis shows that the CNS repertoire had lower ^qD values than those in the CLN, demonstrating lower diversity in the CNS (that is, a more focused repertoire).

trafficking in the MS CNS. We have defined both the characteristics of B cells populating the MS CNS and their relationship to the secondary lymphoid organs. B cells residing in the MS CNS are largely antigen-experienced because they display the principal characteristics of mature effector B cells. Members of these clones are not exclusively confined to the CNS but also occupy secondary lymphoid organs, which directly drain the CNS. Furthermore, these multicompartment clonal members appear to circulate between the CNS and periphery, with most parental clonal members arising in the periphery. These data collectively suggest that MS CNS B cells encounter antigen and gain experience in secondary lymphoid tissue. These B cells then dispatch colonists that populate the CNS and then continue trafficking between the CNS and periphery. Although our data implicate peripheral compartments as the initial site for most B cell maturation, we do not exclude that maturation may continue to occur within the CNS. B cells populating the meninges often form organized structures that emulate germinal centers present in lymph nodes (*3*, *4*, *12*). It is conceivable that such structures are those in which CNS-resident B cells encounter antigen and mature. Rare tissue collections that include these structures, paired with both lesions containing B cells and peripheral lymphoid tissues, present an ideal setting for the study of their contribution.

Much of our previous work and that of others have relied on autopsy tissue (*4*, *10*, *28*) for the examination of immune cell infiltrates in MS CNS tissue. Biopsy tissue from living patients is rarely acquired, and the small size of tissue considerably limits investigations. Historical archives include fixed tissue rather than fresh-frozen. Contemporary deep sequencing approaches cannot be reliably performed using fixed tissue. Fresh-frozen autopsy tissue is typically derived from older individuals with an advanced and progressive form of MS. Given that the repertoires here were constructed from autopsy-derived tissue, we were not able to assign sequence data to particular B cell phenotypes. Such approaches would be possible through flow cytometry–based sorting of fresh samples before sequencing. We are limited from extending the interpretation of our data to all types of MS given that most of our tissues were derived from subjects in the progressive stage. However, we did include a single case of RRMS in a younger subject. Furthermore, a recent study (*15*) demonstrated that the CSF B cell population is linked to B cells in the peripheral blood in younger patients with early RRMS. This study and ours together indicate that B cell traffic, both in and out of the CNS, can occur both at early and later stages of the disease. Although the multicompartment clonal lineages provide clear evidence that the clonally expanded B cells that populate the CNS are related to B cells in the peripheral lymphoid

