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

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Abstract

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

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Author contributions: The original hypothesis was conceived by DAH and KCO. The study was initiated and designed by DAH, JHNS, SHK and KCO. The Sanger sequencing and tissue preparation for next-generation sequencing was performed by JHNS, BAS and AN. SR and DP performed the immunohistochemistry and DP interpreted the slides. Tissue was identified, collected and characterized by JDL, RQH, AJH and RMN. The next-generation sequencing PCR technology was developed by GC and FV and performed by FV and WD. The sequence data analysis pipeline was conceived and developed by GY, JAVH and SHK. The sequencing data analysis was performed by GY, JAVH and SHK. The collective data was interpreted by GY, JAVH, FV, SHK, DAH and KCO. JHNS, RQH and JDL supported the work with key suggestions and editing the manuscript. The manuscript was written by GY, JAVH, SHK, DAH and KCO. SHK, DAH and KCO co-directed the project.

Competing interests: D.P. both consults for and receives speaking fees from Biogen. KCO received speaking fees from EMD-Serono. The other authors declare they have no competing interests.

Data and materials availability: The sequence data for this study have been deposited in the NCBI Sequence Read Archive (SRA). The BioProject accession number is PRJNA248475.

Supplementary Materials
Materials and Methods

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. In this study, 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 clonal families were more often found in the draining CLNs. More mature clonal family 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 impact 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). While 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 (OCB) 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 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). Intracлонаl variants, that represent steps in the antigen-driven affinity maturation process, are also present in MS CNS compartments (10, 13). OCB 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 IgG representing the OCB 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 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, ofatumumab) or impact the intrathecal B cell compartment by inhibiting lymphocyte transmigration into the CNS (anti-VLA4; natalizumab)

We reasoned that CNS B cells in patients with MS may gain antigen experience and mature in lymph nodes associated with the CNS, namely the 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 implicates 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 (EAE) (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 re-construct B cell lineages across tissues. Founding members of clonal families were more often found in the draining CLNs, while more mature members derived from these founders were observed both in the draining CLNs and the CNS. 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 draining secondary lymphoid tissue.

Results

Characterization of tissue specimens

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 approximately 1,300 sequence reads, which reduced to 754 sequences used for analysis when PCR generated repeats were removed. We identified a total of 9 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 divided into two or three independent heavy chain repertoires. Approximately 32 million total raw sequence reads were collected from the 32 separate repertoires. Quality control processing and selecting unique reads that each represent a single mRNA molecule reduced the data to approximately 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 clonal 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% 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 the vast majority of the 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 multi-compartment 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 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%–13%. The isotype distribution in the choroid plexus specimen from subject M5 was 72%, 5% and 23% 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 employed an algorithm (BASELINE) that quantifies selection by analyzing mutation patterns in experimentally derived immunoglobulin 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, the majority of B cells were distributed into clonal families (Table 2). Both the CLNs and CNS compartments included clonally expanded B cells with many representing more than half of all the B cells. While 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 non-switched, experienced memory cells. We found that IgM sequences were often parts of clonal groups 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 non-switched 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 immunoglobulin heavy chain variable (IGHV) family and immunoglobulin heavy chain joining (IGHJ) gene usage by calculating odds 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. 2A and 2B). 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, S8 and S9). The usage of IGHJ genes (Fig. 2B and S10) was heavily biased toward IGHJ4 in the 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 the CLN (Fig. 2C), both in terms of fewer total clones ($q=0$) and less dominance by larger clones ($q>1$). 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.

Ancestral clonal family 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 populations that span multiple compartments, B cells that resided in both the CNS and CLN behaved as ancestors (i.e., they tended to produce progeny that undergo further rounds of somatic hypermutation). Conversely, their counterparts residing in a single tissue compartment often represent terminal nodes of the lineage tree (i.e., any progeny did not accumulate additional mutations). Fig. 4B further illustrates the ancestral character of multi-compartment B cells by showing that these B cells are more likely to be observed as ancestors than expected by chance. Taken together, this implies that local expansion of B cell populations regularly occurs following trafficking between compartments.

