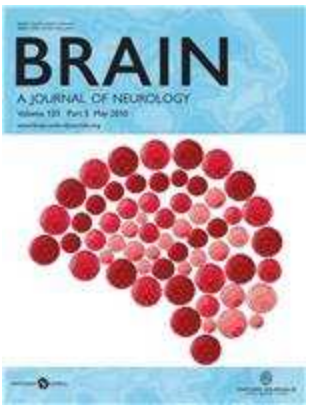

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Related B cell Clones Populate the Meninges and Parenchyma of Patients with Multiple Sclerosis.

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SUMMARY

In the central nervous system of patients with multiple sclerosis, B cell aggregates populate the meninges, raising the central question as to whether these structures relate to the B cell infiltrates found in parenchymal lesions or instead, represent a separate central nervous system immune compartment. We characterized the repertoires derived from meningeal B cell aggregates and the corresponding parenchymal infiltrates from brain tissue derived primarily from patients with progressive multiple sclerosis. The majority of expanded antigen-experienced B cell clones derived from meningeal aggregates were also present in the parenchyma. We extended this investigation to include 20 gray matter specimens containing meninges, 26 inflammatory plaques, 19 areas of normal appearing white matter and cerebral spinal fluid. Analysis of 1,833 B cell receptor heavy chain variable region sequences demonstrated that antigen-experienced clones were consistently shared among these distinct compartments. This study establishes a relationship between extraparenchymal lymphoid tissue and parenchymal infiltrates and defines the arrangement of B cell clones that populate the central nervous system of multiple sclerosis patients.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by demyelination of the brain and the spinal cord (Hafler *et al.* , 2005, Noseworthy *et al.* , 2000, Lucchinetti *et al.* , 1998, Filippi and Rocca, 2005, O'Connor *et al.* , 2001). Although most of the tissue damage is found within the parenchyma, several recent studies have implied that extraparenchymal structures, such as the meninges, may play a role in the disease pathology. The first of these seminal studies demonstrated that organized B cell structures, reminiscent of germinal centers, are present in the cerebral meninges of patients with MS (Serafini *et al.* , 2004, Magliozzi *et al.* , 2007). These tertiary lymph node-like structures include proliferating B cells with a network of follicular dendritic cells and appear to be primarily present in the meninges and sporadically in the parenchyma (Prineas, 1979). The second major finding showed that the choroid plexus, a distinct meningeal structure, is the port through which Th17 lymphocytes enter the CNS where they then initiate the experimental autoimmune encephalomyelitis (EAE) (Reboldi *et al.* , 2009). While these studies point toward a role for the meninges in the inflammatory response, it is not known whether the B cells present at these extraparenchymal sites are related to those which populate the CNS and potentially contribute to the pathophysiology of MS. Through analysis of the B cell repertoires derived from meningeal B cell aggregates and the corresponding parenchymal infiltrates from brain tissue derived primarily from patients with progressive multiple sclerosis, we defined the organization of antigen experienced B cell populations present in distinct locations of the MS CNS. This study provides new evidence for a correlation between the extraparenchymal sites and the clonal B cell population that infiltrates MS CNS tissue.

MATERIAL AND METHODS

Specimens

Plaque, adjacent (a)-normal appearing white matter (NAWM) or distant (d)-NAWM and a-gray matter or d-gray matter were dissected and bisected at autopsy from eleven subjects with clinically defined MS. Seven of the eleven subjects had a secondary progressive clinical course, three had a chronic progressive course and one had a relapsing clinical course. Half of the bisected samples were placed in 10% buffered formalin then stored at room temperature and half were immediately snap-frozen then stored at -80°C. The CSF of MS-7 was removed post-mortem, centrifuged then the isolated cell pellet and supernatant were stored at -80°C. The clinical features of each MS patient and the respective brain specimens are summarized in Supplementary Table 1. Non-MS controls included two white matter and gray matter samples derived from an individual (autopsy) without history of any neurological disease and the peripheral blood from three subjects without neurological diseases. Local human research internal review boards approved all human subject related work.

Histology and immunohistochemistry

Immunohistochemical analysis was performed on either paraffin-embedded formalin-fixed (PEFF) or frozen tissue as previously described (Willis *et al.* , 2009). Primary anti-human CD3, CD20, CD138 and CD68 (all from Dako) were applied for 1h to the specimens MS4-MS11. Slides were washed in Tris-HCl (50mM, pH 7.4) and incubated with Dako Cytomation Envision kit according to the manufacturer's instructions. After further washing, immunoperoxidase staining was developed using DAB chromogen and counterstained with hematoxylin. Luxol Fast Blue (LFB) was used to identify the areas of demyelination. Due to the limited size of the

meningeal B cell aggregates, on MS1, MS2 and MS3 brains it was possible to perform only the CD20 staining.

Immunoglobulin variable region cloning and B cell repertoire analysis

Immunoglobulin (Ig) variable region heavy chain (VH) libraries were constructed from two non-consecutive sections of each specimen (15 x 20 mm area; 14μm thickness). RNA was extracted from tissue sections using the RNeasy Kit (QIAGEN) according to the manufacturer's instructions. From the total RNA, cDNA was synthesized and human Ig variable region genes were amplified as previously described (Wang and Stollar, 1999), with minor modifications described by our group (Willis *et al.* , 2009).