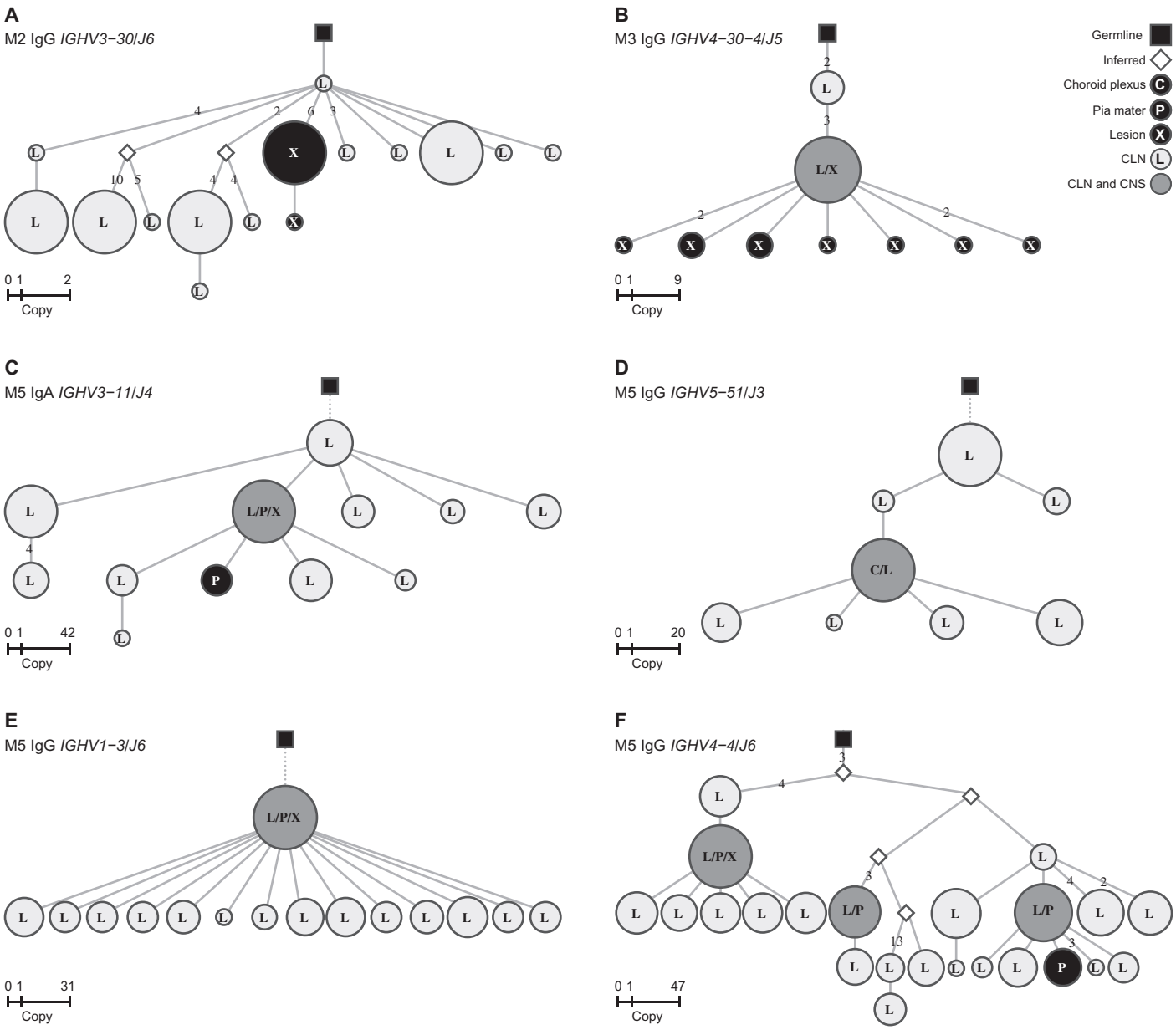

Fig. 3. Multicompartment lineage trees illustrate that B cells traffic between the CLN and CNS. (**A** to **F**) Representative lineage trees are shown for subjects M2 (A), M3 (B), and M5 (C to F). Lineage trees are shown with the germline sequence at the root (black square). The number of somatic mutations accumulated from one node to the next is shown on each edge (branch) of the tree; an unlabeled edge corresponds to either 1 mutation (solid line) or 0 mutations (dotted line). Observed unique B cell sequences (nodes of the tree) are each annotated with text representing the tissue in which they were observed. The size (area) of each node is proportional to the number of unique mRNA sequences (number of unique molecular identifiers) identified with the same nucleotide sequence.

tissue, the conclusions concerning the directionality of B cell trafficking are based on a statistically significant enrichment. Direct proof for the model proposed herein would require in vivo tracking of individual B cells in humans, which cannot be performed with current technology.

The role of B cells in the MS CNS

It is not entirely clear how B cells contribute to MS pathology. The major effector functions of B cells include antibody production, antigen presentation to T cells, and cytokine production. B cells can express proinflammatory cytokines such as interleukin-6 (IL-6), IL-12, tumor necrosis factor, and lymphotoxin and can perform an anti-inflammatory regulatory role through expression of IL-10 (*29*). In the context of the MS CNS, it is not known whether they function as APCs or produce pro- or anti-inflammatory cytokines. Moreover, it is not entirely clear how B cell subsets populate the MS CNS. Memory B cells are present, and they share the space with plasmablasts and plasma cells. Naïve cells, based on collective antibody sequencing repertories, appear to be a very minor fraction.

