Cortical grey matter demyelination can be induced by elevated pro-inflammatory cytokines in the subarachnoid space of MOG-immunized rats

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A substantial proportion of cases with secondary progressive multiple sclerosis have extensive inflammation in the leptomeninges that is associated with increased subpial demyelination, neuronal loss and an exacerbated disease course. However, the mechanisms underlying this extensive subpial pathology are poorly understood. We hypothesize that pro-inflammatory cytokine production within the meninges may be a key to this process. Post-mortem cerebrospinal fluid and dissected cerebral leptomeningeal tissue from patients with multiple sclerosis were used to study the presence of tumour necrosis factor and interferon gamma protein and messenger RNA levels. A novel model of subpial cortical grey matter demyelination was set up in Dark Agouti rats and analysed using quantitative immunohistochemistry. Increased expression of the pro-inflammatory cytokines tumour necrosis factor and interferon gamma was found in the meninges of cases with secondary progressive multiple sclerosis exhibiting tertiary lymphoid-like structures. Injection of tumour necrosis factor and interferon gamma into the subarachnoid space of female Dark Agouti rats pre-immunized with a subclinical dose of myelin oligodendrocyte glycoprotein mimicked the pathology seen in multiple sclerosis, including infiltration of lymphocytes (CD4+ and CD8+ T cells and CD79+ B cells) into the meninges and extensive subpial demyelination. Extensive microglial/macrophage activation was present in a gradient from the pial surface to deeper cortical layers. Demyelination did not occur in control animals immunized with incomplete Freund's adjuvant and injected with cytokines. These results support the hypothesis that pro-inflammatory molecules produced in the meninges play a major role in cortical demyelination in multiple sclerosis, but also emphasize the involvement of an anti-myelin immune response.

Keywords: demyelination; multiple sclerosis; microglia; immune-mediated demyelination; neuroinflammation

Abbreviations: EAE = experimental autoimmune encephalomyelitis; IFNγ = interferon gamma; MOG = myelin oligodendrocyte glycoprotein; SPMS = secondary progressive multiple sclerosis; TNF = tumour necrosis factor

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Introduction

Recent neuropathology studies have re-emphasized earlier observations of the extensive nature of cortical grey matter damage in multiple sclerosis (Peterson et al., 2001; Bó et al., 2003; Kutzelnigg et al., 2005) and, together with longitudinal neuroimaging findings (Calabrese et al., 2012), have suggested that this widespread pathology plays a major role in the accumulation of neurological disability and cognitive dysfunction. Damage to the cortical grey matter in multiple sclerosis is characterized by leuco- cortical, intracortical and subpial demyelination, the latter of which can be extensive and extend over multiple gyri (Peterson et al., 2001; Bo et al., 2003; Kutzelnigg et al., 2005; Magliozzi et al., 2007). Unlike white matter lesions, subpial grey matter demyelination is not associated with a significant perivascular peripheral immune cell infiltrate but rather is accompanied by widespread microglial activation (Peterson et al., 2001; Brink et al., 2005; Kutzelnigg et al., 2005; Magliozzi et al., 2010). The absence of significant numbers of peripheral immune cells and the predominantly subpial localization of the demyelination suggests an influence of the CSF in the overlying subarachnoid space (Kutzelnigg et al., 2005; Lassmann, 2010).

The presence of immune cell infiltrates in the cerebral leptomeninges of secondary and primary progressive multiple sclerosis brains at post-mortem is a common finding and is extensive in cases characterized by active and widespread cortical demyelination (Guseo and Jellinger, 1975; Kutzelnigg et al., 2005; Magliozzi et al., 2007; Dal Bianco et al., 2008; Kooi et al., 2009; Lovato et al., 2011; Choi et al., 2012), although there may not be a direct spatial relationship between the infiltrates and areas of demyelination (Kooi et al., 2009). Recently, substantial meningeal inflammation has also been demonstrated to accompany subpial demyelination in cortical biopsies of early stage multiple sclerosis brains (Lucchinetti et al., 2011), thus demonstrating that it is not restricted to the later progressive stages. In addition to diffuse meningeal inflammatory infiltrates (Kutzelnigg et al., 2005; Kooi et al., 2009; Howell et al., 2011), B cell aggregates with some characteristics of tertiary lymphoid structures are reported to be present in the subarachnoid space in a substantial proportion of cases with secondary progressive multiple sclerosis (SPMS) (Serafini et al., 2004; Magliozzi et al., 2007, 2010; Howell et al., 2011). Increased meningeal inflammation is associated with more extensive grey matter demyelination, increased numbers and activation state of microglia in the cortical layers closest to the pia mater and damage to the glia limitans (Magliozzi et al., 2010; Howell et al., 2011). In support of imaging studies showing a good correlation between grey matter atrophy, cortical lesion load and clinical progression (Fisniku et al., 2008; Calabrese et al., 2012), we have shown that the disease milestones of age at progression, wheelchair use and death are reached at an earlier age in multiple sclerosis cases with more extensive grey matter pathology and meningeal inflammation (Magliozzi et al., 2007; Howell et al., 2011). In addition, an increasing loss of cortical neurons could be correlated with both the extent of meningeal inflammation and shorter time to clinical milestones of progression (Magliozzi et al., 2010; Reynolds et al., 2011).

