

Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis

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Meningeal inflammation in the form of ectopic lymphoid-like structures has been suggested to play a prominent role in the development of cerebral cortical grey matter pathology in multiple sclerosis. The aim of this study was to analyse the incidence and distribution of B cell follicle-like structures in an extensive collection of cases with secondary progressive multiple sclerosis with a wide age range and to determine their relationship to diffuse meningeal inflammation, white matter perivascular infiltrates and microglial activation. One hundred and twenty three cases with secondary progressive multiple sclerosis were examined for the presence of meningeal and perivascular immune cell infiltrates in tissue blocks and/or whole coronal macrosections encompassing a wide array of brain areas. Large, dense, B cell-rich lymphocytic aggregates were screened for the presence of follicular dendritic cells, proliferating B cells and plasma cells. Ectopic B cell follicle-like structures were found, with variable frequency, in 49 cases (40%) and were distributed throughout the forebrain, where they were most frequently located in the deep sulci of the temporal, cingulate, insula and frontal cortex. Subpial grey matter demyelinated lesions were located both adjacent to, and some distance from such structures. The presence of B cell follicle-like structures was associated with an accompanying quantitative increase in diffuse meningeal inflammation that correlated with the degree of microglial activation and grey matter cortical demyelination. The median age of disease onset, time to disease progression, time to wheelchair dependence and age at death all differed significantly in these cases when compared with those without B cell follicle-like structures. Our findings suggest that meningeal infiltrates may play a contributory role in the underlying subpial grey matter pathology and accelerated clinical course, which is exacerbated in a significant proportion of cases by the presence of B cell follicle-like structures.

Keywords: B cell follicle; clinical disability; grey matter lesion; microglia; neuropathology

Abbreviations: F+ = follicle-like positive; F– = follicle-like negative; SPMS = secondary progressive multiple sclerosis

Introduction

Secondary progressive multiple sclerosis (SPMS) is defined clinically by the progressive accumulation of neurological dysfunction following a period of relapses and remissions (Compston and Coles, 2008). Pathologically, SPMS is characterized by the presence of chronic demyelinated lesions in the white matter, diffuse inflammation, axonal loss, changes to the normal appearing white matter and substantial cortical grey matter pathology (Peterson *et al.*, 2001; Bo *et al.*, 2003*b*; Kutzelnigg *et al.*, 2005; Zeis *et al.*, 2008; Frischer *et al.*, 2009; Howell *et al.*, 2010; Magliozzi *et al.*, 2010), the latter being suggested as an important determinant of neurological disability (Calabrese *et al.*, 2010*a*). The frequency of new white matter lesion formation and the number of infiltrating peripheral immune cells is decreased in the progressive phase of the disease and it is suggested that the immune response becomes largely confined to the CNS behind a relatively intact blood–brain barrier (Lassmann *et al.*, 2007; Mehl *et al.*, 2008).

We have previously described the presence of ectopic B cell follicle-like structures in the cerebral meninges of a proportion of cases with SPMS and suggested that these could be an important site of immune cell activation and expansion in chronic disease (Serafini *et al.*, 2004, 2007; Magliozzi *et al.*, 2007, 2010). In autoimmune conditions, such as rheumatoid arthritis, Sjögren's syndrome, myasthenia gravis and autoimmune thyroiditis, chronic inflammation within the target organ is frequently accompanied by the generation of ectopic B cell follicles containing organized B and T cell areas reminiscent of those present in secondary lymphoid tissues (Armengol *et al.*, 2001; Magalhaes *et al.*, 2002; Aloisi and Pujol-Borrell, 2006; Carragher *et al.*, 2008). The pathophysiological role of these ectopic or tertiary lymphoid tissues is still unclear, but it is thought that these structures may represent a local source of pro-inflammatory mediators, autoantibodies and self-reactive T cells (Aloisi and Pujol-Borrell, 2006; Carragher *et al.*, 2008).

The subarachnoid and perivascular spaces of the CNS are sites of T and B cell (re)activation, where circulating lymphocytes establish contacts with antigen-presenting cells at vascular loci, similar to that which occurs in lymphoid organs (Bartholomaeus *et al.*, 2009; Kivisakk *et al.*, 2009). The presence of abundant lymphocytic infiltrates in the meninges and perivascular areas in progressive multiple sclerosis (Hassin, 1921; Guseo and Jellinger, 1975) and the finding of lymphatic capillaries and reticular cells, key elements of lymphoid-like tissues, inside the perivascular cuffs of white matter lesions (Prineas, 1979), have been previously described. In agreement with our description of the presence of B cell follicle-like tissues in the post-mortem multiple sclerosis brain (Serafini *et al.*, 2004, 2007; Magliozzi *et al.*, 2007, 2010), analysis of patient CSF has identified the recapitulation of all features of B cell development, normally restricted to lymphoid organs (Corcione *et al.*, 2004). Furthermore, clonally related B lymphocytes are seen in demyelinated lesions, meninges and CSF, suggesting that a local expansion of B-cell clones occurs and persists over time within the CNS (Colombo *et al.*, 2000; Owens *et al.*, 2003; Lovato *et al.*, 2011).

The presence of an extensive subpial ribbon-like pattern of demyelination in cases with SPMS with prominent meningeal inflammation (Kutzelnigg *et al.*, 2005; Magliozzi *et al.*, 2007; 2010), and the almost total absence of lymphocytes and macrophages in cortical lesions (Bo *et al.*, 2003*a, b*), precludes a role for perivascular inflammation and suggests that an immune response in the inflamed meninges might play a role in the pathogenesis of the underlying grey matter pathology (Peterson *et al.*, 2001; Kutzelnigg *et al.*, 2005; Magliozzi *et al.*, 2007; Dal Bianco *et al.*, 2008). Both the diffuse inflammatory cell infiltrates and the ectopic lymphoid-like tissues of the meninges represent a source of pro-inflammatory cytokines (Magliozzi *et al.*, 2010), lytic enzymes (Serafini *et al.*, 2007) and immunoglobulins that could contribute to the damaging environment. Consistent with this, cases with meningeal B cell follicle-like structures have more extensive grey matter demyelination and oligodendrocyte loss, greater cortical atrophy and increased numbers of activated microglia in the outer cortical layers. The elevated inflammatory milieu is accompanied by damage to the glia limitans and a gradient of neuronal loss, greatest in the superficial cortical lamina nearest the pial surface (Magliozzi *et al.*, 2010). Cases that lack significant meningeal inflammation and detectable follicle-like structures have far less severe cortical pathology. However, it is important to note that a number of other studies have failed to identify such structures or recognize a role for meningeal inflammation in mediating subpial pathology, so this issue requires clarification (Kooi *et al.*, 2009; Torkildsen *et al.*, 2010).

The mechanisms involved in cortical grey matter pathology have been hitherto little studied. Therefore, there is a need to further explore the extent and role of meningeal inflammation and B cell follicle-like structures in the pathogenesis of multiple sclerosis. Here, we report an analysis of 2760 tissue blocks and 48 whole coronal macrosections from 123 post-mortem SPMS brains to definitively describe the location and frequency of ectopic lymphoid-like structures, and the relationship that exists between elevated meningeal inflammation, grey matter demyelination and progression of disease.

Materials and methods

Criteria for selecting cases

Tissue blocks and whole coronal brain sections for this study were provided by the UK Multiple Sclerosis Tissue Bank at Imperial College, London, UK. All tissues were collected with fully informed consent via a prospective donor scheme with ethical approval by the National Research Ethics Committee (08/MRE09/31). One hundred and twenty-three cases with SPMS were selected to ensure a wide age range at death (median age at death = 58, age range 30–83 years; Table 1) that reflects the entire SPMS cohort in the UK Multiple Sclerosis Tissue Bank (median age = 60, range 30–96 years, $n = 450$). The diagnosis of multiple sclerosis was confirmed based on the patient history (summarized by R.N.) and a detailed neuropathological analysis (provided by B.R., S.M.G. and F.R.). Individual case details, including the age at which an individual received disease-modifying therapies ($n = 22$) and the duration of the therapy (if known), are noted in Supplementary Table 1.

Table 1 Characteristics of the SPMS cases examined

Cohort	Sex	Age onset	Age progressive	Age died	Disease duration
123	78 F: 45 M	30 ± 6.5 years (9–64)	40.5 ± 6 years (20–66)	58 ± 10 years (30–83)	25 ± 8 years (2–52)

Median ± half interquartile range. Range shown in brackets.
F = female; M = male.

Post-mortem tissue and block selection

Tissue blocks (2 × 2 × 1 cm) were prepared from whole coronal slices dissected immediately on brain retrieval and fixed in 4% paraformaldehyde for a minimum of 12 h and either processed for paraffin embedding or cryoprotected in 30% sucrose in preparation for cryosectioning at 10 µm. Whole coronal bi-hemispheric macrosections were prepared from 16 brains, for which we had no prior information on the extent of inflammation or demyelination, that had been whole fixed by immersion in formalin for a minimum of 6 weeks (range 6 weeks–57 months, median = 25 months). Three 1 cm thick coronal slices were taken from each brain at the following levels: (i) anterior septum; (ii) posterior caudate including motor cortex; and (iii) occipital horn of the lateral ventricles, thus representing a large number of sampled forebrain areas. Coronal 1 cm slices from these three regions were dehydrated, wax impregnated under vacuum and whole bi-hemispheric (10 µm) macrosections cut on a Reichart-Jung tetrandr for standard histological and immunohistochemical assessment.