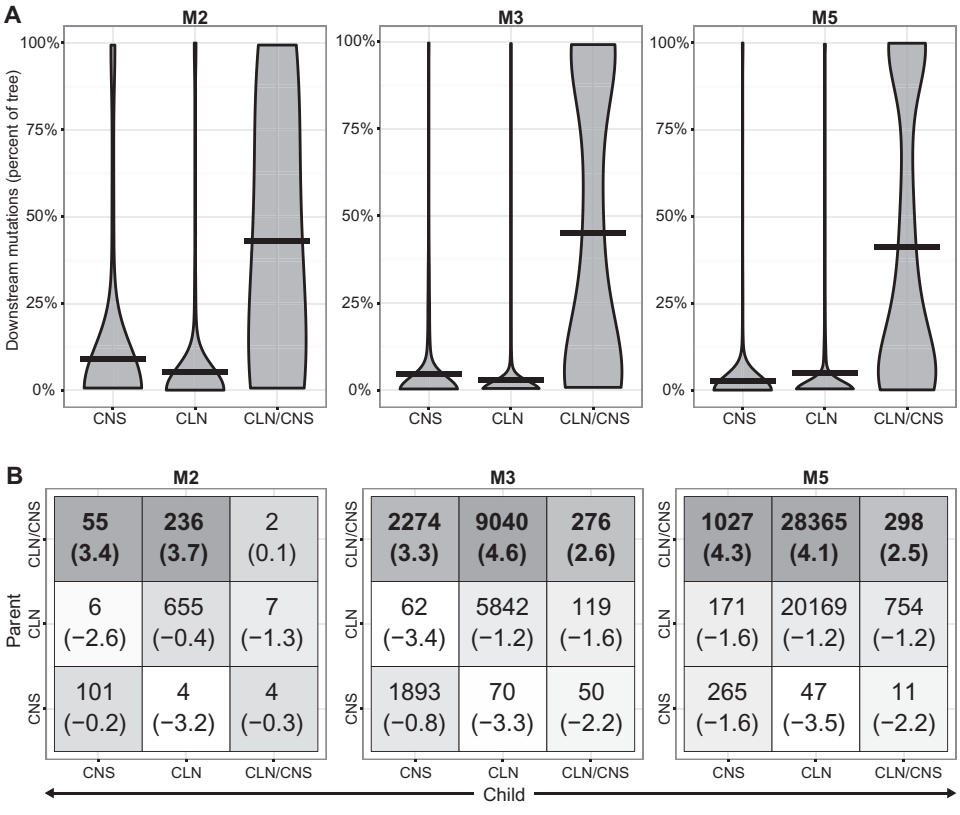

Fig. 4. Trafficking between CNS compartments and the CLN often occurs early in clonal expansion. Sequences were classified according to where they resided; CNS, CLN, or CLN/CNS (multicompartment) and lineage trees were constructed for all clones that contained at least one CNS or multicompartment sequence. Two separate analyses demonstrate that multicompartment B cells are more often observed as ancestors than those that resided in a single compartment. (**A**) For each lineage, the fraction of somatic mutation events accumulated by progeny of CNS, CLN, or multicompartment sequences was determined. The distribution over all lineages, along with the mean fraction (black horizontal bar), is shown as a violin plot for each subject. Increasing values on the *y* axis represents the propensity of a clonal member to produce offspring. The width of the plot for the CNS, CLN, or multicompartment sequences is proportional to the fraction of clonal members that produced descendants. A column that is wide only on the bottom indicates that few ancestors were among the clones. A column that is wide through its height indicates that many of the clonal members were ancestral because they produced further ancestors. (**B**) The set of all expanded (>10 unique sequences) multicompartment lineage trees from each subject was analyzed to identify direct parent-child relationships among sequences from different compartments (numbers in each box). Statistically significant relationships, determined through permutation of node compartment labels (n = 2000), are indicated in bold text. Numbers in parentheses indicate the effect strength, defined as the base 2 logarithm of the number of observed over the number of expected edges (branch in a lineage tree) of each type. These values designate whether the observation is more than (positive) or less than (negative) expected. Each edge type is shaded according to this effect strength, with darker colors indicating more edges (branches) than expected. Thus, the positive effect strength values observed only in the cases in which the parent node resided in two compartments indicate that these shared sequences more often gave rise to daughters than those restricted to a single compartment.