The VH libraries were examined for evidence of clonal expansion, somatic mutation and isotype distribution. The variable region cloning procedure captures the 5' end of the Ig constant region, allowing the Ig isotype to be determined. VH sequences were analyzed using software available from the human variable region database on the ImMunoGeneTics (IMGT) web site http://imgt.cines.fr (Ford *et al.* , 1994). This analysis afforded identification of the most homologous germline segments (VH, DH, and JH) and allowed determination of the extent of somatic mutation relative to germline and the presence of insertions or deletions. The first 10 codons of framework 1, being primer coded, were excluded from this analysis. Allelic polymorphism was not considered in the assessment of somatic mutation, because the Ig variable gene alleles have very few such nucleotide substitutions (Cook and Tomlinson, 1995) and the IMGT database includes various alleles for alignment (Lefranc, 2001). Chimeric molecules arising from PCR amplification artifacts were not included in any analysis (Ford *et al.* , 1994).

Clonal expansion

Clones were identified through their invariably unique CDR3 sequences. Identical sequences derived from separate (non-consecutive) tissue sections defined clonal expansion, whereas identical sequences within one tissue section library were considered to be the product of PCR amplification rather than expanded clones, as the two cannot be reliably distinguished. Two or more sequences were considered to be derived from clonally related B cells, which we term clonal variants, if they had identical CDR3 and differed by at least two different somatic mutations in the VH region.

VH gene family usage

Perturbations of VH gene family usage are indicative of antigen-driven stimulation. Accordingly, we compared the usage of the different VH gene families in meninges, plaques, NAWM, and CSF of the MS cases with that of the B cells in the peripheral blood of three control subjects. Differences in VH gene family usage were measured using Fisher's Exact test with p values less than 0.005 considered significant.

RESULTS

To examine the role of the extraparenchymal meningeal lymph-like structures in MS, we selected meningeal specimens containing B cell aggregates and corresponding parenchymal white matter areas isolated from distinct locations of MS brain tissues (Fig 1). In order to identify the B cell clones present in each specimen, we generated B cell receptor (BCR) VH libraries from two non-consecutive sections of each secondary progressive MS specimen and of a control brain from a subject without neurological disease (Fig 2A). By examining the repertoires derived from the meningeal aggregates and the corresponding parenchymal infiltrates, we observed similar features of antigen experience for the B cell clones populating each area (Fig 2B). The relative clonal expansion of the B cells in the meningeal aggregates and in the parenchymal infiltrates was 24% and 28% respectively. The mutation frequency in the VH sequences (number of nucleotide and amino acid mutations accumulated) was similar in the two areas. The isotype distribution showed that approximately 90% of the clones in both locations used the IgG isotype and the remainder the IgM isotype (Fig 2B). This ratio was substantially different from that found in peripheral blood of healthy subjects where the IgG/IgM ratio is typically 15:85 (Klein *et al.* , 1998) further highlighting that these B cells were antigen experienced.

Through analyzing the distribution of B cell clones in the meningeal aggregates and the corresponding parenchymal infiltrates, we found that the majority of B cells were exclusive to each location (Fig 2C). However, several clones were shared between the meningeal aggregates and parenchymal infiltrates in MS1 and MS2, but not in MS3. When our analysis was restricted to only expanded, antigen-experienced clones, 71% and 89% of the expanded B cell clones

present in the meningeal aggregates of MS1 and MS2 were present in the parenchymal infiltrates. Seven representative VH sequences with their relative clonal variants shared between meningeal B cell aggregates and parenchyma in MS1 and MS2 subjects are represented in Fig 2D.

These data then led us to investigate how B cells populate additional anatomical locations of the MS CNS, including the meninges, plaques, NAWM and CSF. To this end, we built BCR VH libraries from 20 gray matter samples containing meninges, 26 plaques, 19 NAWM sections and one CSF from 11 different MS brains (Supplementary Table 1 and 2; Supplementary Fig 1A) totaling 1,833 individual sequences. The cases studied were derived from secondary and chronic progressive MS and a single case representing the relapsing remitting MS course. Consistent with the immunohistochemical analysis, the amplification of BCR VH regions highlighted the presence of B cells not only in plaques, but also in all NAWM and in the gray matter specimens that showed sparse meningeal CD20+ and CD138+ cells by immunohistochemistry (Supplementary Fig 1B, Supplementary Table 2). The B cell clones present in these different areas possessed the characteristics of antigen experience in terms of clonal expansion, number of mutations in the BCR VH, and isotype distribution (Fig 3A). Among the 1,833 sequences (421 clones) analyzed only four clones (one in each of four cases MS3, MS7, MS10, MS11) carried germline sequences without mutations in the BCR VH sequence. All the other clones carried nucleotide mutations in the VH sequences. Perturbations of VH gene family usage, also indicative of antigen-driven stimulation, were present not only in plaques and CSF as previously described (Qin *et al.* , 1998, Owens *et al.* , 1998, Owens *et al.* , 2001, Baranzini *et al.* , 1999, Smith-Jensen *et al.* , 2000), but also in meninges and NAWM. The VH gene family usage in the

peripheral blood B lymphocytes (PBB) of control subjects reflected the relative distribution of the VH gene germline in the different families (Supplementary Table 3). These data also independently confirmed that the RT-PCR protocol used in this study did not artificially skew the repertoire.

While the majority of B cells were exclusive to a single region (Fig 3B), a number of clones were shared among different anatomical areas, including a clone that populated the meninges, plaque and CSF (Fig 3C). When our analysis was restricted to only expanded, antigen-experienced clones, the fraction of B cells that were shared among distinct locations ranged from 39% to 62% in the 11 MS brains examined (Fig 3B, Supplementary Table IV). There was no clone shared among different subjects, which confirmed the absence of cross contamination in the cloning procedures.