The above studies suggest that the subarachnoid space of the cerebral meninges, in particular in the deep sulci, represents a microenvironment with low CSF flow that would favour the retention, growth and lymphoid organization of inflammatory cells and the production of a highly inflammatory milieu that could affect the viability of the underlying cortical cells (Lassmann, 2010; Magliozzi et al., 2010). Here we have evaluated the transcript frequency and protein levels of two key proinflammatory cytokines, tumour necrosis factor (TNF) and interferon gamma (IFNγ), previously implicated in demyelination, in post-mortem meningeal tissue and CSF of patients with multiple sclerosis with low and high levels of meningeal inflammation. Furthermore, we have stereotactically injected TNF and IFNγ into the subarachnoid space of the sagittal sulcus of Dark Agouti rats immunized subclinically with myelin oligodendrocyte glycoprotein (MOG) and demonstrate that they induce meningeal inflammation accompanied by subpial demyelination and a gradient of microglial activation that closely mimics multiple sclerosis cortical pathology.

Materials and methods

Human tissue samples

Post-mortem brain tissue from six cases with follicle-positive SPMS, six with follicle-negative SPMS and six controls with no neurological disease was obtained from the Multiple Sclerosis Society Tissue Bank at Imperial College and Dr Isidro Ferrer (Servicio Anatomia Patologica, Hospital Belvitge, Barcelona, Spain). These cases had been previously characterized for the presence or absence of meningeal lymphoid-like structures (Magliozzi et al., 2010; Howell et al., 2011). CSF from these cases was also collected post-mortem and stored at −80°C until use. The clinical and neuropathological features of cases with SPMS and control subjects are shown in Supplementary Table 1. All post-mortem multiple sclerosis tissues were obtained through a UK prospective donor scheme with full ethical approval (08/MRE09/31).

Immunohistochemistry and immunofluorescence of human tissue

Cryosections from snap-frozen blocks and formalin-fixed paraffin embedded sections (10μm) were immunostained with monoclonal or polyclonal antibodies (listed in Supplementary Table 2) as described previously (Magliozzi et al., 2007; Howell et al., 2011). Antibody binding was visualized using ABC streptavidin peroxidase (Vector Labs) or with Alexa Fluor® 488- or Cy3-conjugated secondary antibodies (Jackson Laboratories).

Quantitative reverse transcription polymerase chain reaction

One snap frozen tissue block with intact meninges from the precentral gyrus (motor cortex) was analysed for each multiple sclerosis and control case. The outlines of the meninges were scored on the tissue block with a scalpel blade and 50–150 mg tissue was isolated by cryosectioning. RNA was extracted using the RNA Mini Lipid Tissue Kit (Qiagen) and quantified using a Nanodrop ND1000 spectrophotometer. Two-step real-time reverse transcriptase PCR analysis of specific genes was carried out using the QuantiNova SYBR Green kit (Qiagen).
out using the Quant iTech reverse transcription kit with integrated genomic DNA removal, the Quant iTech SYBR Green kit and Quant iTech primer assays (Qiagen), as previously described (Durrenberger et al., 2010). Complementary DNA was then stored at −80% until required. Quantitative reverse transcriptase-PCR was performed using the Stratagene MX3000P system with software version 4.01. For each sample, 25 µl reactions were set up in triplicate with 2.5 µl of TNF (Q701079561) and INFγ (Q700000525) primer assays, respectively using the following cycling protocol, 95°C for 15 min, 40 cycles with a 3-step program (94°C for 15 s, 55°C for 30 s, and 72°C for 30 s) and a final melting curve analysis with a ramp from 55°C to 95°C. GAPDH was used as normalizer and a calibrator as baseline. Relative expression was generated using integrated software.