Identification of B cell follicle-like structures in progressive multiple sclerosis

Screening protocol

Each case was examined for the presence of lymphoid-like structures. Initially paraffin-embedded slides, taken as part of the routine neuropathological assessment and stained with Luxol Fast Blue and Cresyl Fast Violet or haematoxylin and eosin, were examined for the presence of meningeal and perivascular infiltrates (Fig. 1A). Brain areas examined for all the cases included the superior frontal gyrus (sampled 1 cm rostral to the temporal pole), the cingulate gyrus at the level of the nucleus accumbens, the superior temporal gyrus and hippocampal blocks at the level of the lateral geniculate body, the parietal cortex 1 cm caudal to the splenium of the corpus callosum and the precentral gyrus at the level of the pulvinar nucleus of the thalamus. The primary visual (striate) and non-striate occipital cortices were sampled 1.5 cm rostral to the occipital pole. To supplement the study of paraffin-embedded sections, additional fixed-frozen cryosections were prepared from tissue blocks sampled from a wide range of brain areas. In the event that stained slides lacked preserved meninges, additional sections/blocks were sampled. In all instances, only slides containing an intact meningeal compartment were considered for assessment of infiltrates.

Previous studies have shown that cases containing ectopic B cell follicle-like structures have an associated increase in parenchymal, perivascular and meningeal inflammatory infiltrates reflecting the ongoing inflammatory activity of disease at the time of death (Magliozzi *et al.*, 2007). Therefore, on screening haematoxylin and eosin-stained sections from each tissue block, an index of inflammation was assigned to

each case based on the maximum density of meningeal and/or perivascular infiltrates seen (Fig. 1A). Cases without a single example of a moderate meningeal or perivascular infiltrate (score '0', Fig. 1; equivalent of <5 cells per infiltrate in any portion of perivascular or meningeal space using a ×20 objective) noted in the analysis of histological sections from a minimum of 12 tissue blocks were recorded as negative for the presence of follicle-like structures (F–), and no further blocks were examined for these cases. For cases presenting at least a single example of moderate cellular infiltration (+, Fig. 1; equivalent to an infiltrate of 5–50 loosely packed cells in perivascular or meningeal areas), a further 10–18 fixed-frozen tissue blocks from forebrain areas containing cortical sulci were sampled. Only tissue blocks (paraffin-embedded or fixed-frozen blocks) containing substantial (++) meningeal or perivascular infiltrates present as a dense cluster of small, round lymphocytic cells (>50 cells) resembling potential follicle-like structures were processed further for anti-CD20 immunohistochemistry. We did not perform CD20 immunostaining on tissue sections containing moderate (+) infiltrates, because the definition of B cell follicle-like structures requires the presence of immune cell aggregates. It should be noted that infiltrates designated as 'moderate, +' could possibly represent the start or end of larger cellular accumulations in some instances. Therefore, it is possible that our investigation may have underestimated the degree of maximal inflammation, or indeed the overall incidence of follicle-like positive (F+) SPMS. If no significant aggregates of CD20+ B cells (<10 CD20+ cells with loosely packed configuration) were evident in the extended sampling of cases of interest (++) rated cases, they were characterized as follicle negative (number of blocks screened for cases identified as follicle negative 22–30). Screening for follicle-like structures in cases where whole coronal macrosections were available was performed on both the whole coronal sections and on additional small tissue blocks taken as part of the routine neuropathological examination, to ensure that the same areas were sampled for all 123 cases.

Immunohistochemical identification of B cell follicle-like structures

Cases with multiple sclerosis were categorized as follicle-like positive SPMS if at least one aggregate of CD20+ B cells was identified together with the presence of CD35+ follicular dendritic cells; proliferating Ki67+ CD20+ cells; and IgA-, -G-, -M+ plasmablasts/plasma cells. The equivalent incidence of lymphoid-like aggregates per 4 cm² tissue block was calculated by expressing the number of F+ blocks as a percentage of the total number of tissue blocks sampled per case. Cases were identified as F+ on the basis of the presence of a single ectopic B cell follicle-like structure in the brain tissue blocks examined.

Paraffin-embedded sections were de-waxed and rehydrated and fixed-frozen cryosections were air dried before commencement of immunostaining. To best preserve the delicate meningeal structure, antigen retrieval was performed in a household vegetable steamer, which caused less tissue disruption in comparison to other forms

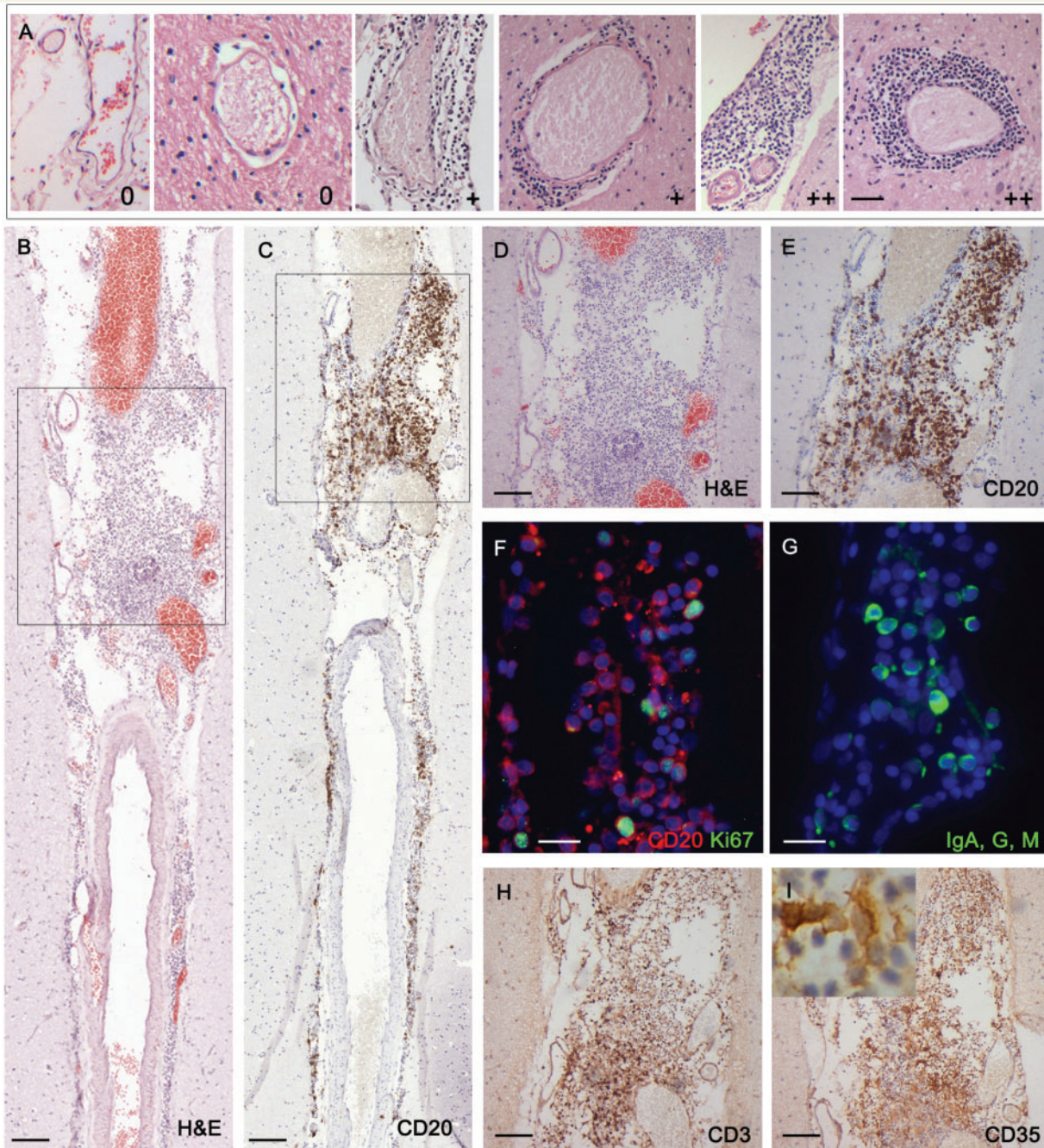


Figure 1 Screening for the presence of follicle-like structures. (A) Tissue blocks ($n = 12$) were examined by heamatoxylin and eosin histology and scored based on the degree of perivascular and meningeal infiltrates (score 0, mild; +, moderate and ++, substantial). Cases containing at least a single tissue block harbouring at least one moderate perivascular or meningeal cellular aggregate (B and D) were examined further (additional 10–18 tissue blocks per case), while cases without even such moderate inflammation (score 0) were regarded as follicle negative at this point and were not sampled further. Tissue blocks containing notable follicle-like aggregates were immunostained for CD20 to determine the extent of B cell infiltration (C and E) and subsequent sections were stained for the presence of proliferating Ki67⁺/CD20⁺ B cells (F), immunoglobulin A, G, M⁺ plasma cells/plasmablasts (G), CD3⁺ T cells (H) and CD35⁺ follicular dendritic cells (I). Scale bars: (A) = 50 μ m; (B and C) = 400 μ m; (D), (E), (H) and (I) = 200 μ m; (F and G) = 20 μ m.

of heat-induced antigen unmasking. The primary antibodies used in this study are detailed in Supplementary Table 2 and immunostaining was performed as previously described (Magliozzi *et al.*, 2007, 2010).