DISCUSSION

The presence of immunoglobulin in the CNS of patients with MS is a hallmark of the disease. Accordingly, humoral immunity is thought to play an important role in the autoimmune response and the development of demyelinated plaques. Several recent studies imply that extraparenchymal structures, such as the meninges, also play a role in MS immunopathology. The presence of B cell follicle-like structures in the cerebral meninges of some multiple sclerosis patients supports such a role. It is not clear whether these meningeal aggregates harbor antigen experienced B cells and their relationship to the B cells known to partially comprise parenchymal infiltrates is also not understood. Identifying a relationship between the immune cells that populate these distinct compartments would further define the role that the cerebral meninges may play in MS pathophysiology. In this study, the detailed molecular analysis of the BCR variable region demonstrates that antigen experienced B cell clones are shared between the meningeal aggregates and the corresponding parenchyma. Notably, a high percentage of the expanded, antigen-experienced B cell clones present in the meningeal aggregates of two of the three brains examined were also present in the corresponding parenchymal infiltrates. Interestingly, the distance between the meningeal and parenchymal specimens in the third brain that showed no overlap was considerably higher than that in the others. Possibly indicating that B cell trafficking within the CNS may be constrained by distance.

It has been recently described in the EAE model that peripheral CCR6+ Th17 lymphocytes, critical for initiating parenchymal damage, can use meningeal structures such as the choroid plexus to enter the CNS (Reboldi *et al.* , 2009). Because the CCR6 ligand CCL20 is constitutively expressed in the epithelial cells of choroid plexus in mice and humans, these

structures are suggested to control the immune surveillance of the human CNS as well. The meningeal B cell aggregates that we identified are likely to be the same structures previously identified as germinal center-like tertiary lymph nodes (Serafini *et al.* , 2004). Following negative selection, proliferation and maturation in these structures, expanded antigen experienced B cells may then migrate to the parenchymal sites and possibly contribute to tissue damage. Although we could not determine the direction in which B cell traffic in the MS brain, this scenario represents one of the possible schemes of B cell maturation and infiltration in the MS meninges and parenchyma.

We also defined the arrangement of antigen experienced B cells present in distinct locations of the MS CNS providing insight into the manner by which the B cells populate the tissue. The analysis of the B cell repertoire in different anatomical locations showed the presence of antigen experienced B cells in all areas of the MS CNS. Interestingly, even though the NAWM showed a more restricted number of clones in comparison to the plaques and meninges, the B cells present in this area nevertheless showed features of antigen experience similar to the B cells present in plaques and meninges. These features included clonal expansion, somatic mutations in the BCR VH region, isotype switching and a skewed VH gene family repertoire. The antigen experienced B cells present in NAWM might represent an early inflammatory event that occurs prior to parenchymal tissue damage. It follows that such B cells may gain antigen experience within extra-parenchymal compartments, such as the meninges or the periphery, and then subsequently populate sites in the MS brain.

While the majority of B cell clones present in MS CNS tissue resided in a single exclusive location, a number of antigen-experienced B cell clones were shared among different locations. We observed shared B cell clones in multiple locations of each MS brain, including multiple meningeal areas, plaques, NAWM and CSF. The meningeal B cell aggregates were not dissected from the surrounding tissue. Consequently, these B cell libraries may have included sequences from those very few B cells (confirmed through immunohistochemistry) found in the grey matter. Still, these libraries accurately represented the meningeal infiltrate because the majority of the sequences were most likely be derived from the B cell follicles that contain large numbers of cells. As B cell clones were shared between the CNS tissue and the CSF, these data indicate that sampling of CSF B cells can provide insight into parenchymal B cell populations that are often proximal to tissue damage.

Our data complement the work of Junker and colleagues who demonstrated the presence of a pervasive T-cell response in distinct regions of MS brain comprised of "private" T cell clones unique for each brain region and "public" T cell clones shared in multiple sites of the brain (Junker *et al.* , 2007). These data collectively indicate that both CNS B and T cells may be targeting a CNS antigen. Furthermore, our data define a relationship between meningeal structures and MS parenchymal tissue. We propose that the B cell distribution in the MS brain may be due to a hierarchy of the B cell clones involved in the disease, some of them dominant and possibly involved in the lesion formation in multiple sites, and others exclusively involved in a single site of inflammation. One possible scenario concerning B and T cell migration is that at the initial phases of the disease, distinct B and T clones from extra-parenchymal sites such as peripheral lymph nodes or meningeal lymphoid structures populate different anatomical

locations of MS brain. A subpopulation of these immune cells may subsequently diffusely seed to multiple locations of CNS through the CSF circulation and contribute to the tissue damage (Fig 3D). In conclusion, following the recent evidence that supports a primary role for the meningeal structures in T cell infiltration and B cell proliferation and maturation, this study provides new evidence for a direct correlation of extraparenchymal sites to the clonal B cell population that infiltrates MS CNS tissue.

FIGURE LEGENDS

Figure 1

MS brain contains a prominent B cell infiltrate within the meninges and parenchyma.

Immunohistochemistry identified meningeal B cell aggregates (CD20 staining), with follicle-like appearance, in a subset of MS brain specimens (A, 200x; B, 400x). CD20 staining also identified B cell infiltration in MS plaques (C), and scattered B cells adjacent to vessels in NAWM (D). Gray matter from MS brain contained a sparse B cell infiltrate within the meninges but not in the parenchyma of these specimens (E, meninges; F parenchyma).

Figure 2

Subsets of meningeal B cells are clonally related to those present in the parenchyma of the MS brain.