Clonal lineages that span multiple compartments likely originate in lymphoid tissue

In order to determine the origin site of multi-compartment 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 (i.e., appearing closest to the germline sequence). We found that multi-compartment 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). As the origin of multi-compartment 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 the majority of 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 the majority of sequences are CLN) with statistically significant enrichment at $p < 4.3 \times 10^{-5}$ for all three subjects combined (Table 3). While 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.

A proposed model describing B cell population of the CNS

Antigen-experienced, clonally expanded B cells reside in both the CNS and secondary lymphoid organs (Fig. 5 and 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. Clonal family members, that represent the more evolved products of maturation, are more stationary as 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 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 as they display the principal characteristics of mature effector B cells. Members of these clones are not confined exclusively to the CNS but also occupy secondary lymphoid organs, which directly drain the CNS. Furthermore, these multi-compartment clonal members appear to circulate between the CNS and periphery, with the majority of parental clones arising in the periphery. These data collectively suggest that in the MS CNS B cells encounter antigen and gain experience in secondary lymphoid tissue. These B cells then dispatch colonists that populate the CNS then continue trafficking between the CNS and periphery. Although our data implicate peripheral compartments as the initial site for the majority of 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 prior to sequencing. We are limited from extending the interpretation of our data to all types of MS given that the majority of our tissue was derived from subjects in the progressive stage. However, we did include a single case of relapsing remitting MS (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 taken together indicate that B cell traffic, both in and out of the CNS, can occur both at early and later stages of the disease. While the multi-compartment clonal lineages provide clear evidence that the clonally expanded B cells that populate the CNS are related to B cells in the peripheral lymphoid 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 function of B cells includes antibody production, antigen presentation to T cells, and cytokine production. B cells can express pro-inflammatory cytokines such as interleukin (IL)-6, IL-12, tumor necrosis factor (TNF) 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 antigen presenting cells (APC) 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 repertoires, appear to be a very minor fraction.

It has been clearly demonstrated that B cells present in MS tissue and CSF contribute to production of antibody (14, 30). While 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. Interestingly, 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 products, fully mature antibodies.

Some parallels to acute CNS inflammation such as that seen in viral encephalitis can be drawn. In those instances OCB 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 OCB, 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 immunoglobulin from the expanded CNS B cell clones along with innovative antigen screening technology, which should include post-translational modifications, non-protein entities such as lipids and high-throughput arrays to screen viruses, bacteria and other exogenous antigen sources, may provide needed insight into antigen specificity.

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 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, ofatumumab) is among the most effective MS therapies and these findings describe how it may impact the CNS.

Natalizumab is a monoclonal antibody that blocks immune cell migration into the CNS. Increased peripheral B cell frequencies observed post 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 has diminished OCB (42, 43). A subset of those responding favorably to the treatment has reduced CSF immunoglobulin and B cells (44). These studies suggest that in some patients the immunoglobulin and OCB are dependent on B cells trafficking from the periphery while in others long-lived B cells that reside in a CNS niche may produce the OCB. 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 (1) determine whether clonally expanded B cells that populate the CNS are related to B cells in the peripheral lymphoid tissue, (2) determine whether B cells traffic between the periphery and the CNS, (3) describe the traffic direction, and (4) ascertain the compartment(s) in which B cells experience affinity maturation. This study did not require blinding or randomization as 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 clonal families 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 primary amplification bias were both greatly reduced by use of molecular bar codes 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 CDR3 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 clonal groups on the basis of identical IGHV gene, IGHJ gene, and junction length, with a weighted interclonal 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 (49). The analysis of lineage tree topologies was performed using standard graph traversal algorithms provided by the igraph R package (50).