It has been demonstrated that B cells present in MS tissue and CSF contribute to production of antibodies (*14*, *30*). Although these antibodies may be disease-related and participate in the autoimmune pathology, their antigen specificity has not been clearly demonstrated. A number of groups, including our own, continue to search for the antigens that may drive these B cells. Use of recombinant IgG derived from CNS-resident B cell clones (*31*–*33*) and CSF-derived IgG (*34*, *35*) both represent pragmatic approaches. The possibility remains that these CNS B cells are not specific for a single antigen. They may be targeting numerous antigens exposed during tissue injury. B cells accumulate in solid tumors and autoimmune tissue, including the MS meninges, where they often organize as clusters termed tertiary lymphoid structures. The characteristics, such as the accumulation of antigen-experienced B cells, are shared between these different autoimmune tissue compartments and tumors. Those in tumors (*23*) are strikingly similar to those in the damaged muscle tissue of patients with myositis (*36*) and the joints of individuals with Lyme arthritis (*37*). One explanation for this activity is that antibodies are locally produced in response to tissue damage that occurs throughout the disease course. That is, the persistent B cell infiltration perhaps reflects the ongoing nature of tissue injury in MS and a chronic immune response to such injured tissue. Both our group and others report the presence of numerous intraclonal variants (*10*, *13*–*15*, *21*, *30*), indicating the persistence of an ongoing process of affinity maturation, rather than the presence of a more final product, fully mature antibodies.

Some parallels to acute CNS inflammation such as that seen in viral encephalitis can be drawn. In those instances, OCBs that recognize a viral insult (*38*) are produced, and B cell infiltrates have characteristics identical to those in the MS CNS (*39*). However, when the insult is cleared, the active immune response reflected by the OCBs disperses. Conversely, in MS, it appears to remain in a chronically evolving, active state. It is possible that this is the product of a chronic infection. The actively replicating human virome that is held in-check by a normally functioning immune system (*40*) is still likely to provoke B cell responses. It is possible that a non-MS antigen-specific response may be the target of the CNS-infiltrating B cells. Supporting this possibility is the conspicuous proportion of IgA-producing B cells we found, which suggests that the response may be linked to an exogenous microbial antigen. Using the recombinant Ig from the expanded CNS B cell clones along with innovative antigen-screening technology, which should include posttranslational modifications, nonprotein entities such as lipids, and high-throughput arrays to screen viruses, bacteria, and other exogenous antigen sources, may provide needed insight into antigen specificity.

Table 3. Analysis of lineage tree founder cells by compartment. *P* values are provided for testing the hypothesis that founders are multi-compartment (CNS/CLN) versus single compartment (CNS or CLN), as well as CNS versus CLN.

Subject	Compartment	Total nodes, % (*n*)	Percentage of lineages (*n*)		*P*
			Founders	Unmutated founders	
M2	CNS/CLN	2.7 (32)	18.7 (6)	3.1 (1)	0.00324
	CNS or CLN	97.3 (1,133)	81.3 (26)	0	0.99250
	CNS	9.7 (113)	12.5 (4)	0	0.85100
	CLN	87.6 (1,020)	68.8 (22)	0	0.14750
M3	CNS/CLN	3.5 (739)	45.0 (139)	0.3 (1)	<0.0005
	CNS or CLN	96.5 (20,196)	55.0 (170)	0	>0.9995
	CNS	19.3 (4,034)	8.7 (27)	0	>0.9995
	CLN	77.2 (16,162)	46.3 (143)	0	<0.0005
M5	CNS/CLN	3.7 (2,374)	29.5 (425)	7.1 (103)	<0.0005
	CNS or CLN	96.3 (62,220)	70.5 (1,017)	1.2 (18)	>0.9995
	CNS	2.4 (1,534)	1.0 (15)	0	>0.9995
	CLN	93.9 (60,686)	69.5 (1,002)	1.2 (18)	<0.0005
Combined	CNS/CLN				$<3.74 \times 10^{-5}$
	CNS or CLN				>0.9999
	CNS				>0.9999
	CLN				$<4.34 \times 10^{-5}$