Quantification of protein levels in cerebrospinal fluid

Concentrations of TNF and INFγ in post-mortem CSF were determined by ELISA using commercially available kits (R&D systems Ltd. Quantikine HS human TNF/TNFαF1A immunoassay and Quantikine human INFγ immunoassay, respectively). Snap frozen CSF samples from six cases with SPMS characterized as follicle-positive, six SPMS follicle-negative and six controls, were thawed, centrifuged at 1300g at 4°C for 3 min and 200 µl of supernatant, mixed with 50 µl of appropriate assay diluent, added in triplicate according to the manufacturer’s protocol. Optical densities were recorded at 490 nm with wavelength correction at 650 nm, and the concentration of cytokine in pg/ml calculated from the standard curves (range 0.25–16 pg/ml) prepared on the same assay plate. Mean ± standard error of the mean (SEM) cytokine concentrations were compared by non-parametric Kruskal-Wallis analysis and Dunns post-test.

Animals

Eight-week-old female Dark Agouti rats (160 g; total of n = 132) were obtained from Harlan UK and kept in groups of five in a 12 h light/dark cycle with food and water provided ad libitum. All animal experiments were carried out under approval from the UK Home Office.

Induction of experimental autoimmune encephalomyelitis

In order to determine a subclinical MOG immunization dose, rats were anaesthetized with isoflurane and immunized intradermally at the base of the tail (dorsal aspect) with either 5 µg (n = 6), 25 µg (n = 6) or 50 µg (n = 6) recombinant mouse MOG (amino acids 1–119, corresponding to the external Ig-like domain) diluted in sterile PBS and emulsified in an equal volume of incomplete Freund’s adjuvant (Difco; 100 µl total volume), or with PBS alone emulsified in incomplete Freund’s adjuvant (n = 6). Recombinant mouse MOG was expressed in Escherichia coli and purified as described previously (Reynolds et al., 2002). In addition, naïve age-matched animals (n = 3) that received no treatment were used for determining baseline anti-MOG immunoglobulin levels as well as meningeal and neuronal cell counts. Rats were weighed and examined daily and clinical signs scored on a neurological scale as described previously (Papadopoulos et al., 2006).

Intracerebral injection of cytokines

Rats were immunized as above with recombinant mouse MOG in incomplete Freund’s adjuvant (n = 78) or incomplete Freund’s adjuvant alone (n = 27) and stereotactic surgery was performed at 20–23 days post-immunization under isoflurane anaesthesia (Supplementary Fig. 1). A 2 mm hole was drilled in the skull in the midline 0.9 mm caudal to bregma. A finely calibrated glass capillary (approximate diameter 30 µm) attached to a 26G 10 µl Hamilton syringe was inserted to a depth of 2.5 mm below the dural surface. The rats were then injected with 1 µl of a cytokine mixture composed of 1.25 µg recombinant rat TNF (R&D Systems) and 75 ng recombinant rat INFγ (Peprotech) in PBS (n = 64), or PBS alone (n = 17), at a rate of 0.20 µl/min. In a separate experiment, rats immunized with MOG in incomplete Freund’s adjuvant or incomplete Freund’s adjuvant alone were subsequently stereotactically injected, as above, with TNF alone or INFγ alone (n = 6 for each group). India ink was added as a tracer and did not alone give rise to any pathological changes. The needle was left in place for 5 min to allow diffusion of sample from the area and then slowly withdrawn.

Immunohistochemistry

Animals were perfused through the left ventricle with PBS followed by 4% paraformaldehyde under terminal anaesthesia at 1, 3, 5, 7, 10, 14 or 21 days post-cytokine injection. The brain was extracted, post-fixed in 4% paraformaldehyde overnight at 4°C before cryo-protecting in 30% sucrose (w/v) and freezing in isopentane on dry ice. Coronal cryosections (10-µm thickness) were cut and stored at −20°C. Immunohistochemistry/immunofluorescence was performed as described previously (Magliozzi et al., 2007; Howell et al., 2011) using monoclonal and polyclonal antibodies listed in Supplementary Table 2. Tiled digital images of sections were obtained at ×4 or ×10 magnification and single high power images at ×40, using a Nikon Eclipse 80i microscope with QImaging Qi-Cam digital camera, and analysed using Image Pro-PLUS 6 software (Media Cybernetics). Captured images were coded to ensure quantification was performed in a blinded manner.

Quantification of demyelination

Immunofluorescence tiled images of sections stained with anti-MOG antibody were used to quantify demyelination. Greyscale images were processed and demyelinated areas of tissue were selected manually as an area of interest, and the area calculated in Image Pro-Plus. Three sections per animal were analysed.