Statistical Analyses

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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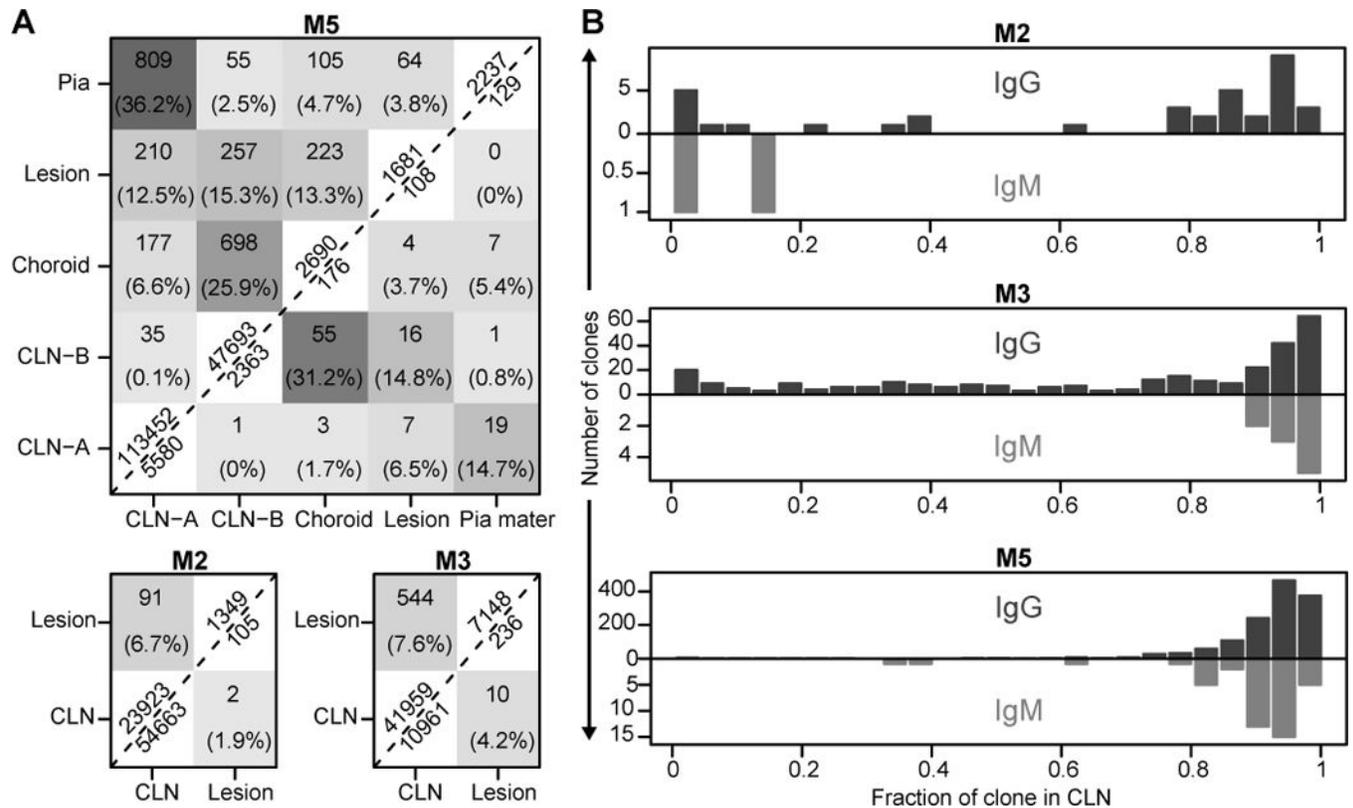


Fig. 1. B cell antibody repertoire sequencing demonstrates that clonally expanding B cells populate the MS CNS and draining CLNs

(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) Column graphs 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.