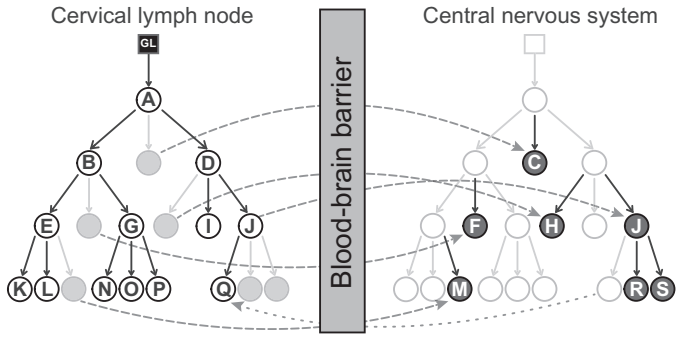

Fig. 5. Proposed model of B cell maturation and migration observed between the CLN and CNS. A reconstruction of an Ig lineage from a hypothetical clone is shown on both sides of the BBB. GL refers to germline cells and letters refer to different antibody sequences (intraclonal variants represented as nodes) in a single clone. Empty nodes represent sequences that are not empirically observed in a particular compartment. Mature clonally expanded (IgM, IgG, or IgA) B cells traffic between the CLN and the CNS compartments by crossing the BBB indicated by the dashed arrow. Cells originate (node GL) and clonally expand (node A) in the CLN before trafficking into the CNS. However, this process begins early in the clonal expansion process because less mature offspring (for example, nodes C and H) from the CLN can reside in the CNS. Several possibilities exist where the B cells can migrate back and forth through the BBB. The proposed scenario suggests that periphery B cell clones migrate from the CLN to the CNS while undergoing additional clonal expansion. Clonally expanded (IgM, IgG, and/or IgA) B cells within the CNS can then traffic back into the peripheral tissue and undergo additional clonal expansion.

Relevance to current MS therapeutics

The CNS immune response in MS, particularly that of B cells, has been considered to be sequestered. Specifically, it has not been clear whether the B cell activity in the MS CNS is independent of that in the periphery, particularly the lymph nodes. This raised the question as to how therapeutics that may be limited to acting peripherally can affect changes in the CNS. Our findings, along with a recent study showing that CSF B cells exchange with those in the periphery (*15*), suggest that treatments affecting B cells in the periphery have consequences for those that populate the CNS. B cell depletion (anti-CD20; rituximab, ocrelizumab, and ofatumumab) is among the most effective MS therapies, and these findings describe how it may affect the CNS.

Natalizumab is a monoclonal antibody that blocks immune cell migration into the CNS. Increased peripheral B cell frequencies observed after treatment suggest that natalizumab perturbs homing and migration through secondary lymphoid tissue (*41*). MS patients treated with natalizumab show a decrease in $CD4^+$ T cells in the CSF, and a subset of patients have diminished OCBs (*42*, *43*). A subset of those responding favorably to the treatment have reduced CSF Ig and B cells (*44*). These studies suggest that in some patients, the Ig and OCBs are dependent on B cells trafficking from the periphery, whereas in others, long-lived B cells that reside in a CNS niche may produce the OCBs. Collectively, studies of natalizumab and our findings described here, along with investigations describing an overlap between CSF and peripherally circulating B cells, indicate that B cells in the peripheral blood, lymph nodes, and CNS traffic between these compartments. These findings clarify how peripherally acting therapeutics, such as natalizumab, may disrupt the B cell–mediated immune response in the CNS.