Whole hemispheric coronal sections

In order to study the global extent of cortical and white matter demyelination, the relationship between meningeal lymphoid-like

structures and cortical demyelination and the distribution of lymphoid-like aggregates, we performed histology and immunohistochemistry on bi-hemispheric coronal sections (48 sections), representing three separate coronal forebrain slices (as represented in schematics in Fig. 2)

prepared from a cohort of 16 available whole brains (as detailed in Supplementary Table 1). Analysis of demyelination was restricted to bi-hemispheric sections as quantification of lesion areas in sections from small tissue blocks is hindered by the fact that lesions, particularly

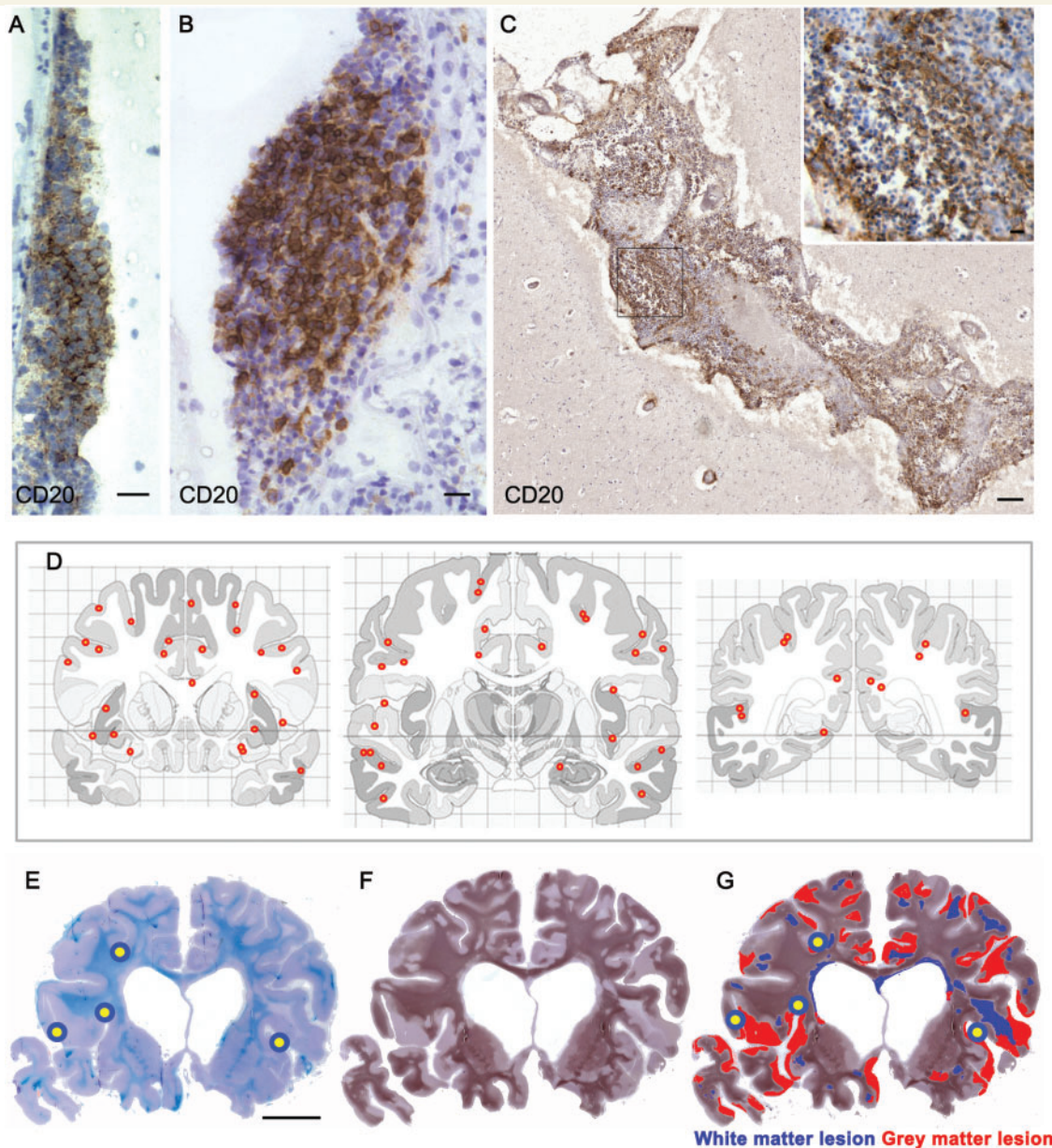


Figure 2 Follicle-like structures are variable in size and anatomical location. Aggregates of B cells in follicle-like structures can vary greatly in overall size and cell density from relatively modest (A and B) to large and extensive aggregates filling an entire cerebral sulcus (C). (D) Schematics represent the three coronal levels analysed per F+ SPMS case where whole coronal sections were available (nine cases) onto which the location of the 61 separate meningeal follicle-like aggregates identified were plotted (red dots). Our analysis demonstrates that follicle-like structures are frequently associated with the deep infoldings of the cerebral sulci and are widely distributed throughout the extent of the sampled forebrain. Analysis of whole-brain coronal sections stained with Luxol Fast Blue/haematoxylin (E, to identify areas of white/grey matter) and myelin oligodendrocyte glycoprotein immunohistochemistry (F, which best illustrates areas of grey matter demyelination), revealed extensive areas of white matter and cortical grey matter demyelination in some cases (blue mask highlights white matter lesions, red mask highlights grey matter demyelination, G). By highlighting the location of meningeal B cell follicle-like structures identified in this slice, we are able to demonstrate that meningeal aggregates are found adjacent to areas of subpial grey matter demyelination in the left medial and inferior frontal gyri, and insular gyri of both hemispheres, although grey matter demyelination was also evident some distance from such structures (G). Scale bars: (A), (B) and inset in (C) = 20 μ m, (C) = 200 μ m, (E–G) = 20 mm.

subpial lesions, often extend over several centimetres and cannot be studied in their entirety within individual blocks.

Quantification of white matter and grey matter demyelination

To determine the extent of white matter and grey matter demyelination, serial coronal sections stained by both Luxol Fast Blue and haematoxylin (which most clearly delineates areas of white matter) and myelin oligodendrocyte glycoprotein immunohistochemistry (to detect grey matter demyelination) were compared. Images were captured by scanning at 1200 dpi using a flat-bed scanner and areas of white matter lesions and grey matter lesions (sum of all subpial, intracortical and leucocortical demyelinated grey matter) manually outlined on the digitized images by reference to microscopic examination of the stained tissue preparations. Areas of demyelination from three whole coronal sections taken from three separate 1 cm brain slices (Fig. 2) per case were highlighted by applying a colour mask using Adobe Photoshop CS2 (Adobe Systems) and total section area (mm²), white matter area, grey matter area and the proportion of total white matter and grey matter demyelination calculated.

Quantification of meningeal and parenchymal inflammation

Meningeal, grey matter parenchymal and white matter perivascular inflammation was quantified on region-matched sections of superior frontal gyrus (sampled 1 cm rostral to the temporal pole), the superior temporal gyrus, hippocampus (both at the level of the lateral geniculate body) and the primary visual (striate) cortex (sampled 1.5 cm rostral to the occipital pole) from 12 F+, 12 F– and six controls (including the 9 F+ and 7 F– cases where whole brains were available; see Supplementary Table 1 for details). Inclusion of the cases where whole coronal sections were available allowed us to compare inflammation to global measures of demyelination in these cases (as described above). CD3⁺, CD20⁺ and CD68⁺ cell counts were determined in four non-continuous fields ($\times 200$, total area imaged 0.29 mm²) of preserved portions of meninges in tissues directly overlying regions of normal appearing grey matter and grey matter lesions (eight sampled fields per block, four blocks per case), determined on serial sections stained with myelin oligodendrocyte glycoprotein. Cell numbers were expressed as mean number per mm of intact meninges. Parenchymal CD68⁺ microglial density was quantified from four $\times 200$ images captured from each of the underlying subpial normal appearing grey matter and grey matter lesions, ensuring the pial surface was present in every captured image to ensure that an even depth of cortex was sampled for each case. Cell density was expressed as mean CD68⁺ cells/mm². White matter perivascular T and B cell infiltrates were quantified from four vessels (veins and/or arteries) in cross-section presenting with a thin tunica media and with a total area (vessel and perivascular space) >0.003 mm² on microscopic examination of previously characterized normal appearing white matter and white matter lesion centres (active or chronic active lesion status determined by major histocompatibility complex-II and myelin oligodendrocyte glycoprotein immunohistochemistry). The mean density of perivascular T or B lymphocytes per mm² of measured perivascular space ($n = 8$ vessels) was calculated and the mean values per case (four tissue blocks) compared.