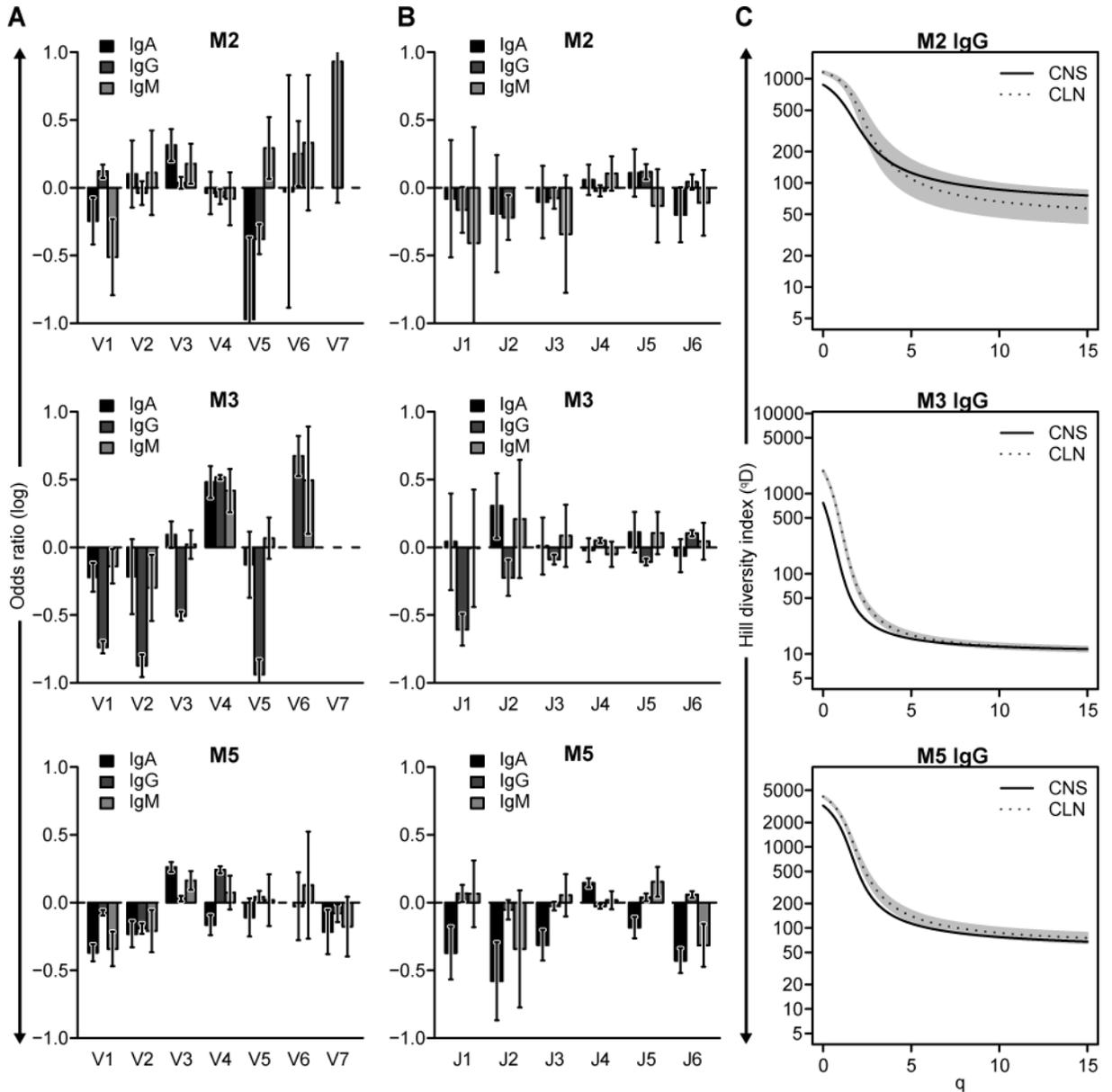


Fig. 2. The antibody repertoire in the CNS and CLN share features

Usage of both (A) IGHV families and (B) IGHJ genes were compared between the CNS and CLN. For each subject, the odds 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 sub-sampled to the number of sequences in the smallest sample and the Hill diversity index was calculated independently in 1,000 equally spaced q values between 0 and 15. As q varies from 0 to infinity the diversity (qD) depends

less on rare species and more on common ones, 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, while 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 (i.e., a more focused repertoire).