Quantification of immune cells

Lymphocytes and macrophages were quantified on serial sections immunostained for CD4, CD8, CD79a, IBA1 and CD68 antibodies. Cell numbers in the meninges were counted bilaterally in an area from the base of the sagittal sulcus to the position of the lateral blood vessels present in the meninges. Images were converted to grey scale (8bpp) and the area of interest was selected manually. The number of CD8+ and CD79+ cells was counted automatically through the application of set parameters, which were optimized before analysis (pixel intensity 0–80, cell roundness 1–4 and total area 20–400 pixels). The total number of infiltrating cells was then divided by the total area to give the total cells/mm². Three sections per animal were analysed. To ensure reliable counts the number of CD4+ and CD68+ ‘Iba1+’ cells were counted manually using a Nikon E800 microscope at ×10 magnification.
Quantification of central nervous system cells

To visualize cortical layers, sections were labelled by double fluorescence with NeuN and GluR2/3 antibodies. GluR2/3 receptors are present on pyramidal cells in layer II-III, V and VI. The thickness of cortical layers positioned lateral to the sagittal sulcus (the medial anterior cingulate cortex) stained by GluR2/3 was then measured in three locations from the corpus callosum to the sagittal sinus using ImagePro Plus analysis software. The thickness of layers II-III and IV did not differ between experimental groups. Neurons (NeuN) and activated microglia/macrophages (IBA1/CD68) were quantified in each individual layer.

Statistical analysis

GraphPad Prism was used in all cases to present data and conduct statistical analysis. All data represent the mean ± SEM. Group comparisons were analysed by one-way ANOVA with Tukey correction. For correlated data, Pearson’s rank correlation was used to assess the slope of the correlation and significance. For gene expression analysis individual group comparisons were made using the Mann-Whitney non-parametric test. A P-value of <0.05 was considered significant in all cases.

Results

Detection of TNF and IFNγ in the meninges and cerebrospinal fluid of cases with SPMS

Extensive areas of subpial demyelination in SPMS brains (Fig. 1A) were associated with meningeal inflammation, including the presence of immune aggregates containing T and B cells (Fig. 1B and C) that have previously been characterized as tertiary lymphoid-like structures (Serafini et al., 2004; Magliozzi et al., 2007, 2010; Howell et al., 2011). Subpial areas of demyelination were characterized by increased MHC Class II expression in microglia in the upper cortical layers (Fig. 1D). In keeping with previous studies (Serafini et al., 2007), double immunostaining revealed that IFNγ was expressed by a proportion of cells in the inflamed meninges, the majority of which were CD3+ T cells (Fig. 1E). TNF was found to be expressed in the inflamed meninges by cells with monocyte/macrophage morphology and some microglia in the upper grey matter layers (Fig. 1F). Quantitative reverse transcription PCR analysis of RNA extracted from meningeal tissue showed that TNF gene expression was significantly upregulated in both follicle-positive (average fold change = 4.35) and follicle-negative cases with SPMS (average fold change = 1.45; Fig. 1G) compared to controls, with a much greater difference in follicle-positive cases with SPMS. There was an accompanying significant increase in the TNF protein concentration in follicle-positive SPMS post-mortem CSF compared with controls (4.08 pg/ml versus 0.25 pg/ml; Fig. 1G) and follicle-negative SPMS CSF (4.08 pg/ml versus 1.47 pg/ml). IFNγ gene expression was also significantly upregulated in the meninges of follicle-positive cases compared with controls (average fold change = 30.07; Fig. 1G) but not in follicle-negative SPMS cases. CSF IFNγ concentration was higher in follicle-positive SPMS compared with follicle-negative cases, and controls (5.62 pg/ml versus 4.17 and 3.57 pg/ml) but did not reach significance (Fig. 1H).