The BCR VH region was amplified from two non-consecutive sections (first and third sections) from matched meningeal (containing B cell aggregates) and parenchymal (containing infiltrates) specimens derived from three MS brains (MS1, 2, 3)(A). No BCR VH region was amplified from healthy control brain containing no detectable B cell infiltrate by immunohistochemistry (A). Sequence analysis of the BCR VH region demonstrated that the B cells populating both compartments appeared antigen experienced as indicated by clonal expansion, significant somatic mutation and isotype distribution (B). Sequence comparison between meningeal and parenchymal B cell clones demonstrated that while most B cells localized exclusively to one area (unique), a small but significant proportion of B cells were present in both locations in MS1 and MS2 brains (shared; C). The distance between the meningeal and parenchymal specimens in each brain is indicated on the top of the columns. Analysis of the subset of B cells that were

clonally expanded revealed that 70% and 90% of the expanded clones populated both the meningeal aggregates and parenchymal plaques of MS1 and MS2 respectively (C). Variable region sequence alignments of seven representative B cell clones shared between meningeal aggregates and parenchymal infiltrates in MS1 and MS2 are shown (D). The CDR regions are indicated by vertical bars.

Figure 3

Clonally related B cells populate the meninges, plaques, NAWM and CSF in the MS CNS.

Analysis of the B cell repertoires derived from the meninges, plaques, NAWM and CSF of eleven MS brains demonstrated the characteristic features of antigen experience for the B cells present in all locations (A). Sequence comparison between the BCR repertoires derived from different locations determined that the majority of the B cells resided exclusively in one area, however a small proportion of clones were shared among different locations (B). Only the MS3 case did not show any shared clones. Analysis of the subset of B cells that were clonally expanded revealed that 39% to 62% of these clones populated different locations within the MS CNS (B). Representative examples of sequence alignments from seven B cell clones shared among CNS compartments, including meninges, plaque, NAWM and CSF (C). Distinct B cell clones, activated in extra-parenchymal sites such as peripheral lymph nodes or meningeal lymphoid structures, might populate different anatomical locations of MS brain and potentially contribute to the tissue damage. Further diffusion and seeding of a subset of B cells to multiple locations of the CNS may occur through the CSF circulation (D).

Supplementary Fig 1

Immunohistochemical and molecular characterization of the immune cell infiltrates in different areas of MS brain.

Immunohistochemical analysis was performed on 17 gray matter (containing meninges), 23 plaque and 19 NAWM specimens derived from nine MS brains to characterize lesion activity, axonal loss, demyelination and to enumerate immune cell infiltrates (Supplementary Table 2) (A). All plaques contained regions of demyelination, while all NAWM and gray matter specimens were normally myelinated (Luxol Fast Blue staining, LFB, a, b, c). CD3 staining showed the presence of several CD3 positive cells in the meninges, particularly in the cerebral sulci, of all the gray matter specimens examined. No CD3 positive T cells were identified in the parenchyma of the same specimens (d). In contrast, abundant perivascular and parenchymal T cell infiltrates were detected in plaques, while scattered CD3 positive cells associated with the vessels were observed in the NAWM (e, f: CD3 positive cells are indicated by arrows). CD68 staining showed microglia activation in NAWM areas as well as in plaques and in gray matter parenchyma (g, h, i: CD68 positive cells are indicated by arrows). The PCR products of BCR VH chains from different areas of MS specimens are shown (B). A band of 350 bp is shown from two non-serial sections of MS meningeal infiltrates (MS4-3), MS plaques (MS4-1 and MS4-2, section 1 and 3) and MS NAWM (MS4-1 and MS4-2, section 1 and 3).

APPENDIX

L.L. performed experiments, collected and analyzed the data. LL, KCO, DAH, and SNW wrote the paper. KCO, DAH and LL conceived the experimental approach and designed the study. SJR performed the immunohistochemistry along with TC. OH and RR performed immunohistochemistry and characterised and contributed human tissue specimens. Other authors assisted with data collection.

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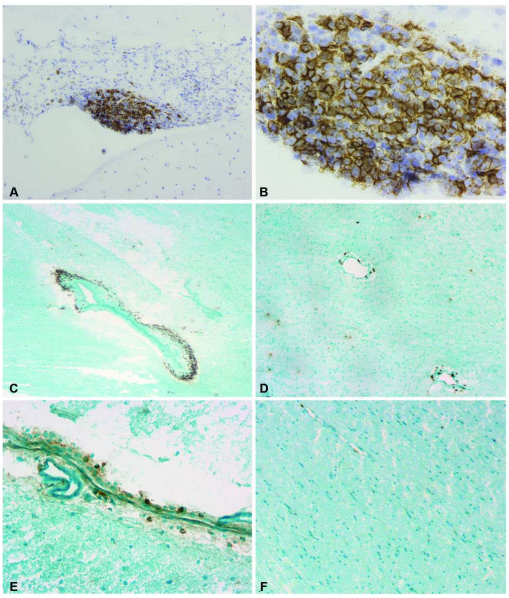
Lovato - Figure 1