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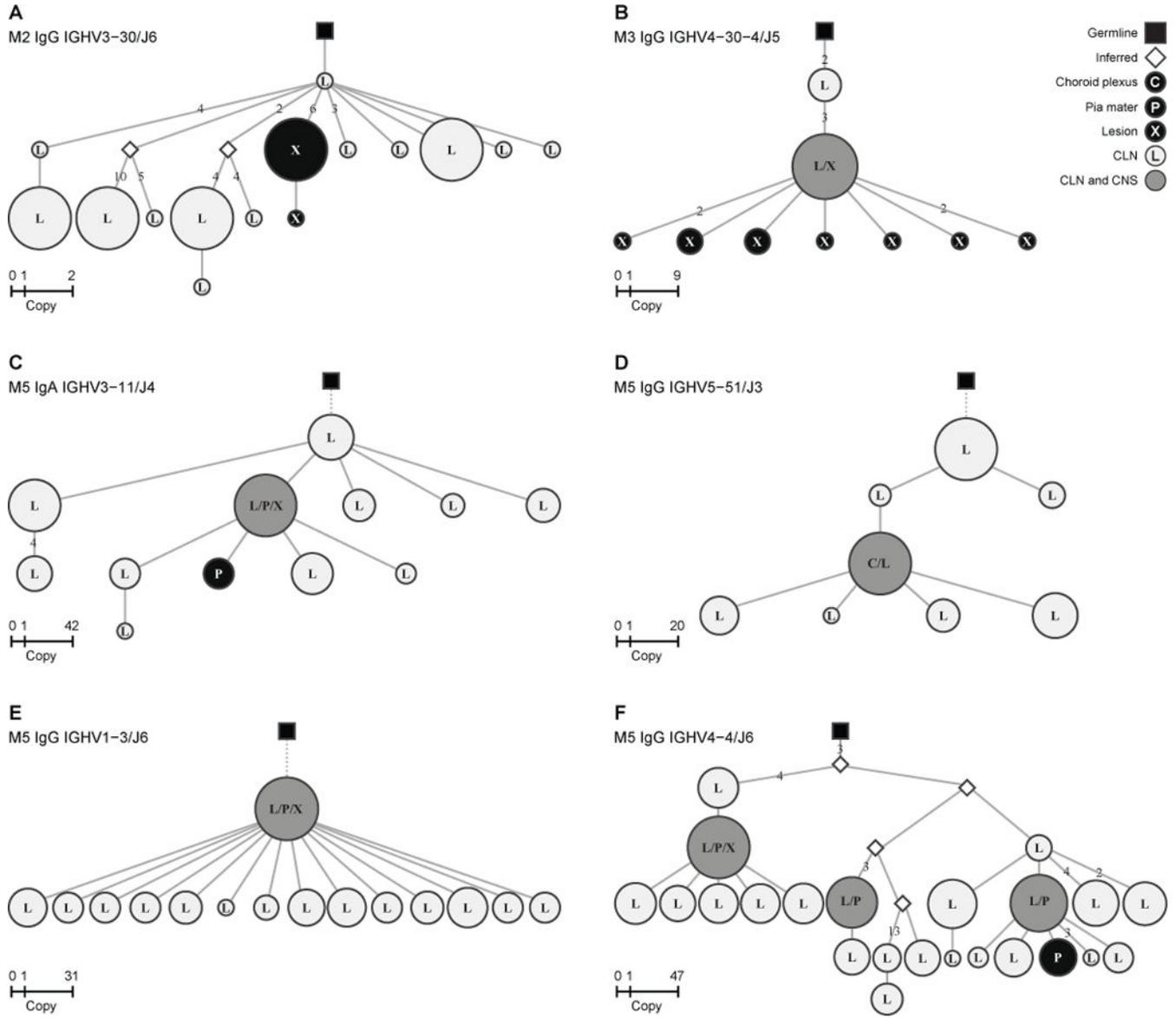


Fig. 3. Multi-compartment lineage trees illustrate that B cells traffic between the CLN and CNS
 Representative lineage trees are shown for subject M2 (A), M3 (B) and M5 (C-F). Lineage trees are shown with the germline sequence at the root (black square). The number of somatic mutations accumulated from 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 UIDs) identified with the same nucleotide sequence.