Subarachnoid injection of TNF and IFNγ leads to subpial demyelination

Injections into the subarachnoid space of the sagittal sulcus could be reproducibly performed, as indicated by the location of India Ink particles in the sagittal sulcus (Supplementary Fig. 1C–F). Immunization with 5 μg recombinant mouse MOG did not elicit clinical signs of experimental autoimmune encephalomyelitis (EAE) in Dark Agouti rats, or lead to T cell infiltration and demyelination (data not shown). However, a peripheral anti-MOG humoral response identical to animals with clinically defined EAE was detected by ELISA 3 weeks post-immunization (Supplementary Fig. 2). Double-labelling immunofluorescence for MOG (myelin) and Iba1 (microglia/macrophages) revealed extensive subpial demyelination in animals immunized with recombinant mouse MOG and injected 21 days later with TNF and IFNγ (Fig. 2A). Demyelination typically extended from the corpus callosum to the dorsal surface of the cortex (Fig. 2A) and from the pial surface into layer II–III in the post-genual medial anterior cingulate cortex (Fig. 2B). No demyelination was observed in control animals immunized with incomplete Freund’s adjuvant and injected with TNF and IFNγ (Fig. 2C), or immunized with recombinant mouse MOG and injected with PBS (Fig. 2D). The pattern of demyelination was very similar to that described for subpial grey matter demyelination in SPMS cortex (Fig. 2E). The demyelination was characterized by loss of myelin basic protein (MBP) (Fig. 2F) and MOG (Fig. 2A and B) immunostaining of myelin, but preservation of neurofilament protein positive axons (Fig. 2E). Brains sectioned in the sagittal plane revealed the maximal extent of demyelination to be 1.1 mm either side of the injection site in the rostral/caudal plane (Supplementary Fig. 3). A small area of demyelination in layer I was first evident after 3 days and reached its maximum extent at 7 days post-injection (Fig. 2H). Demyelination was significantly less at 10 days and no longer evident at 14 and 21 days post-injection. A much reduced area of demyelination was seen at 7 days when MOG immunized animals were injected with either TNF (0.22 mm² ± 0.05) or IFNγ (0.47 mm² ± 0.25) alone, when compared to animals injected with both (1.22 mm² ± 0.36), which was more than additive (Fig. 2I).

Microglial activation after subarachnoid injection of TNF and IFNγ

In animals immunized with recombinant mouse MOG and injected with TNF and IFNγ, demyelination was closely associated with an increased number of Iba1+ microglia with a highly activated morphology at 7 days post-injection, particularly at the leading edge of the lesion where they were intimately associated with MOG+ myelin sheaths, but amoeboid macrophages were not present (Fig. 3A and B), similar to observations in multiple sclerosis cortical lesions (Peterson et al., 2001). Animals immunized with incomplete
Figure 1. Expression of cytokines in the meninges of SPMS cases. Large areas of subpial cortical demyelination could be visualized by anti-MOG immunohistochemistry in follicle-positive SPMS cases (A), accompanied by the presence of meningeal infiltrates (asterisks). Meningeal infiltrates contained large numbers of CD3+ T cells (B) and CD20+ B cells (C). (D) Activated MHC class II expressing microglia were seen in increased numbers in areas of subpial demyelination. (E) IFNγ expression was seen in CD3+ T cells in the meninges. (F) TNF-expressing cells with monocyte/macrophage morphology (arrow) could be seen in the meninges amongst CD3+ T cells. A significant increase in TNF messenger RNA levels was detected in the meninges of follicle-positive and follicle-negative cases with SPMS compared with controls (G, n = 5 cases per group) and the concentration of TNF protein in post-mortem CSF was significantly increased in follicle-positive cases with SPMS compared with both controls and follicle-negative SPMS (H, n = 6 cases per group). IFNγ gene expression was also increased in the meninges of follicle-positive cases with SPMS when compared to controls, but not in follicle-negative cases with SPMS (I), whereas levels of IFNγ protein were not significantly different between groups. Data represent mean ± SEM. Statistics; G and I Mann-Whitney Test; H and J one-way ANOVA with Tukey corrections *P < 0.05, **P < 0.01. Scale bars: A = 1 mm; B and C = 50 μm; D = 200 μm; E and F = 20 μm.
Figure 2 Subpial demyelination in Dark Agouti rats immunized with 5 μg recombinant mouse MOG (rmMOG) after injection of TNF and IFNγ into the sub-arachnoid space at the sagittal sulcus. Immunofluorescence for myelin (MOG = green) revealed extensive demyelination in the upper layers of the cortex (A) coupled with high levels of microglia/macrophage activity at 7 days (B; Iba1 = red). Animals immunized with incomplete Freund’s adjuvant (IFA) and injected with cytokines exhibited meningeal inflammation but no demyelination (C) and animals immunized with recombinant mouse MOG and receiving a PBS injection showed no demyelination or microglia/macrophage activity at 7 days (D). (E) The extent of the cortical demyelination in the Dark Agouti rat was similar to that seen in subpial lesions in the follicle-positive SPMS brain. (F) The presence of primary demyelination was indicated by the absence of MBP immunostaining but continued presence of neurofilament protein-positive axons at 7 days. Demyelination was maximal 7 days post-cytokine injection and had resolved by 14 days post-injection (H; n = 4–10 per time point). Reduced demyelination was found in a subset of animals injected with recombinant mouse MOG and injected with either TNF or IFNγ alone (I; n = 6 per group). Data are presented as mean ± SEM. Statistics: one-way ANOVA with Tukey post test. *P < 0.05, **P < 0.01. Scale bars: A, C, D = 1 mm; B = 250 μm; E = 3 mm; F = 100 μm.
Freund’s adjuvant and injected with cytokines had increased numbers of microglia with a less activated phenotype in the upper layers of the cortical grey matter cortex (Fig. 3C and D), whereas those in animals immunized with recombinant mouse MOG and injected with PBS were sparsely distributed with a resting ramified phenotype (Fig. 3E and F). Microglial activation was already present throughout the cerebral cortex at 1 day post-injection in both MOG and incomplete Freund’s adjuvant immunized animals injected with cytokines (Supplementary Fig. 4A–D), particularly in layer I, indicating a post-surgical activation. Microglial numbers then increased from Day 3 to 7 post-injection in layers I–III in both groups of animals injected with cytokines, but to a much greater extent in recombinant mouse MOG-immunized animals (Supplementary Fig. 4C and E). The number of CD68+ Iba1+ activated phagocytic microglia (Fig. 4A and B) was elevated in layer I in all experimental conditions at Day 1 (Fig. 4C), but this did not extend to layer II–III (Fig. 4D). The number of CD68+ Iba1+ cells was then maximal at 7 days post-cytokine injection in recombinant mouse MOG immunized animals in layers I and II–III (Fig. 4D) and there was clear evidence of a gradient of microglial activation, with the highest levels in layer I, which was also evident to a lesser extent in animals immunized with incomplete Freund’s adjuvant and injected with cytokines (Fig. 4E). This was followed by a substantial decline in CD68+ Iba1+ cell numbers between 7 and 14 days (Fig. 4C and D). The number of CD68+ Iba1+ cells in the cortical parenchyma of MOG immunized animals strongly correlated with the extent of demyelination (Pearson R = 0.74 at P < 0.01) at 7 days post-cytokine injection (Fig. 4F).