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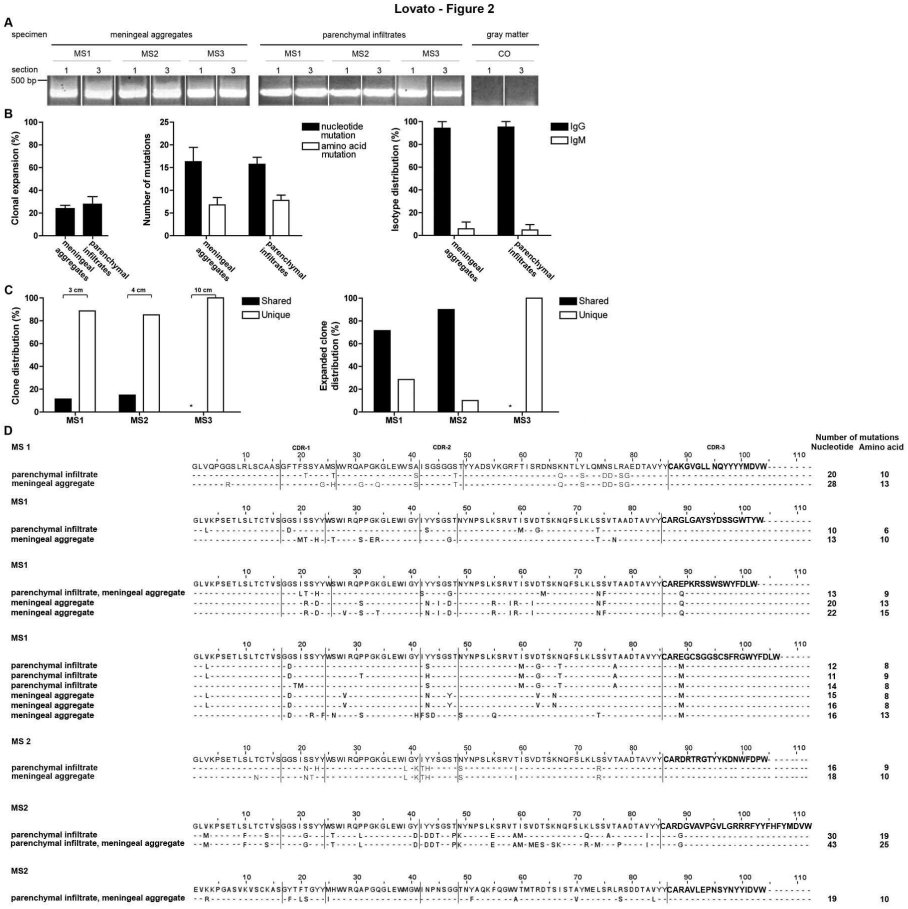

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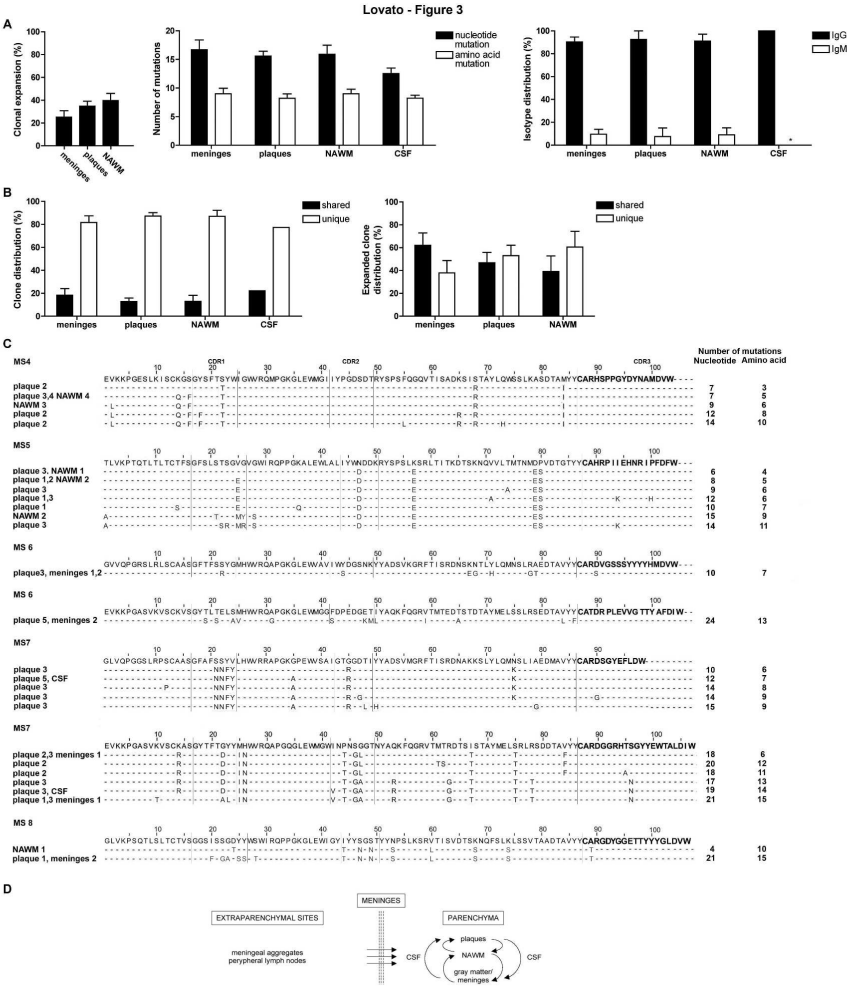


Clonally related B cells populate the meninges, plaques, NAWM and CSF in the MS CNS. Analysis of the B cell repertoires derived from the meninges, plaques, NAWM and CSF of eleven MS brains demonstrated the characteristic features of antigen experience for the B cells present in all locations (A). Sequence comparison between the BCR repertoires derived from different locations determined that the majority of the B cells resided exclusively in one area, however a small proportion of clones were shared among different locations (B). Only the MS3 case did not show any shared clones. Analysis of the subset of B cells that were clonally expanded revealed that 39% to 62% of these clones populated different locations within the MS CNS (B). Representative examples of sequence alignments from seven B cell clones shared among CNS compartments, including meninges, plaque, NAWM and CSF (C). Distinct B cell clones, activated in extra-parenchymal sites such as peripheral lymph nodes or meningeal lymphoid structures, might populate different anatomical locations of MS brain and potentially contribute to the tissue damage. Further diffusion and seeding of a subset of B cells to multiple locations of the CNS may occur through the CSF circulation (D).