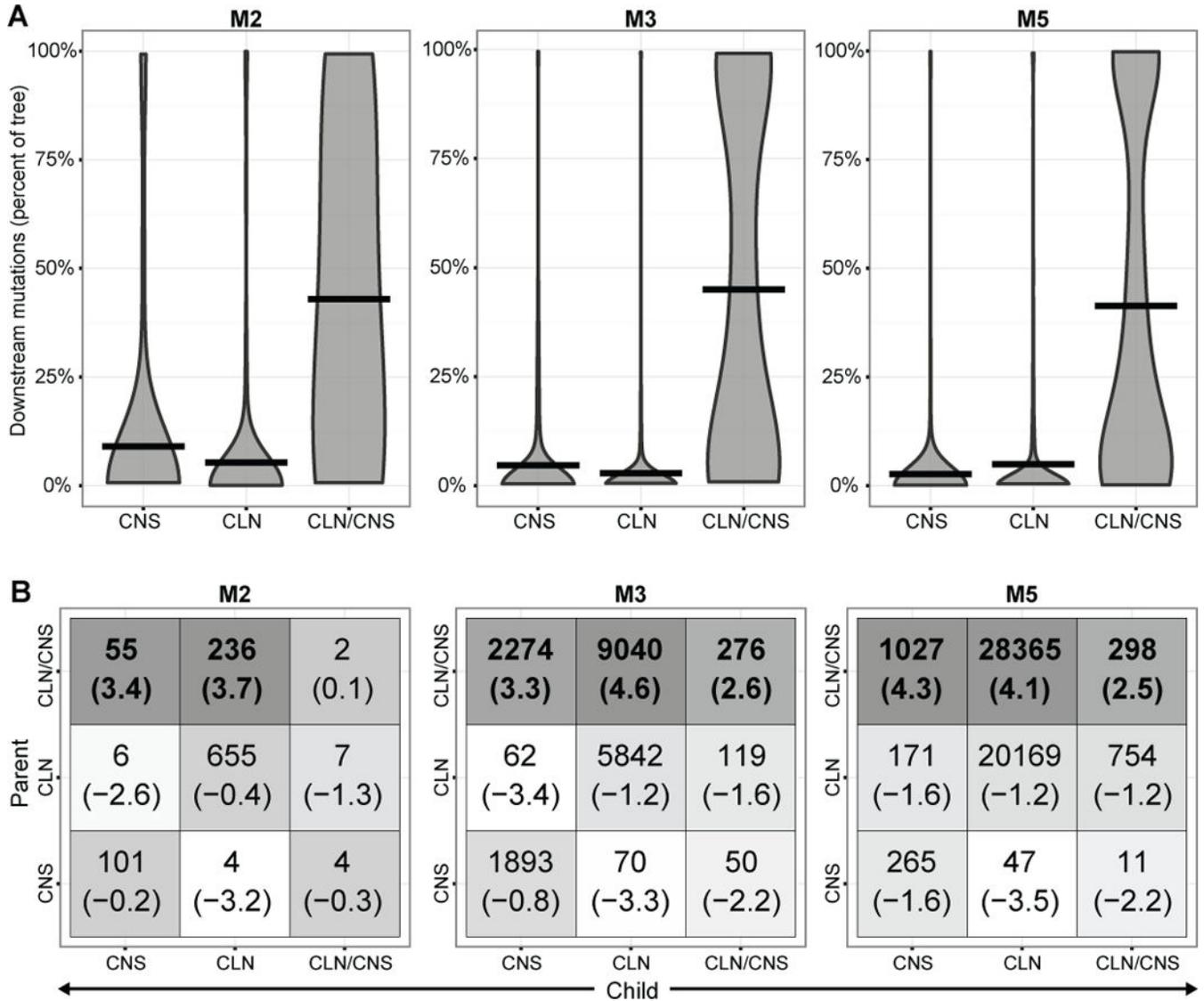


Fig. 4. Trafficking between CNS compartments and the CLN often occurs early in clonal expansion

Sequences were classified according to where they resided; CNS, CLN or CLN/CNS (multi-compartment) and lineage trees were constructed for all clones that contained at least one CNS or multi-compartment sequence. Two separate analyses demonstrate that multi-compartment 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 multi-compartment 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 family member to produce offspring. The width of the plot for the CNS, CLN or multi-compartment 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 as they produced further ancestors. **(B)** The set of all expanded (>10 unique sequences) multi-compartment 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 indicates that these shared sequences more often gave rise to daughters than those restricted to a single compartment.