Assessment of disease milestones and their association with pathological measures

The number of clinical relapses in the first 2 years from disease onset, the time to conversion to secondary progression, the time at which the patient required the use of a wheelchair and the age at death were compiled from the detailed clinical histories (data from records provided by the patient's physician and from hospital letters) by a consultant neurologist with a specialist interest in multiple sclerosis (R.N.). The availability of general practitioner and hospital notes allowed the cross-checking of information for confirmation. The completeness of patient records were graded from 0 (no information available) to 5 (complete records available). The majority of patient records were graded 5 and 117 out of 123 (95%) had records of ≥ 4 . If the completeness of the available data was graded ≤ 3 , these data were not included in our analysis. Clinical milestones were compared with follicle status (F– or F+) and the cumulative index of forebrain perivascular/meningeal inflammation (0–3) from the screening of tissue blocks for all 123 cases examined (score 0–2 as detailed above, with the inclusion of inflammatory score 3 = substantial inflammation and the presence of B cell follicle-like aggregates).

Image analysis and experimental details

Tissue sections were analysed on a Nikon E1000M microscope using brightfield or epifluorescence imaging (Nikon Instruments Inc.) with a digital camera (QImaging). Digitized images were analysed using Image ProPlus (Media Cybernetics) and ImageJ (<http://rsb.info.nih.gov/ij/>) and prepared in Adobe Photoshop for publication. All quantifications were performed with the observer blinded to case identification and follicle-like status.

Data analysis and correlations

Data were handled in Excel (Microsoft Office 2007) and compared using GraphPad PRISM (Ver. 5.0, GraphPad Inc.) or SPSS statistics (version 19, IBM Inc.). Data were expressed as median \pm half interquartile range or mean \pm SD or SEM where noted. Statistical comparisons were made using the non-parametric Mann–Whitney test, Fisher's exact test, Cox proportional hazards model, Wald test or ANOVA and suitable post-test as detailed in the 'Results' section. Testing for correlation between two groups used the Spearman's test and survival curves were plotted using the Kaplan–Meier method.

Results

The definition and identification of B cell follicle-like structures in multiple sclerosis

The majority of cases with SPMS (107/123, 87%) contained at least one example of a moderate inflammatory infiltrate. Sections from tissue blocks identified as containing substantial (+ +, Fig. 1A) perivascular or meningeal inflammatory infiltrates (64/123, 52%) were subsequently immunostained for CD20 to determine the extent of the B cell component of these aggregates

(Fig. 1B–E, Supplementary Table 1). Identification of B cell aggregates as B cell follicle-like structures by immunohistochemical staining for Ki67⁺/CD20⁺ proliferating B cells (Fig. 1F) and IgA, G and M⁺ plasma cells (Fig. 1G), T cells (Fig. 1H) and CD35⁺ follicular dendritic cells (Fig. 1I) revealed their presence in 40% (49/123) of cases. Positive identification of these cellular constituents was the minimum requirement for our definition of a follicle-like aggregate, and the presence of a single follicle-like structure defined a case as F+.

It is important to note that death with associated systemic infection was a common event in both the F– and F+ SPMS groups (45/74 of the F– SPMS cohort and 29/49 of the F+ cohort), which did not associate with the incidence of meningeal follicle-like structures (Fisher's exact test, $P = 1.0$). Post-mortem delay, which can profoundly affect tissue inflammation, was not different between the two groups (F– SPMS 18.9 ± 1.3 and F+ SPMS 21.9 ± 2 h; $P = 0.21$).

B cell follicle-like structures are heterogeneously distributed throughout the brain

There was a wide variation in the number of constituent immune cells in the follicle-like aggregates (Fig. 2A–C) and the frequency of such structures per case. Follicle-like structures could be variable in size; small follicle-like structures, such as that shown in Fig. 2A, typically consisted of 50–75 positive B cells in a single section (for example, 63 CD20⁺ B cells are present in the single section in Fig. 2A) and at their largest could consist of hundreds of cells in a single 10 μ m thick section. On one occasion, an entire cortical sulcus in a section appeared packed with CD20⁺ B cells (Fig. 2C; 655 CD20⁺ B cells). Follicle-like aggregates were predominantly found in the shielded confines of the deep cerebral sulci, in particular in cingulate, insula, temporal and frontal areas, and only twice were noted at the crown of a gyrus. The overall incidence of follicle-like structures per 4 cm² tissue block screened was 18% (range 5–67%). Due to the variable incidence and size of follicle-like structures, it is possible that we have underestimated the true proportion of cases with SPMS that harbour them. Unfortunately, it would only be possible to definitively characterize a case as F– multiple sclerosis by the careful screening of serial sections of the entire blocked brain and spinal cord tissue.

Analysis of whole coronal sections from a collection of 16 cases with SPMS not previously examined, allowed us to identify nine cases that matched our criteria of containing B cell follicle-like aggregates. From a total of 27 whole bi-hemispheric sections, 61 separate lymphoid-like aggregates were noted. Plotting the location of these structures onto schematic images of these brains at the coronal planes sampled demonstrates their wide distribution throughout the cerebral cortex (Fig. 2D).

B cell follicle-like structures were always located in close association with underlying subpial pathology (Fig. 2E–F). However, the converse was not the case and ribbons of subpial demyelination were also seen that were not spatially associated with follicle-like structures in the sections we examined (Fig. 2G), although this

does not preclude that such structures were not in close association in adjacent sections. Subpial demyelination was also observed to a lesser extent in F– SPMS (Fig. 3).

Follicle-like positive secondary progressive multiple sclerosis cases have more extensive grey matter demyelination

Whole coronal sections were analysed to determine the degree of forebrain white matter and grey matter demyelination in F– and F+ SPMS (Fig. 3). Representative myelin oligodendrocyte glycoprotein immunostained coronal macrosections illustrate the degree and location of grey matter (red colour mask) and white matter (blue colour mask) demyelination in two F– SPMS (Fig. 3A and C) and two F+ SPMS (Fig. 3B and D) cases. Cortical lesions, predominantly in the form of subpial grey matter demyelination, but also involving intracortical and leucocortical locations, were seen in the cingulate gyrus, superior and inferior frontal sulci, insula and in the temporal lobes, in agreement with the previous publications (Kutzelnigg *et al.*, 2005; Vercellino *et al.*, 2005). Periventricular white matter lesions were commonly noted at the angles of the lateral ventricles and in the centrum semiovale, which was often markedly reduced in volume, while the lateral ventricles were frequently enlarged (Fig. 3B and D). Quantitative assessment revealed that F+ cases with SPMS had a 3-fold greater area of total demyelination (follicle-like positive SPMS = $18.3 \pm 2.7\%$; follicle-like negative SPMS = 5.3 ± 1.4 ; $P = 0.002$) in sampled whole coronal sections (Fig. 3E). There was a 6-fold increase in total grey matter lesion area (follicle-like positive SPMS = 12.2 ± 2.3 ; follicle-like negative SPMS = $1.9 \pm 0.7\%$; $P = 0.003$) in F+ SPMS in comparison to F– cases (Fig. 3F). The white matter lesion area was ~2-fold greater in F+ SPMS, but this was not statistically significant (F+ SPMS = $6.0 \pm 1.1\%$; F– SPMS = $3.3 \pm 1.1\%$; $P = 0.1$).

Follicle-like positive secondary progressive multiple sclerosis cases exhibit greater meningeal, grey matter and white matter inflammation

Quantitative analyses of meningeal and white matter infiltrates were performed in order to better understand the relationship between global inflammation and demyelinating pathology in the cases identified as F+ in comparison to F– SPMS and controls (Fig. 4A). Although there was no significant difference in the density of CD68⁺, CD3⁺ or CD20⁺ immune cell infiltrates (Fig. 4B) in the meninges overlying normal appearing grey matter when compared with grey matter lesion areas (data not shown), in agreement with the previous reports (Kutzelnigg *et al.*, 2005; Kooi *et al.*, 2009), the total macrophage, T and B cell infiltration of the meninges (irrespective of the spatial relationship to grey matter lesions) was significantly increased in F+ cases with SPMS in comparison to F– and control cases (Fig. 4C–E). It should be noted that areas of meningeal inflammation selected