169x169mm (600 x 600 DPI)

Subject	Age (years) Gender	Clinical course	Post-mortem interval (hours)	Number of anatomical regions examined			
				gray matter	plaque	NAWM	CSF
MS1	77 F	secondary progressive	7	1	1+	-	-
MS2	59 F	secondary progressive	21	1	1+	-	-
MS3	43 M	secondary progressive	26	1	1+	-	-
MS4	38 F	relapsing remitting	9.5	3	4	4	-
MS5	65 M	chronic progressive	29.4	2	3	3	-
MS6	43 F	chronic progressive	< 2	2	5	2	-
MS7	39 F	chronic progressive	17.5	2	3	2	1
MS8	78 M	secondary progressive	15.5	2	2	2	-
MS9	75 M	secondary progressive	12.9	2	2	2	-
MS10	53 F	secondary progressive	10.3	2	2	2	-
MS11	75 F	secondary progressive	13.8	2	2	2	-

Lovato - Supplementary Table 1

Clinical and demographic features of MS patients from which the specimens were derived.

For each patient the number of samples per area (plaques, NAWM, grey matter and CSF) is specified. For MS-1, 2, 3 the disease duration was 21, 39 and 18 years, for MS-6 and MS-7 it was 20 and 13 years. For the other cases the clinical data was not available. + Parenchymal infiltrates.

Subject	area	plaque, NAWM, gray matter *	lesion activity	axonal loss (%)	demyeli-nation **	microglia / macrophages ***	T cells (CD3) ***	B cells (CD20) ***	plasma cells (CD138) ***
MS1	1	gray matter containing meningeal aggregates						+++	
		plaque +						+++	
MS2	1	gray matter containing meningeal aggregates						++++	
		plaque +						++++	
MS3	1	gray matter containing meningeal aggregates						+++	
		plaque +						++	
MS4	1	a-gray matter	-	0	-	-	-	-	-
		plaque	+	80	+	++++	++	+++	++
		a-nawm	-	0	-	+	+	+	-
	2	a-gray matter	-	0	-	-	-	-	-
		plaque	+	40	+	+++	+	+++	+
		a-nawm	-	0	-	-	-	+	-
	3	a-gray matter (cbl)	-	0	-	-	-	-	-
		plaque (cbl)	+	30	+	+++	++	++	+
		a-nawm (cbl)	-	0	-	+	+	+	-
	4	plaque	-	20	+	+++	++	++	-
		a-nawm	-	0	-	-	+	+	-
MS5	1	a-gray matter	-	0	-	-	-	-	-
		plaque	+	20	+	++	+	+++	-
		a-nawm	-	0	-	-	-	+	-
	2	a-gray matter	-	0	-	-	-	-	-
		plaque	+	70	+	+++	++	+	+
		a-nawm	-	0	-	-	-	+	-
	3	plaque	+	30	+	++	+	+	-
		a-nawm	-	0	-	-	+	+	-
MS6	1	d-gray matter	-	0	-	-	-	-	-
		d-nawm	-	0	-	++	+	+	-
	2	d-gray matter (cbl)	-	0	-	-	-	-	-
		d-nawm (cbl)	-	0	-	++	+	+	-
	3	plaque	+	20	+	++	+++	+++	-
	4	plaque	+	30	+	++	+++	+++	-
	5	plaque	-	50	+	+	++	++	-
	6	plaque	+	20	+	+	++	++	-
	7	plaque	-	30	+	+	+++	+++	+

MS7	1	d-gray matter	-	0	-	+	+	-	-
	2	d-gray matter	-	0	-	+	+	-	-
	3	plaque	+	20	+	++	++++	+++	-
	4	plaque	+	30	+	-	++++	+++	-
	5	plaque	+	20	+	+	++++	++++	-
	6	d-nawm	-	0	-	-	+++	-	-
	7	d-nawm	-	0	-	+	+++	+	-
MS8	1	a-gray matter	-	0	-	+	+	+	-
		plaque	-	100	+	-	+++	++++	-
		a-nawm	-	0	+	-	+++	+	-
	2	a-gray matter	-	0	-	-	+	-	-
		plaque	-	100	+	-	+	+	-
		a-nawm	-	0	+	-	+	-	-
MS9	1	a-gray matter	-	0	-	+	+++	+	-
		plaque	-	80	+	-	+++	+	-
		a-nawm	-	0	-	-	+++	+	-
	2	a-gray matter	-	0	-	+	+	-	-
		plaque	-	80	+	-	+++	+	-
		a-nawm	-	0	-	-	++	-	-
MS10	1	a-gray matter	-	0	-	+	+	-	-
		plaque	-	100	+	-	++	+	-
		a-nawm	-	0	-	-	+	-	-
	2	a-gray matter	-	0	-	++	++	-	-
		plaque	-	100	+	-	++	-	-
		a-nawm	-	0	-	-	-	-	-
MS11	1	a-gray matter	-	0	+	+	+	-	-
		plaque	-	75	+	-	++	++	-
		a-nawm	-	0	+	-	+	-	-
	2	a-gray matter	-	0	-	-	-	-	-
		plaque	-	75	+	-	++	+	-
		a-nawm	-	0	-	-	+	-	-

Lovato - Supplementary Table 2

Pathological features of gray matter, plaque and NAWM specimens of MS brains.