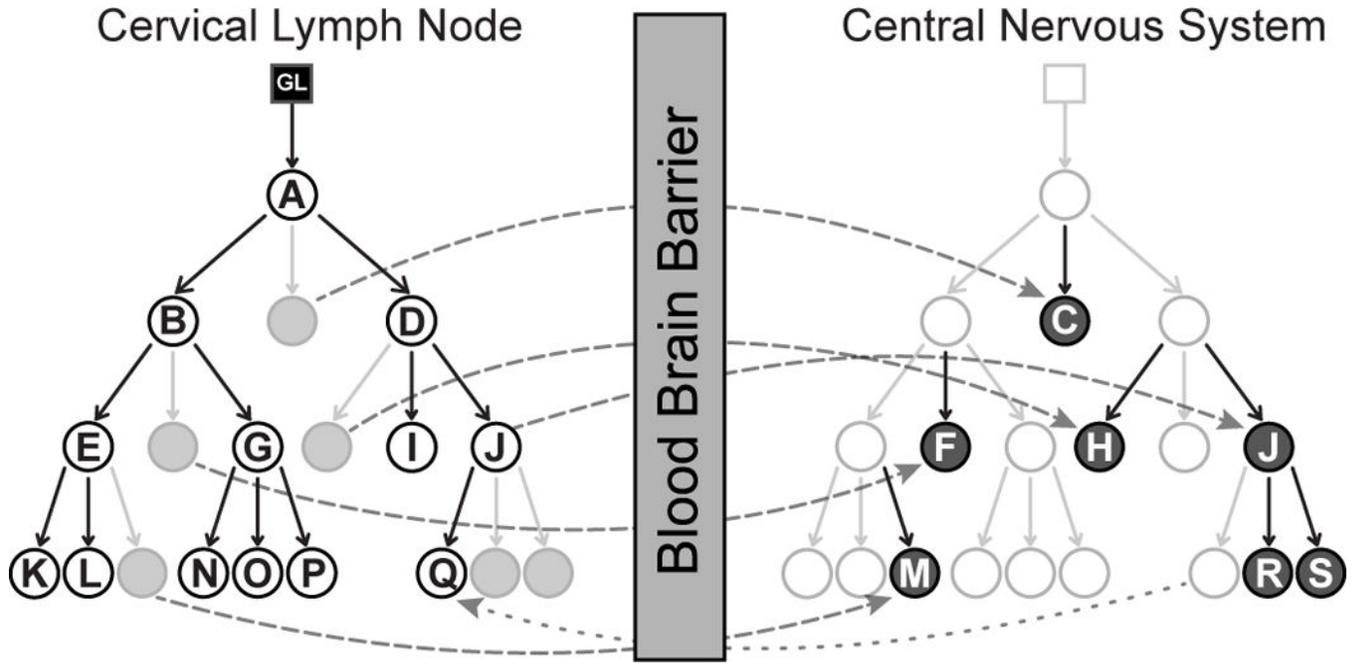


Fig. 5. Proposed model of B cell maturation and migration observed between the CLN and CNS
 A reconstruction of an immunoglobulin lineage from a hypothetical clone is shown on both sides of the blood brain barrier (BBB). GL refers to germline cells and letters refer to different antibody sequences (clonal variants represented as nodes) in a single clonal family. 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 as less mature offspring (e.g. node 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.

Table 1

Subject demographics and cellular characteristics of the CNS tissue.

Subject	Disease state	Age	Gender	Histopathologic findings
M1	Relapsing remitting MS	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.

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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) vs. single compartment (CNS or CLN), as well as CNS vs. CLN.

Subject	Compartment	Total Nodes	Percentage of lineages (number)		P Value
			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				<3.74×10 ⁻⁵
	CNS or CLN				>0.9999
	CNS				>0.9999
	CLN				<4.34×10 ⁻⁵