Meningeal inflammation after TNF and IFNγ injection

In cytokine injected animals, T and B lymphocytes (Fig. 5A–C) were initially observed close to meningeal blood vessels. CD4+ and CD8+ T cells were scattered in very small numbers throughout the cortical parenchyma (not shown), but CD79+ B cells were rarely observed. CD4+ and CD8+ T cell and CD79a+ B cell numbers in the meninges were maximal 1 day post-injection in all experimental conditions, decreased at Day 3, increased again at Day 7 and decreased thereafter (Fig. 5F–H). Each cell type was present in elevated numbers at 7 days after cytokine injection compared with naive animals and recombinant mouse MOG/PBS controls and returned to naive levels at 14 days post-injection. Interestingly, no significant difference in the numbers of T and B cells was seen between MOG and incomplete Freund’s adjuvant immunized animals injected with cytokines. IgG+ plasma cells were found sparsely distributed in the meninges surrounding the injection site (Fig. 5D). Iba1+ CD68+ monocytes/macrophages were present in the meninges overlying the areas of intense microglial activity associated with cortical pathology (Fig. 5E and I). Increased numbers of macrophages were already seen in the meninges at 1 day in all three animal groups relative to naive controls, with maximal numbers at 7 days in both MOG and incomplete Freund’s adjuvant immunized animals injected with cytokines (Fig. 5I). Interestingly, there was no difference between these two animal groups. For all immune cells there appeared to be a strict compartmentalization between the meninges and the cortical parenchyma (Fig. 5).

Immunoglobulin and complement deposition and oligodendrocyte pathology in subpial lesions

The deposition of immunoglobulin G (IgG) on myelin sheaths was detected using double-immunofluorescence for IgG and
myelin proteins. As a positive control, IgG and C9 could be clearly detected in demyelinating spinal cord lesions in Dark Agouti rats immunized with 50 μg recombinant mouse MOG (Supplementary Fig. 5), compatible with the involvement of antibody- and complement-mediated mechanisms in recombinant MOG-induced EAE in the Dark Agouti rat (Storch et al., 1998). In the naive cortex, IgG could be detected in the meninges and lining cerebral blood vessels (Fig. 6A–C). In some lesions IgG deposits could be detected on myelin at the lesion edge 7 days post-cytokine injection (Fig. 6D–F) in MOG-immunized animals. C9 complement deposits were detected in the meningeal space of naive animals, in a similar fashion to IgG (Fig. 6G–I) and in some areas of demyelination at 7 days post-cytokine injection in animals immunized with recombinant mouse MOG.
This deposition occurred in a perivascular region and on myelin at the leading edge of the area of demyelination. APC + oligodendrocyte cell bodies expressing activated caspase 3, an indicator of the apoptotic cascade, were seen in small numbers prior to demyelination at 3 days post-cytokine injection (Fig. 6M–O).