For each specimen we evaluated lesion activity, axonal loss, demyelination, activated microglia/macrophages and lymphocyte composition of the infiltrates. The gray matter specimens of MS1, 2, 3 cases showed structurally organized aggregates of B cells at the meningeal layers. The B cell aggregates were considered *bona fide* ectopic follicles-like structures, previously described. Only the CD20 staining was performed on these three cases because of the limited size of tissue. All plaques contained regions of demyelination (LFB staining), while all NAWM specimens were normally myelinated. CD3 staining showed the presence of abundant T cell infiltrates in all the plaques while scattered CD3 positive cells were observed in the NAWM. In the MS4-MS11 brains several CD3 positive cells were identified in the meninges. The majority of the plaque and NAWM infiltrates were mostly composed of T lymphocytes, including when plentiful B cell infiltrates were present. The CD138 staining showed the presence of plasma cells in 5 plaques and in 4 gray matter specimens. The majority of the plaques (12/19) were defined as active due to the presence of abundant perivascular infiltrates, with a different level of axonal loss (20-80%). * a: adjacent; d: distant; cbl: cerebellum. ** The presence or absence (+/-) of myelin was evaluated by Luxol Fast Blue staining (LFB). *** The quantitation of each region is indicated as follow: + 1-3 cells per 100X field, ++ 4-6 cells per 100X field, +++ 6-10 cells per 100X field, ++++ more than 10 cells per 100X field. $^{+}$ parenchymal infiltrates.

Subject	Specimen (n)	V gene usage (%)						
		1	2	3	4	5	6	7
	gray matter							
MS1	1	9	8	33	50 *	0 *	0	0
MS2	1	6	0	76 *	18	0 *	0	0
MS3	1	0 *	17	33	50 *	0 *	0	0
MS4	4	0 *	0	33	67 *	0 *	0	0
MS5	3	0 *	0	33	67 *	0 *	0	0
MS6	2	33	0	67 *	0 *	0 *	0	0
MS7	2	11	5	26	58 *	0 *	0	0
MS8	2	13	0	62	25	0 *	0	0
MS9	2	0 *	36 *	21 *	36 *	7	0	0
MS10	2	30	0	60	10	0 *	0	0
MS11	2	9	9	46	27	9	0	0
	plaque							
MS1	1^{+}	0 *	0	43	57 *	0 *	0	0
MS2	1^{+}	7	0	43	43 *	7	0	0
MS3	1^{+}	12	25 *	38	25	0 *	0	0
MS4	4	24	10	43	9	14	0	0
MS5	3	0 *	7	40	0 *	47 *	0	6
MS6	5	27	3	47	13	9	1	0
MS7	3	29	19	42	0 *	10	0	0
MS8	2	0 *	12	62	25	0 *	0	0
MS9	2	0 *	7	57	36 *	0 *	0	0
MS10	2	40 *	0	40	20	0 *	0	0
MS11	2	21	0	36	36 *	7	0	0

		Brain						
	NAWM							
MS4	4	8	8	31	38 *	15	0	0
MS5	3	8	8	67 *	8	8	0	0
MS6	2	9	0	64	9	18	0	0
MS7	2	0 *	0	44	44 *	12	0	0
MS8	2	10	0	40	40 *	1 *	0	0
MS9	2	17	17	0 *	66 *	0 *	0	0
MS10	2	17	0	33	17	33 *	0	0
MS11	2	8	8	25	42 *	17	0	0
	CSF							
MS7	1	7	28 *	65 *	0 *	0 *	0	0
HD PBB #	3	16	7	44	14	15	4	0
Germline distribution		15	9	42	28	3	1	2

Lovato - Supplementary Table 3

VH gene family usage in the MS CNS areas.

We observed a skewed VH gene family usage in all of the MS brain areas, including meninges, plaques, NAWM and CSF. The VH gene family usage in the peripheral blood B lymphocytes of control subjects reflected the relative distribution of the VH gene germline in the different families.

* statistically different than PBB of healthy donors ($p<0.005$, Fisher's exact test).

the values are expressed as mean of the three subjects

$^{+}$ parenchymal infiltrates

PBB: Peripheral Blood B lymphocytes

Subject	Compartment	Number of clones	Expanded clones	Shared clones and corresponding distribution			Total number of clones per each subject
MS1	meninges	30	7	5	a	meninges, plaque	42
					b	meninges, plaque	
					c	meninges, plaque	
					d	meninges, plaque	
					e	meninges, plaque	
	plaque	17	7	5	a	meninges, plaque	
					b	meninges, plaque	
					c	meninges, plaque	
					d	meninges, plaque	
					e	meninges, plaque	
MS2	meninges	17	5	4	a	meninges, plaque	27
					b	meninges, plaque	
					c	meninges, plaque	
					d	meninges, plaque	
	plaque	14	4	4	a	meninges, plaque	
					b	meninges, plaque	
					c	meninges, plaque	
					d	meninges, plaque	
MS3	meninges	10	2	0			35
	plaque	25	4	0			
MS4	meninges *	3	2	2	a	meninges 3, NAWM 4	27
					b	meninges 3, NAWM 4	