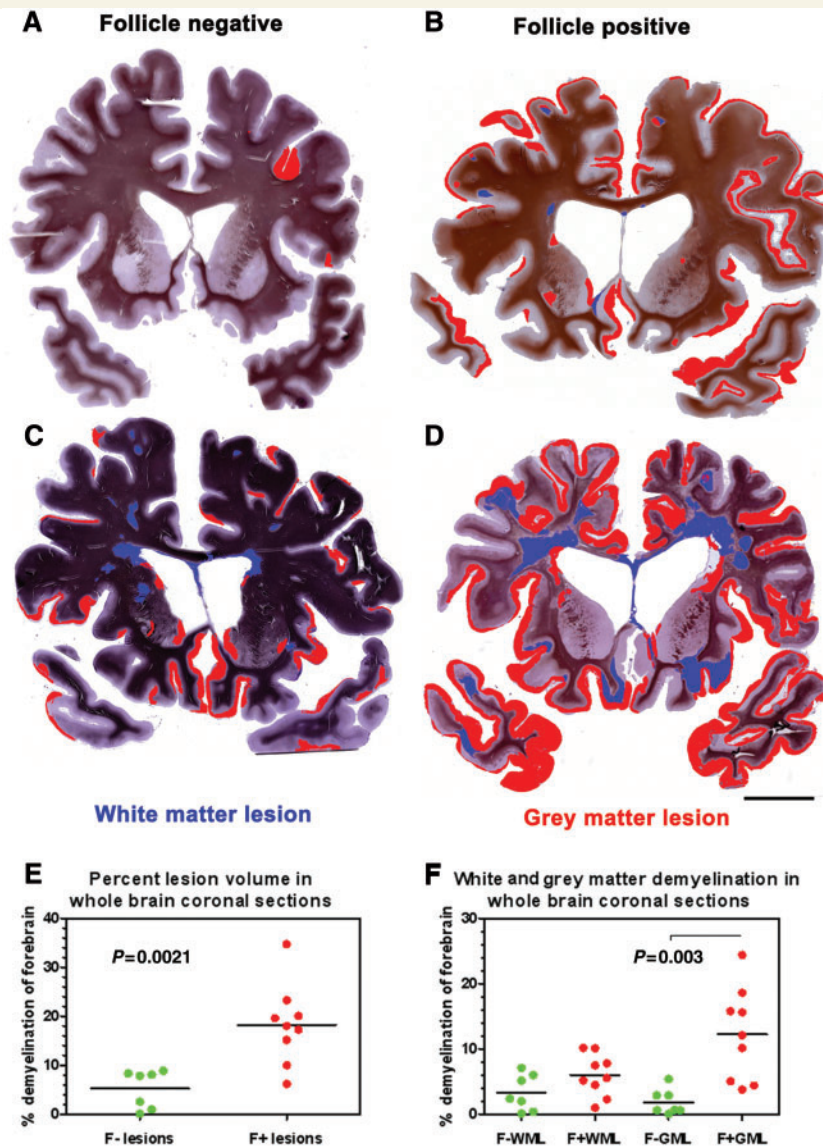


Figure 3 Cortical demyelination, but not white matter demyelination, is significantly increased in F+ SPMS. Representative myelin oligodendrocyte glycoprotein immunostained sections in which colour masks have been applied to illustrate the variable extent of white matter (blue) and grey matter (red) pathology in F– (A and C) and F+ (B and D) SPMS. Note that although grey matter lesions (GML) are evident in both groups, in general the area of demyelinated subpial cortical grey matter was greatly increased in the F+ cohort. (E and F) Quantitative analysis of white matter lesion (WML) and grey matter lesions load from seven F– and nine F+ cases revealed F+ SPMS to have significantly greater area of forebrain demyelination (E) and ~6-fold greater grey matter lesions area in comparison to F– cases (F). (E) Mann–Whitney test, (F) ANOVA and Dunn’s multiple comparison post test. Scale bars: (A–D) = 20 mm.

for quantification of cell numbers did not include B cell follicle-like aggregates as these structures were not present in the regions sampled. Therefore, these data represent the increased density of non-follicle-associated infiltrates, generally diffusely distributed throughout the meninges, in cases known to harbour such structures. Quantification of CD3⁺ and CD20⁺ perivascular infiltrates in white matter lesion centres and normal appearing white matter in the same F+ and F– cases with SPMS revealed lymphocytic infiltrates to be significantly increased in F+ cases in comparison to F– cases with SPMS and age-matched control white matter (Fig. 4F).

Global meningeal inflammatory infiltrates correlate with the extent of cerebral grey matter inflammation and demyelination

Quantification of parenchymal CD68⁺ microglia in the subpial normal appearing grey matter and grey matter lesions revealed F+ cases with SPMS to have significantly increased microglial density in comparison to F– SPMS and controls (Fig. 5A and B). Non-parametric Spearman correlations were performed to investigate

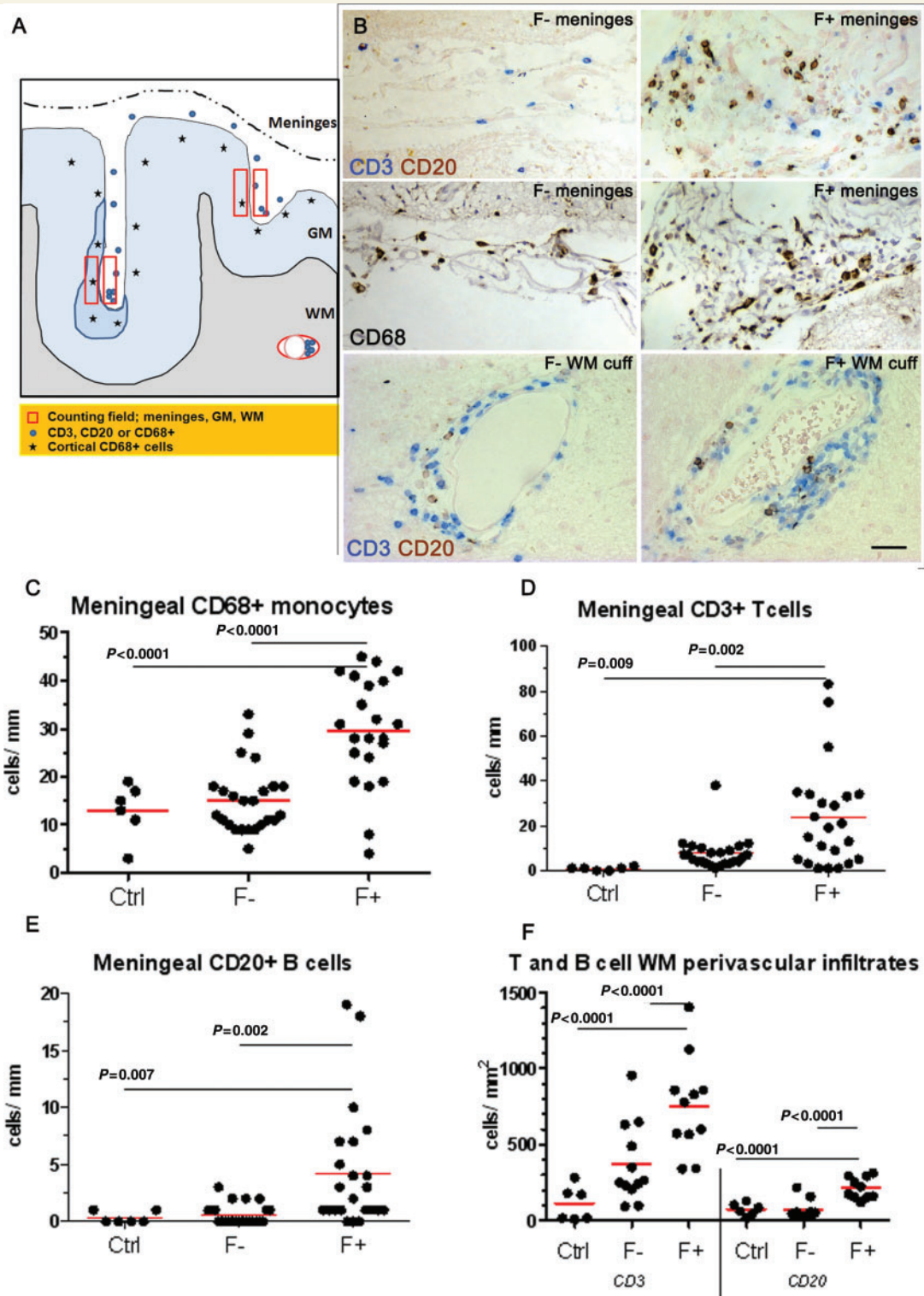


Figure 4 Meningeal and parenchymal inflammation is significantly increased in F+ cases. Quantitative analysis of CD3⁺, CD20⁺ and CD68⁺ inflammatory infiltrates of the intact meninges adjacent to normal and demyelinated grey matter (GM), and of perivascular CD3⁺ and CD20⁺ infiltrates of the normal and demyelinated white matter (WM) were performed as illustrated (A) on 12 F- and 12 F+ cases (four blocks per case) in comparison to six control cases. Meningeal and white matter perivascular inflammation was increased in F+ cases in comparison to F- (B). The total number of CD68⁺ monocytes, CD3⁺ T cells and CD20⁺ B cells per mm length of intact meninges was significantly increased in the F+ group in comparison to the F- cohort (C–E). The density of perivascular white matter lymphocytes (mm²) was significantly increased in the F+ cohort in comparison to F- and control groups, where only modest CD3⁺ T cell and CD20⁺ B cell infiltrates were noted (F). ANOVA and Dunn's multiple comparison post test (C–F). Scale bars: (B) = 30 μm. Ctrl = control.