MATERIALS AND METHODS

Study design

This study was designed to determine whether B cells, known to populate the MS CNS, matured in the peripheral lymphoid tissue. Matched tissue specimens from MS CNS and draining CLNs were collected from five different MS autopsies. The core of the investigative approach was the comparison of the B cell repertoire between the CNS and peripheral lymphoid tissue from the same autopsy. We explored these antibody repertoires to (i) determine whether clonally expanded B cells that populate the CNS are related to B cells in the peripheral lymphoid tissue, (ii) determine whether B cells traffic between the periphery and the CNS, (iii) describe the traffic direction, and (iv) ascertain the compartment(s) in which B cells experience affinity maturation. This study did not require blinding or randomization because neither would have provided an advantage for the interpretation or analysis of the data. Sanger sequencing was first used to establish whether members of B cell clones residing in the CNS were also present in a matched draining CLN. Then, next-generation sequencing was used to construct B cell antibody repertoires so that deeper analysis could be carried out to define how members of clones were represented in the CNS and periphery and how they

trafficked between these two compartments. Repertoires were built from separated sections of the same tissue specimen so that identical members of expanded B cell clones could be identified. Sequencing error, inherent to the high-throughput platform, and primer amplification bias were both greatly reduced by use of molecular barcodes that uniquely identified each mRNA molecule, thereby affording the generation of a consensus sequence representing a B cell clone member. The analysis first probed whether members of clones, identified by common *IGHV/J* gene usage and similarity in their junction region sequence, could be identified in both the periphery and CNS to ascertain whether B cells were exclusive to either compartment. Lineage trees were constructed for clones that spanned the periphery and CNS to provide information on B cell trafficking patterns. A permutation-based method was applied to test hypotheses on the most likely location of the founding cells for these clones and on the migration patterns of the cells over the course of clonal expansion.

High-throughput repertoire sequence analysis

Detailed methods for raw read processing, clonal assignment, diversity analysis, and lineage reconstruction can be found in Supplementary Materials. Briefly, raw high-throughput sequencing reads were quality-controlled, assembled, and filtered using pRESTO (*45*). V(D)J germline segments were determined using IMGT/HighV-QUEST (*46*). Functional V(D)J sequences were assigned into clones based on identical *IGHV* gene, *IGHJ* gene, and junction length, with a weighted intraclonal distance threshold of 3 using the substitution probabilities previously described (*47*). Repertoire diversity was characterized using the Hill diversity index (*48*) with uniform resampling to correct for sequencing depth. Lineage trees were inferred via maximum parsimony with PHYLIP version 3.69 (*49*). The analysis of lineage tree topologies was performed using standard graph traversal algorithms provided by the igraph R package version 0.7.1 (*50*).

Statistical analyses

Confidence intervals for *IGHV/J* family and gene usage were estimated using the Wald method for log risk ratios. Statistical significance of edge relationships and founder occurrence for lineage tree topologies were performed via permutation tests. Combined (meta-analysis) *P* values were calculated using Stouffer's method.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Distribution for the expected frequency of different types of edges in the lineage trees.
Fig. S2. Distribution of the expected frequency for different lineage tree founder compartments.
Fig. S3. Characterization of MS lesions and infiltration.
Fig. S4. Conventional sequencing shows that B cells present in the MS brain parenchyma and choroid plexus are clonally related to those present in the secondary lymphoid organs.
Fig. S5. Alignment of variable regions from representative B cell clones.
Fig. S6. Mutation frequency distribution among isotypes.
Fig. S7. Sequences from the CNS and CLN both show significant evidence of antigen-driven selection.
Fig. S8. *IGHV* family usage in the CNS and CLN.
Fig. S9. *IGHV* gene usage in the CNS and CLN.
Fig. S10. *IGHJ* gene usage in the CNS and CLN.
Fig. S11. Complete set of multicompartment lineage trees with unmutated single-tissue founders.
Fig. S12. Possible models of B cell expansion observed between the CLN and CNS.
Table S1. Nucleotide substitution matrix for clonal assignment.
Table S2. High-throughput sequencing results.
Table S3. Distribution of Ig isotypes.

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