Neurons are not lost in an acute model of cortical demyelination

Cortical layers were visualized and delineated by NeuN/GluR2/3 immunofluorescence and neurons counted in cortical layers I and II-III of the cingulate cortex (Supplementary Fig. 6A,B). Neurons in
layer I were relatively sparse and there was a non-significant trend towards reduced cell numbers at 7 days in animals immunized with recombinant mouse MOG and injected with cytokines compared to naive animals and controls (Supplementary Fig. 6C). There was no significant difference in the number of neurons across experimental conditions, or time points in cortical layers II–III (Supplementary Fig. 6D).

Discussion

In this study we have provided evidence that the levels of TNF and IFNγ gene and protein expression in the meningeal/CSF compartment increases with the degree of meningeal inflammation and the presence of tertiary lymphoid-like structures in the multiple sclerosis brain. This is in agreement with previous studies demonstrating the
presence of IFNγ-expressing CD8+ T cells (Serafini et al., 2007) and TNF-expressing monocytes (Magliozzi et al., 2010) in the inflamed meninges. Both TNF and IFNγ levels in the CSF have been shown to be elevated during acute multiple sclerosis relapses (Maimone et al., 1991) and, although progressive multiple sclerosis is thought to be characterized by a lower level of inflammation, the present data show that expression of pro-inflammatory cytokines in the meningeal/CSF compartment can be increased after many years of progressive disease, with the caveat that it is likely that the protein levels in post-mortem CSF are significantly reduced by the post-mortem delay. We then tested the hypothesis that an increase in proinflammatory cytokines in the CSF could be responsible for subpial pathology in an experimental setting and show that the acute presence of elevated levels of TNF and IFNγ in the subarachnoid space of rats with a subclinical anti-myelin immune response can lead to extensive subpial demyelination.

Rodent EAE models of multiple sclerosis generally show little or no pathology in the cerebral cortex (Storch et al., 1998). Therefore, we sought to reproduce the inflammatory environment of the multiple sclerosis meninges in a controlled manner by the injection of TNF and IFNγ directly into the subarachnoid space. A previous study had shown that the injection of TNF and IFNγ directly into the cortical parenchyma of Lewis rats produced both subpial and intracortical demyelinating lesions, but only in animals first immunized with recombinant MOG protein (Merkler et al., 2006). However, this approach introduced direct injury to the cortex and inevitable damage to the blood–brain barrier and glial limitans. The study of Merkl et al. (2006) suggested that cytokines injected directly into the parenchyma may preferentially drain through subpial areas into the overlying CSF, thereby giving rise to the predominately subpial distribution of lesions. Our study suggests that this may not be necessary for the development of subpial lesions and that cytokines may diffuse into the cortical parenchyma from the subarachnoid space. Thus, acute elevations in TNF and IFNγ can lead to extensive subpial demyelination, either directly or indirectly through other mediators produced as a result of the subsequent meningeal inflammation. The pathology is very similar to that reported for cases with SPMS (Bo et al., 2003; Kutzelnigg et al., 2005; Magliozzi et al., 2007; Gilmore et al., 2009), including the lack of peripheral immune cell infiltration. The depth of penetration of the area of demyelination, usually layers I–III, was very reproducible and in keeping with a single acute event.

Over-expression of TNF in the CNS of transgenic mice has been shown to lead to oligodendrocyte death and primary demyelination through TNFR1 receptor signalling, in the absence of immune cell infiltration (Akassoglou et al., 1998). In addition, IFNγ is able to induce the expression of the TNFR1 receptor in CNS cells (Veroni et al., 2010) and its overexpression in the CNS also leads to demyelination (Horwitz et al., 1997). Recent studies demonstrating the substantial reduction in clinical symptoms of EAE following the inhibition of soluble TNF signalling through TNFR1, accompanied by enhanced remyelination and neuroprotection without a significant difference in inflammation, but not following inhibition of both soluble and transmembrane TNF, would also argue for an important role for soluble TNF in causing oligodendrocyte cell death and demyelination (Brambilla et al., 2011; Taoufik et al., 2011). However, the acute presence of TNF and IFNγ and the accompanying activation of microglia seen following the subarachnoid injection of TNF and IFNγ in incomplete Freund’s adjuvant only immunized animals did not result in demyelination. Demyelination was only seen in animals that had been previously immunized with MOG, indicating the importance of an anti-myelin immune response. One of the clear limitations of the model we describe is that it is acute and resolution of the inflammation and repair occurs rapidly. Thus, it remains to be determined whether the chronic presence of these cytokines in the subarachnoid space would give rise to chronic microglial activation and demyelination in the absence of an anti-myelin autoimmune response or whether the humoral response is still required. Our findings help to explain why subpial demyelination seems to be specific to multiple sclerosis and was not found in other inflammatory CNS conditions with a meningeal component, such as tuberculous meningoencephalitis or HSV-1 encephalitis (Magliozzi et al., 2010; Frischer et al., 2013).