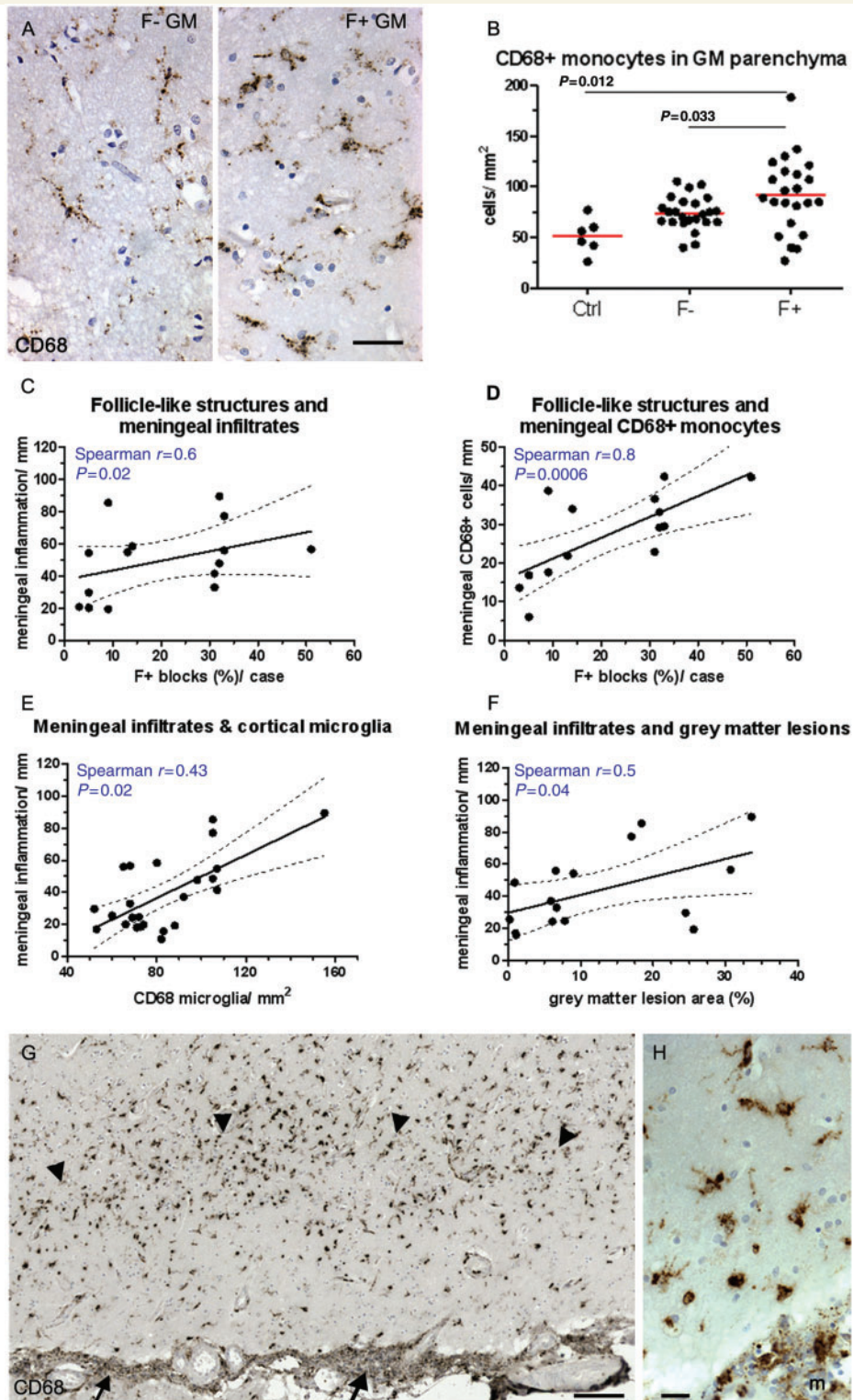


Figure 5 Meningeal inflammation correlates with underlying cortical pathology. The density of parenchymal CD68⁺ microglia/macrophages was significantly increased in cortical fields directly underlying the meninges (see Fig. 4 for counting scheme) in F+ cases in comparison to F- (A and B) and controls. Within the F+ cohort, the relative incidence of follicle-like structures correlated with the total density of meningeal infiltrates (CD3⁺, CD20⁺ and CD68⁺ cells; C), and the density of CD68⁺ monocytes/macrophages in the meninges (D). The sum of meningeal inflammation correlated significantly, albeit modestly, with the density of parenchymal CD68⁺ microglia of the underlying grey matter (GM) parenchyma (E) and with the extent of grey matter demyelination (F), as determined from the subset of cases where whole coronal sections were available. CD68⁺ microglial/macrophage inflammation underlying an inflammatory lymphocytic aggregate in the meninges (arrows in G) in the demyelinated grey matter parenchyma, illustrates the association between meningeal (m) and parenchymal inflammation, which is so marked in F+ SPMS (H). (B) ANOVA and Dunn's post test; (C–F) Spearman's non-parametric comparison. Scale bars: (A and H) = 10 μ m, (G) = 100 μ m. Ctrl = control.

Table 2 Cases harbouring B cell follicle-like structures (F+) at death were common and presented a significantly earlier age of disease onset (years), age at progression, age at which the patient required the use of a wheelchair and age at death

Measure	F– SPMS	F+ SPMS	P-value
Cases (%)	74 (60.2)	49 (39.8)	–
Female (n)	44	34	–
Male (n)	30	15	–
Gender ratio (F/M)	1.47	2.27	0.34
Age of onset	32 ± 5.5	25 ± 6.5	<0.0001
Relapses (first 2 years)	2 ± 1	2 ± 1	0.59
Age at progression	44 ± 6	35 ± 4.5	<0.0001
Age at wheelchair	50 ± 7	37 ± 5	<0.0001
Age at death	62 ± 8.5	48 ± 6	<0.0001

Gender ratio (female: male) or number of relapses in first 2 years of disease did not differ between the groups. Median ± half interquartile range. Mann–Whitney test or Fisher's exact test (gender).

the relationship between follicle-like structures, meningeal inflammation and cortical pathology. In the F+ SPMS cohort, the relative incidence of follicle-like aggregates correlated significantly with the density of total meningeal infiltrates (the sum of CD3⁺, CD20⁺ and CD68⁺ cells; $r = 0.6$, $P = 0.02$; Fig. 5C) and with the density of CD68⁺ macrophages in the meninges ($r = 0.8$, $P = 0.0006$; Fig. 5D). The number of follicle-like structures per section or per case, for those cases for which whole coronal sections were available, did not correlate with the grey matter lesion area ($r = -0.01$, $P = 0.96$ and $r = -0.05$, $P = 0.91$, respectively). However, there was a significant positive correlation, albeit modest, between total meningeal infiltrates and parenchymal CD68⁺ microglial number ($r = 0.4$, $P = 0.02$; Fig. 5E) and total meningeal infiltrates and the extent of forebrain grey matter demyelination ($r = 0.5$, $P = 0.04$; Fig. 5F), for the cases where whole brain macrosections were available ($n = 16$). The presence of parenchymal CD68⁺ cells with an activated morphology closely associated with inflammatory foci (arrowheads) of the meninges illustrates the topographical relationship frequently noted between inflammatory cells in these two compartments (Fig. 5G and H). These data highlight the association that exists between meningeal inflammation in general and cortical pathology.

The presence of B cell follicle-like structures associated with an earlier onset and more rapid conversion to progressive disease and death

We have previously shown that the presence of follicle-like structures is associated with early disease onset and an earlier death (Magliozzi *et al.*, 2007) in comparison to F– SPMS. In our current study of 123 cases with SPMS, the 49 cases identified as F+ differed significantly ($P < 0.0001$) with respect to median age at disease onset, the age at conversion to secondary progressive disease and the age at which the individual required the use of a

wheelchair (Table 2). The median age of death was also significantly younger (48 versus 62 years), indicating that cases harbouring B cell follicle-like structures had a more rapid disabling disease and died younger. In accordance with our quantitative assessments of inflammation in a subgroup of the study cohort (Figs 4 and 5), the complete F+ cohort displayed a greater proportion of cases with an active inflammatory disease at death (based on the observation of early active white matter lesions) in comparison to cases where disease activity was stable (mild inflammation and inactive lesions) based on neuropathological examination ($P = 0.0005$, Fisher's exact test).

By plotting the percentage of cases with SPMS identified as F+ or F– against the age at which their first disease symptoms occurred, we are able to show that the vast majority of individuals who developed disease before the age of 20 years (9/11) were identified as F+ at post-mortem [relative risk of being F+ rather than F– at death if disease onset <20 years = 4.5; 95% confidence intervals (CIs) 1.3–16.3; Fisher's exact test $P = 0.009$], while the majority of individuals who developed multiple sclerosis after 40 years of age (20/24 in total) did not harbour detectable B cell follicle-like structures (relative risk of being F– at death if disease onset >40 years = 5; 95% CIs 2.1–12.4; Fisher's exact test $P < 0.0001$) (Fig. 6A).

Previous work has demonstrated that an older age at disease onset (Vukusic and Confavreux, 2003), male gender (Confavreux and Vukusic, 2006a) and increased numbers of relapses in the first 2 years of disease (Scalfari *et al.*, 2010) are associated with a shortened period between onset of symptoms and disease progression. We were interested in testing if follicle status was an independent variable (as are age of onset, gender and early relapse rate), in affecting our selected landmarks of clinical disease progression. Using Cox proportional hazards modelling, we tested the covariates: age of onset, gender (male = 1), relapses in first 2 years and follicle status (presence = 1) and found that an increased age of onset, male sex, increasing numbers of relapses in the first 2 years and the presence of follicles (F+) shortened disease length (Supplementary Table 3, Fig. 6B; $P < 0.0001$). The presence of follicles also associated with a significantly shortened time from disease onset to progression (Fig. 6C, $P = 0.001$ and Supplementary Table 3) and to wheelchair use. In contrast to the other covariates, male gender and F+ status independently associated with a shortened time from progression to death (Supplementary Table 3 and Fig. 6D; time from progression to death for F+ and F– cases, $P = 0.002$). The period of time from wheelchair to death was not affected by any of the variables assessed (Supplementary Table 3). This analysis demonstrates that the presence of follicles, being independent of other variables known to affect the clinical course, is an important determinant of an accelerated disease and an earlier death in SPMS.