	plaques	20	9	2	c	plaque 2, 3, 4 NAWM 3, 4	
					d	plaque 2, NAWM 2	
	NAWM	8	6	4	a	meninges 3, NAWM 4	
					b	meninges 3, NAWM 4	
					c	plaque 2, 3, 4 NAWM 3, 4	
					d	plaque 2, NAWM 2	
MS5	meninges	3	1	0			26
	plaques	15	10	2	a	plaque 1, 2, 3 NAWM 1	
					b	plaque 1, 2	
	NAWM	9	5	1	a	plaque 1, 2, 3 NAWM 1	
MS6	meninges	12	4	4	a	meninges 1, 2 plaque 3	81
					b	meninges 2, plaque 5	
					c	meninges 1, 2 plaque 3, 5, 6	
					d	meninges 1, plaque 7	
	plaques	64	11	5	a	meninges 1, 2 plaque 3	
					b	meninges 2, plaque 5	
					c	meninges 1, 2 plaque 3, 5, 6	
					d	meninges 1, plaque 7	
					e	plaque 7, NAWM 1	
	NAWM	10	2	1	e	plaque 7, NAWM 1	
MS7	meninges	12	3	2	a	meninges 1, plaque 1, 2, 3, CSF	63
					b	meninges 2, plaque 4	
	plaques	31	13	7	a	meninges 1, plaque 1, 2, 3, CSF	
					b	meninges 2, plaque 4	
					c	plaque 4, 5, NAWM 2	

					d	plaque 3, CSF	
					e	plaque 3, 5, CSF	
					f	plaque 4, 5, CSF	
					g	plaque 4,5	
	NAWM	9	3	1	c	plaque 4, 5, NAWM 2	
	CSF	18	n.a.	4	a	meninges 1, plaque 3, 4, 5, CSF	
					d	plaque 3, CSF	
					e	plaque 3, 5, CSF	
					f	plaque 4, 5, CSF	
MS8	meninges	8	2	1	a	meninges 2, plaque 1, NAWM 1	24
	plaques	8	1	1	a	meninges 2, plaque 1, NAWM 1	
	NAWM	10	4	2	a	meninges 2, plaque 1, NAWM 1	
					b	NAWM 1, 2	
MS9	meninges	14	1	1	a	meninges 2, plaque 1, 2	26
	plaques	7	2	1	a	meninges 2, plaque 1, 2	
					b	plaque 1, 2	
	NAWM	6	3	0			
MS10	meninges	10	2	2	a	meninges 1, plaque 2, NAWM 2	32
					b	meninges 1, plaque 2	
	plaques	20	5	2	a	meninges 1, plaque 2, NAWM 2	
					b	meninges 1, plaque 2	
					c	plaque 1, NAWM 1	
	NAWM	6	2	1	a	meninges 1, plaque 2, NAWM 2	
					c	plaque 1, NAWM 1	
MS11	meninges	12	2	0			38

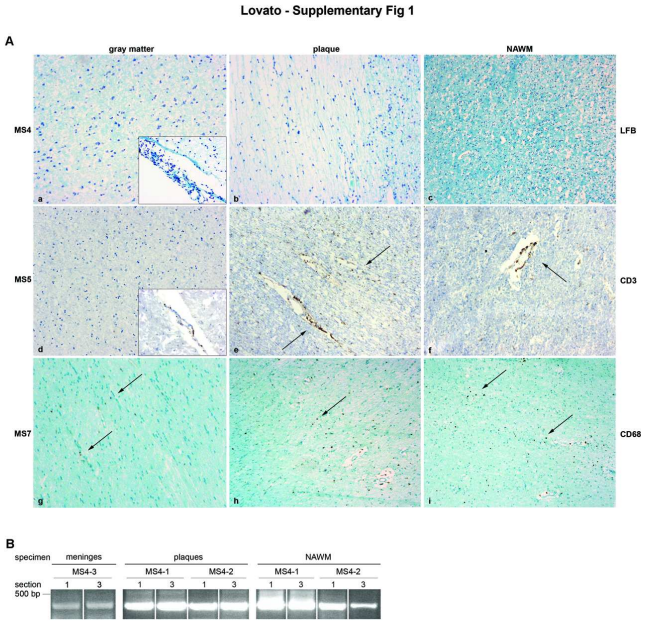
plaques	14	9	0		
NAWM	12	2	1	a	NAWM 1, 2

Lovato - Supplementary Table IV

Distribution of B cell clones in the different compartments of MS CNS.

For each MS brain, the number of different clones per area (meninges, plaques, NAWM and CSF) and the relative fraction of expanded clones and shared clones in multiple areas are shown. The total number of clones per subject is calculated from to the number of different clones per area, subtracting the shared clones.

n.a. not available * VH sequences where amplified only from meninges 3



Immunohistochemical and molecular characterization of the immune cell infiltrates in different areas of MS brain.
Immunohistochemical analysis was performed on 17 gray matter (containing meninges), 23 plaque and 19 NAWM specimens derived from nine MS brains to characterize lesion activity, axonal loss, demyelination and to enumerate immune cell infiltrates (Supplementary Table 2) (A). All plaques contained regions of demyelination, while all NAWM and gray matter specimens were normally myelinated (Luxol Fast Blue staining, LFB, a, b, c). CD3 staining showed the presence of several CD3 positive cells in the meninges, particularly in the cerebral sulci, of all the gray matter specimens examined. No CD3 positive T cells were identified in the parenchyma of the same specimens (d). In contrast, abundant perivascular and parenchymal T cell infiltrates were detected in plaques, while scattered CD3 positive cells associated with the vessels were observed in the NAWM (e, f: CD3 positive cells are indicated by arrows). CD68 staining showed microglia activation in NAWM areas as well as in plaques and in gray matter parenchyma (g, h, i: CD68 positive cells are indicated by arrows). The PCR products of BCR VH chains from different areas of MS specimens are shown (B). A band of 350 bp is shown from two non-serial sections of MS meningeal infiltrates (MS4-3), MS plaques (MS4-1 and MS4-2, section 1 and 3) and MS NAWM (MS4-1 and MS4-2, section 1 and 3).
170x150mm (300 x 300 DPI)