The observation of similar numbers of CD4+ and CD8+ T cells and CD68+ macrophages in the meninges of incomplete Freund’s adjuvant and MOG immunized animals injected with TNF and IFNγ indicates that the injection of the pro-inflammatory cytokines alone is sufficient to induce meningeal infiltration. In addition, the initial activation of microglia after 1–3 days was independent of the immunization status and only became more extensive in MOG immunized animals at the time of maximum demyelination. This suggests that acute non-specific meningeal inflammation and microglial activation alone are insufficient to cause demyelination, which indicates the importance of a cellular and/or humoral immune response to a myelin antigen in the initiation of the demyelination. Our results suggest that both antibody-mediated and direct cell cytotoxicity may contribute to oligodendrocyte death and myelin damage. However, this study did not allow us to distinguish between these two mechanisms of demyelination and further studies are required to determine their relative contribution. Complement and Ig deposition on myelin was not observed in all MOG immunized animals in the current study, but this has previously been reported to be very transitory in nature (Merkler et al., 2006). Studies of subpial lesions in post-mortem tissue have so far demonstrated a lack of complement and Ig deposition (Brink et al., 2005), although this may be because these lesions have not been studied during their acute phase, when inflammatory infiltrates have been demonstrated in the parenchyma (Lucchinetti et al., 2011). However, the compartmentalization seen between meningeal infiltrates and activated microglia in the present study would argue that it is not necessary to have peripheral immune cells infiltrating the cortical grey matter for demyelination to occur even at early stages of disease. Although it is not known whether subpial lesions evolve by a slow ingress of the area of demyelination from the pial surface or whether there is an acute burst of demyelinating activity, the lack of integrity of the glia limitans associated with cortical demyelination in SPMS cases characterized by meningeal inflammation (Magliozzi et al., 2010) would increase the likelihood of a direct action of cytokines derived from the subarachnoid space over a prolonged period.

Our results suggest that if the inflammatory stimulus is transient, then there is resolution of the inflammation and demyelination. Although we have not directly demonstrated remyelination...
after 14 days, the fact that demyelination was extensive at 7 days and almost absent at 14 days, and completely absent at 21 days, is good evidence of a successful reparative process and in agreement with other models of demyelination (Ludwin, 1978; Reynolds and Wilkin, 1993; Franklin et al., 1997; Merkler et al., 2006). The presence of large areas of subpial demyelination extending over multiple gyri in the SPMS brain and the accompanying large immune cell aggregates in the meninges, which in 40% of cases have the characteristics of tertiary lymphoid structures (Howell et al., 2011), does suggest that the subpial cortical pathology seen in SPMS is chronic rather than acute. Therefore, the model that we describe has the major limitation that it does not reproduce the chronic nature of the human disease and may not reflect the pathogenetic mechanisms leading to the chronic pathology in multiple sclerosis. It remains to be seen whether the presence of chronic meningeal inflammation in the rodent would eventually lead to chronic demyelination. The lack of neuronal loss in the outer cortical layers after the acute inflammatory and cytotoxic episode in the current study, in contrast to the substantial neuronal loss in multiple sclerosis cortex (Petersen et al., 2001; Magliozzi et al., 2010), suggests that loss in the multiple sclerosis cortex may occur over a protracted period, possibly decades, due to a chronic inflammatory state. This is in agreement with the slow progressive increase in motor, sensory and cognitive deficits that characterizes most cases of SPMS (Kremenchutsky et al., 2006).

In summary, the presence of increased and organized meningeal inflammation in the brains of SPMS cases is reflected in elevated levels of TNF and IFN-γ expression in the meninges. Injection of TNF and IFN-γ into the subarachnoid space of rats with a pre-existing immune response to myelin leads to extensive meningeal inflammation and subpial demyelination, thus supporting the hypothesis that cytotoxic molecules diffusing from the CSF in the inflamed meninges into the cortical grey matter can be a cause of subpial demyelination in multiple sclerosis.

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Supplementary material

Supplementary material is available at Brain online.

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