The degree of global meningeal inflammation, irrespective of follicle status, correlated with age at disease milestones

Our data suggest that the general degree of inflammation of the meninges, irrespective of whether it is organized into follicle-like

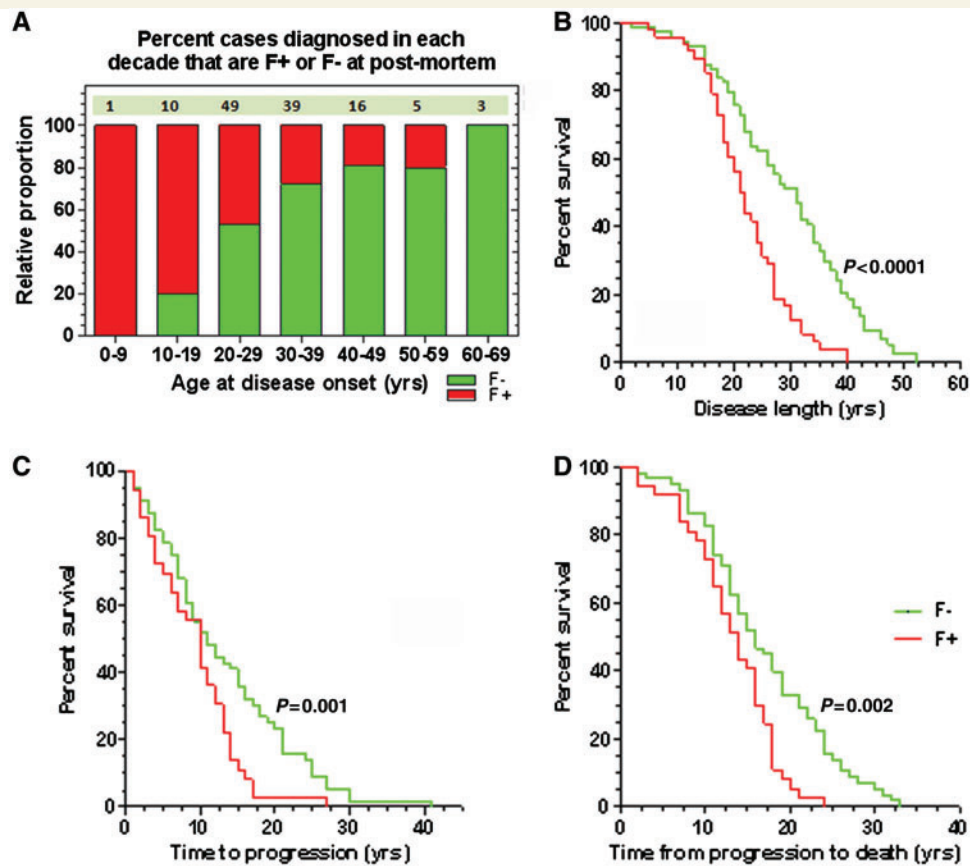


Figure 6 The presence of lymphoid-like structures associated with a more severe disease course. (A) An earlier age of disease onset reflects a greater tendency for the identification of lymphoid-like tissues at post-mortem. The numbers of cases per decade are shown above each bar. (B) Kaplan–Meier survival curve of disease length revealed a significantly shortened mean disease duration (22.2 ± 1.1 and 29.5 ± 1.3 , mean \pm SEM years), (C) shortened time from disease onset to conversion to secondary progressive (8.8 ± 1.0 and 12.7 ± 1.2 years) and (D) a reduced duration of the progressive phase (time from secondary progression to death, 13.0 ± 0.9 and 16.9 ± 1.0 years), for follicle positive (F+) versus follicle negative (F–) cases, respectively.

structures, is an important contributor to cortical pathology and overall disease progression. To test this hypothesis, we separated the study cohort based on the semi-quantitative assessment of meningeal and perivascular inflammation used in our initial screening (refer to the 'Methods' section and Fig. 1). These data (Fig. 7) suggest that increasing inflammation is associated with reaching disease milestones, in particular the early stages of disease progression, at a younger age. F+ cases (inflammation score 3) differed significantly from each of the other inflammation-characterized groups with respect to age of onset, age at progression, age at wheelchair and age at death ($P < 0.05$ in all instances).

Discussion

There is now increasing evidence that meningeal inflammation plays an important role in cerebral cortical grey matter pathology in the progressive stages of multiple sclerosis (Magliozzi *et al.*, 2007, 2010; Pirko *et al.*, 2007; Dal Bianco *et al.*, 2008; Gray *et al.*, 2008; Frischer *et al.*, 2009). Here we definitively show,

using an extensive representative sample of 123 cases from the UK Multiple Sclerosis Tissue Bank, that ectopic B cell follicle-like structures are detectable in the cerebral meninges of a large proportion (40%) of cases with SPMS. The cases with SPMS harbouring these structures in the subarachnoid space displayed a greater global meningeal inflammation that was associated with increased parenchymal grey matter pathology. Our data suggest that the presence of lymphoid aggregates signifies a state of elevated meningeal inflammation that is likely to play a role in causing the globally increased cortical demyelination and reduced time to the onset of significant disability.

Identification of B cell follicle-like aggregates

The description of ectopic meningeal B cell follicle-like structures in the multiple sclerosis post-mortem brain is still the subject of some controversy and a number of other studies have not been able to identify them (Kooi *et al.*, 2009; Torkildsen *et al.*, 2010). The B cell follicle-like structures described by ourselves (Magliozzi *et al.*, 2007,

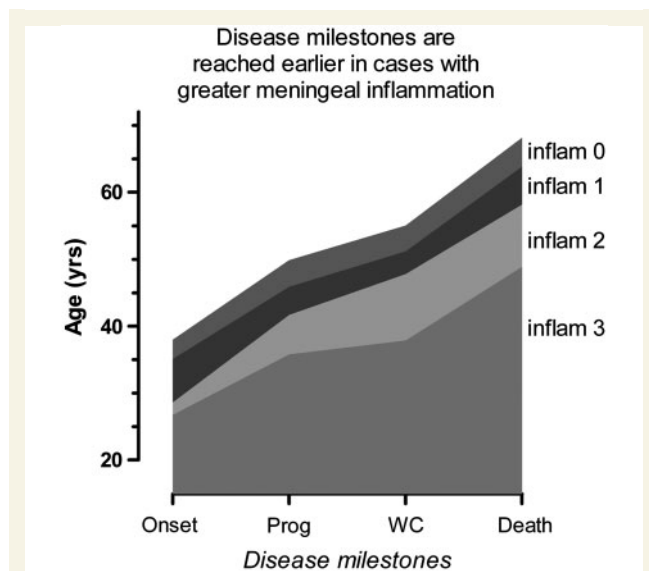


Figure 7 The relative degree of meningeal and perivascular immune cell inflammation associated with a more severe disease course. We separated the study cohort based on a semi-quantitative assessment of meningeal/perivascular inflammation into cases displaying a maximum inflammatory focus of: mild (inflammation score 0, $n = 16$), moderate (inflammation score 1, $n = 43$), severe but in the absence of follicles (inflammation score 2, $n = 15$) and severe inflammation plus follicles (inflammation score 3, $n = 49$). These data reveal that the degree of meningeal and perivascular inflammation associated with arrival at the disease milestones of disease onset, conversion to secondary progressive (Prog), age at wheelchair (WC) and age at death at a younger age (mean age plotted per group). F+ cases (inflammation score 3) differed significantly to the other inflammation-characterized groups with respect to age of onset, age at progression, age at wheelchair and age at death ($P < 0.05$ for all groups versus score 3 group; ANOVA and Dunn's multiple comparison post test).

2010; present study) resemble the leptomeningeal infiltrates noted by Guseo and Jellinger (1975). In a large post-mortem study, they described the presence of meningeal inflammatory accumulations in 41% of cases, which were characterized by extensive inflammation and shorter disease duration. In order to clarify their frequency, distribution and relationship to cortical pathology, we have performed an extensive screen in a large cohort of cases with a mean age of onset and age at conversion to secondary progression typical of the wider SPMS population (Table 1) (Confavreux and Vukusic, 2006b; Kremenchutzky *et al.*, 2006). The present study suggests that extensive sampling of the whole brain is necessary to reveal such structures, and failure by others to find these B cell follicle-like aggregates may be due in part to differing methods of tissue retrieval, preservation and handling that can inadvertently disrupt the meningeal compartment (Aloisi *et al.*, 2010). Although B cell follicle-like structures were noted in all cortical regions sampled, it seems that the deep cortical sulci provide the optimal biological and physical niche for follicle formation and retention. This may reflect both the stability of these deep folds to the physical trauma of brain removal and tissue

preparation and also a reduced CSF flow. Meningeal inflammation in early disease, together with acute inflammation of the brain, might lead to the release of antigens through the drainage of interstitial fluids into the subarachnoid space (Zhang *et al.*, 1992). Protein accumulation, antigen presentation and stimulation of reactive cells may promote a sustained leucocyte presence in the confines of the cerebral sulci that would be conducive to the formation of ectopic lymphoid-like tissue (Aloisi and Pujol-Borrell, 2006).

Ectopic B cell follicle-like structures reflect a greater degree of meningeal inflammation and contribute to the exacerbated subpial cortical pathology

Imaging studies suggest that demyelination, axonal loss and cortical atrophy are all important contributors to the symptoms and progression of multiple sclerosis (De Stefano *et al.*, 2003; Fisher *et al.*, 2008; Fisniku *et al.*, 2008; Calabrese *et al.*, 2010b), although the efficiency of current *in vivo* imaging technologies at resolving pure cortical lesions is very low. The mechanisms of grey matter pathology are poorly understood, as cortical lesions are rarely populated by T cells, B cells or amoeboid macrophages (Peterson *et al.*, 2001). The primary inflammatory component of these lesions is the activated microglia (Kidd *et al.*, 1999; Peterson *et al.*, 2001; Gray *et al.*, 2008), whose density is related spatially and quantitatively to the degree of lymphocyte infiltration of the meninges (Bo *et al.*, 2003b; Dal Bianco *et al.*, 2008). Whether microglial activation is a cause or consequence of cortical injury cannot be determined from our studies, although it should be noted that tumour necrosis factor and inducible nitric oxide synthase expression has been observed in microglia in these F+ cases with SPMS (Magliozzi *et al.*, 2010). Microglial activation, caused by the diffusion of inflammatory mediators from the meninges, could contribute to cortical pathology through the release of cytotoxic molecules. On the other hand, it is also possible that diffuse CD8⁺ parenchymal lymphocytes (Magliozzi *et al.*, 2010) may cause neuronal and oligodendrocyte damage with subsequent microglial activation. It is likely that both direct (from meningeal signals) and indirect (as a consequence of tissue injury) mechanisms of microglial activation occur in the multiple sclerosis cortex. In addition to demyelination, neuronal, synaptic and glial alterations occur in the multiple sclerosis cortex that are likely to contribute to the significant atrophy and associated cognitive impairments suffered by individuals with multiple sclerosis (Kidd *et al.*, 1999; Peterson *et al.*, 2001; Wegner *et al.*, 2006; Vercellino *et al.*, 2007; Magliozzi *et al.*, 2007, 2010; Calabrese *et al.*, 2010a; Schmierer *et al.*, 2010). We have shown that the incidence of B cell follicle-like structures in the cerebral meninges correlates with the general level of diffuse meningeal inflammation and that the severity of meningeal infiltration, irrespective of follicle status, is associated significantly with grey matter demyelination. Thus, the presence of B cell follicle-like structures in SPMS represents a more substantial, but relatively common, inflammatory pathology that represents one end of a continuum of diffuse

global meningeal inflammation, which may be responsible for the more severe clinical disease.

The subarachnoid compartment filled with CSF is an important site for CNS immune surveillance, where a deregulated immune response could become a source of pro-inflammatory mediators and autoreactive cells leading to underlying tissue damage (Shin *et al.*, 1995; Matsumoto *et al.*, 1996; Bartholomaeus *et al.*, 2009; Kivisakk *et al.*, 2009; Ransohoff, 2009). The immune cells of the meninges are only separated from the brain parenchyma by a thin non-continuous layer of pial cells, components of the basal lamina and the end-feet of interlaminar astrocytes (Lopes and Mair, 1974; Peters, 1991). Therefore, the structural nature of the pia mater does not represent a significant barrier to the movement of small molecules (Zhang *et al.*, 1990) and our recent results have demonstrated significant damage to the glial limitans in the presence of meningeal inflammation (Magliozzi *et al.*, 2010). We suggest that the destruction of the underlying cortical grey matter may be caused by the pro-inflammatory milieu caused in part by T and B cell release of cytokines, metalloproteinases and reactive oxygen and nitrogen species into the subarachnoid space (Brown and Sawchenko, 2007; Ransohoff, 2009). The presence of a marked gradient of neuronal and oligodendrocyte damage, greatest in the most superficial cortical lamina, and less so in deeper layers, is strongly supportive of the diffusion of a factor or factors from the meninges (Magliozzi *et al.*, 2010).

Our finding of modest correlations between inflammation of the meninges and the degree of parenchymal grey matter demyelination and microglial activation is suggestive of a role for meningeal inflammation in cortical pathology, although it does not prove causality. It should be noted that it remains possible that meningeal inflammation could occur as a consequence of cortical injury, although this is not supported by the available evidence. It is fully expected that the far less frequent observation of cortical pathology (6-fold less than in F+ SPMS), particularly subpial (Magliozzi *et al.*, 2007), in those cases with mild or moderate meningeal inflammation is due in part to the combined influence of persistent, inflammatory processes in the meninges which, in concert with intraparenchymal inflammatory changes such as the influence of subcortical white matter lesions, retrograde degeneration and chronic microglial activation, may be sufficient to cause cortical damage. Nevertheless, our work adds support to the most plausible hypothesis that subpial lesions are a consequence of inflammatory events within the overlying meninges in a significant proportion of cases with the most severe disease (Kutzelnigg *et al.*, 2005; Magliozzi *et al.*, 2007; Serafini *et al.*, 2007; Lassmann, 2007; Dal Bianco *et al.*, 2008; Moll *et al.*, 2008). In order to test this hypothesis, it will be necessary to create a model of chronic meningeal inflammation overlying the cortical grey matter. Our preliminary investigations have found that the presence of pro-inflammatory cytokines in the subarachnoid space in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis can give rise to meningeal inflammation and extensive subpial demyelination (Gardner *et al.*, 2009).

The presence of plasma cells in the meninges, which are undoubtedly responsible for the oligoclonal bands present in the CSF, suggests a role for pathogenic antibodies in cortical pathology. Although recent studies have shown an absence of complement

activation products in most cortical lesions (Brink *et al.*, 2005), their presence in actively demyelinating subpial lesions has not yet been excluded.

B cell follicles and the persistence of inflammation in progressive multiple sclerosis

The recent description of the presence of shared B cell clones between meningeal, grey matter and white matter compartments from the same brain suggests that subsequent to B cell maturation, which occurs in the CNS (Colombo *et al.*, 2000; Owens *et al.*, 2003; Corcione *et al.*, 2004), these cells go on to populate secondary sites within the brain that could precede the formation of new lesions (Lovato *et al.*, 2011). Therefore, lymphoid-like structures could be important in the continuation and diversification of the inflammatory response by generating new autoreactive B cell clones to spread throughout the tissue.

It has been proposed that lymphoid-like structures forming in the meninges could also represent major sites of Epstein–Barr virus persistence in the multiple sclerosis CNS (Serafini *et al.*, 2007). This scenario would be compatible with the serological evidence linking Epstein–Barr virus infection to multiple sclerosis (Ascherio and Munger, 2007) and with the unique ability of Epstein–Barr virus to infect and immortalize B cells. Epstein–Barr virus infects naïve B cells, which through the expression of viral proteins can undergo germinal centre-like reactions to generate long-lived memory B cells without the need for antigen or T cell co-stimulation (Thorley-Lawson and Gross, 2004). It is proposed that Epstein–Barr virus persistence and reactivation occurs within ectopic lymphoid tissue of the meninges and that cell-mediated targeting of infected B lymphocytes by CD8+ T cells could cause bystander damage of the underlying cortex (Serafini *et al.*, 2007, 2010). However, the identification of Epstein–Barr virus infection of B cells in meningeal and perivascular infiltrates in the multiple sclerosis brain and its role in the pathogenesis remains controversial (Chard *et al.*, 2002; Farrell *et al.*, 2009; Pender, 2009; Sargsyan *et al.*, 2009; Willis *et al.*, 2009; Zivadinov *et al.*, 2009; Lunemann *et al.*, 2010).

The formation of follicle-like structures may be an early event in disease

Meningeal inflammation and the formation of lymphoid-like tissues may not be restricted to the later stages of multiple sclerosis. Such a scenario is supported by: the observation of clonally restricted B cells in the CSF of relapsing–remitting patients (Colombo *et al.*, 2000; Corcione *et al.*, 2004); our findings of follicle-like structures in two cases with short disease duration (5–6 years); and the significant influence of follicle status on the early course of the disease (Supplementary Table 3 and Fig. 7). Our observation of a shorter time from disease onset to progression and to the use of a wheelchair (Expanded Disability Status Scale 7 equivalent), and the fact that cortical grey matter pathology is detectable in the earliest stages of disease (Chard *et al.*, 2002; De Stefano *et al.*, 2003; Dalton *et al.*, 2004; Calabrese *et al.*, 2010a),

supports the hypothesis that meningeal inflammation is a factor in early multiple sclerosis. Interestingly, the period of time from wheelchair use to death did not appear to be influenced by follicle status, suggesting that the formation of such structures in the forebrain, or other events that precede and promote their formation, play an influential role particularly during the early disease course. These observations suggest that the development of imaging techniques to visualize meningeal inflammation, and/or identification of serum and CSF biomarkers to detect its presence, might allow the early identification of those patients likely to follow a more rapid disease progression.

Supplementary material

Supplementary material is available at *Brain* online.

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